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Bafilomycin A_1 induces apoptosis in PC12 cells independently of intracellular pH

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Abstract PC12 cells growth-arrested with bafilomycin A_1 died showing apoptotic chromatin condensation in the nuclei. The bafilomycin A_1-induced chromatin condensation was preceded by neurite outgrowth (NOG), required higher concentrations of bafilomycin A_1 than NOG, and was suppressed by cycloheximide and aurintricarboxylic acid. NH_4Cl (10 mM), another acidotropic pH perturbing agent, neither induced apoptotic chromatin condensation by itself nor suppressed that induced by bafilomycin A_1, suggesting that bafilomycin A_1-induced apoptosis occurs independently of intracellular pH in PC12 cells.

Key words: Bafilomycin A_1; Apoptosis; Vacuolar H^+-ATPase; NH_4Cl; PC12 cell

1. Introduction

Vacuolar type H^+-ATPases (V-ATPases) are present on the membranes of acidic organelles, such as synaptic vesicles, chromaffin granules, platelet-dense granules, secretary granules, lysosomes and the trans-Golgi network, and maintain an acidic environment thereby pumping protons using the energy of ATP hydrolysis [1]. The acidic pH in such organelles is proposed to be responsible for important cellular functions like endocytosis, exocytosis, and intracellular trafficking [2]. V ATPases are also present on the plasma membranes of at least some types of cells (e.g. osteoclasts [3], macrophages [4] and some tumor cells [5]) thereby acidifying the surrounding medium and/or maintaining the cytosolic pH by extruding protons out of the cells. However, their role in cell differentiation, cell growth and cell death remains unknown. Using a recently discovered potent selective inhibitor of vacuolar H^+-ATPase, bafilomycin A_1 [6], we suggested the possible involvement of vacuolar H^+-ATPase in cell differentiation and in cell growth: bafilomycin A_1 not only induced neurite outgrowth (NOG) of PC12 cells [7] and differentiation of M1 cells into macrophage-like phagocytic cells [8] but also arrested cell growth of various cells in culture [9]. Similar observations on the growth inhibitory effect of bafilomycin A_1 have also been reported [10,11]. Furthermore, we reported that bafilomycin A_1 induces apoptosis in PC12 cells [12]. Recently, apoptosis has been reported to be induced in several types of cells by a series of V-ATPase inhibitors [13].

Herein, we report full details on the apoptotic nature of the cell growth inhibition by bafilomycin A_1 in PC12 cells as compared with that by NH_4Cl.

2. Materials and methods

2.1. Materials

Bafilomycin A_1 and mouse 7S nerve growth factor (NGF) were kindly provided by Professor K. Altendorf (University of Osnabrück, Germany) and K. Hayashi (Gifu College of Pharmacy, Japan), respectively. Most of the other chemicals used in this study including Hoechst 33342, Hoechst 33258 and aurintricarboxylic acid (ATA) were obtained from Sigma (St. Louis, MO, USA). Most of the lipophilic compounds used in this study, including bafilomycin A_1, were dissolved in DMSO and applied to cells at a final DMSO concentration of 1%.

2.2. Cell culture

Rat pheochromocytoma PC12 cells (RCB 009) were obtained from RIKEN Cell Bank (Tsukuba, Japan) and cultured in Dulbecco's modified Eagle's medium (Flow Laboratories) supplemented with 10% fetal calf serum (M.A. Laboratory) and 5% horse serum (CosmoBio, Japan) at 37°C in 5% CO_2.

2.3. Measurement of cell viability

Cell viability was assessed by dual fluorescence staining with propidium iodide and fluorescein diacetate (FDA) [14]. Cells plated in 24-well culture dishes were incubated in medium containing propidium iodide (50 nM) and FDA (0.15 nM) at 37°C for 10 min, then washed and observed under epifluorescence microscopy (Olympus, BH2-RFK or IMT-2-21CA3 with B-excitation); viable cells showed green fluorescence due to fluorescein accumulated by the cytosolic hydrolysis of FDA, while the nuclei of dead cells showed red fluorescence by propidium iodide taken up because the permeability barrier to the dye had been lost. All data shown are the averages (mean ± S.D.) of triplicate experiments.

2.4. Measurement of chromosome condensation

Apoptosis was first assessed morphologically as chromatin condensation by staining with Hoechst 33342 (or 33258) [15]. PC12 cells plated in 24-well culture dishes at a density of $3–5 \times 10^4$ cells/ml were cultured under various conditions and then stained with Hoechst dye (10 μg/ml) at 37°C for 1 h, washed once with Dulbecco's modified Eagle's medium by centrifugation (3000 × *g* for 5 min) and observed under epifluorescence microscopy (Olympus, BH2-RFK or IMT-2-

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Abbreviations: ATA, aurintricarboxylic acid; BCECF-AM, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein tetraacetoxymethylester; CHX, cycloheximide; DMSO, dimethylsulfoxide; FDA, fluorescein diacetate; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; NOG, neurite outgrowth; NGF, nerve growth factor; PBS, phosphate-buffered saline

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21CA3 with U-excitation) directly or after fixation with 2% glutaraldehyde. About 150 cells were counted per well, and the percent of cells with chromatin condensation was calculated. All data are shown as the averages (mean ± S.D.) of duplicate or triplicate experiments.

2.5. Detection of DNA fragmentation

Apoptosis was also assessed by fragmentation of DNA into a series of nucleosome sets resulting in ladder formation on agarose gel electrophoresis [16]. PC12 cells plated in 90-mm dishes at a density of about 3×10^5 cells/ml and treated with various conditions for indicated periods were washed with Dulbecco's modified Eagle's medium, scraped from dishes with a rubber policeman, and collected by centrifugation at $2500\times g$ for 5 min. The resultant cell pellets were resuspended in PBS, lysed in a 100 μl lysis buffer (10 mM Tris-HCl (pH 7.4), 10 mM EDTA, and 0.5% Triton X-100) at 4°C for 20 min. After centrifugation at 10000 rpm for 30 min, the supernatants were transferred to conical centrifuge tubes, and treated with RNase A (400 μg/ml, at 37°C for 2 h) and proteinase K (200 μg/ml, at 37°C for 1 h), sequentially. The DNA was applied onto a 2% agarose gel for electrophoresis and stained with 0.5 μg/ml of ethidium bromide for 10 min.

2.6. Neurite outgrowth (NOG) assay

The extent of NOG of PC12 cells plated in 24-well culture dishes at a density of $3–5\times10^4$ cells/ml under various conditions was estimated as described previously [7]. Briefly, a neurite was defined as a process more than 1.5 times longer than the cell body judged from photographs taken under phase contrast microscopy (Olympus, Tokyo). About 150 cells were counted per well, and the percent of cells with neurites was calculated. All data shown are the averages (mean ± S.D.) of duplicate or triplicate experiments.

2.7. Measurement of intracellular pH

Measurement of cytosolic pH was performed essentially according to [9] using 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) [17] as a pH probe. Briefly, after overnight culture of cells in microplates ($1–3\times10^4$ cells/ml), the medium was changed to one containing 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein tetraacetoxymethylester (BCECF-AM), cultured for an additional hour, and the cells were washed with Hanks' solution buffered with 20 mM HEPES (Hanks'-HEPES). Medium with or without drugs was added, then the cells were further incubated for 1 h. After briefly washing the cells with Hanks'-HEPES, fresh medium of the same composition was added and the fluorescence emitted by cells in 5% CO_2 95% air at 37°C was viewed using an IMT-2-21-CA3 inverted fluorescence microscope (Olympus, Tokyo) through a SIT camera, after excitation at 450 nm, and analyzed with an ARGUS-100/pH image processor (Hamamatsu Photonics, Hamamatsu). The cytosolic pH was estimated using standards prepared in situ under the same condition as those for culture. For the measurement of vacuolar (lysosomal) pH, cells were loaded overnight with fluorescein-labeled dextran instead of BCECF and then processed essentially as described above for the measurement of fluorescence emitted from lysosomes [18,19].

3. Results

3.1. Induction of chromatin condensation in PC12 cells by bafilomycin A_1

Both bafilomycin A_1 (≥100 nM) and NH_4Cl (≥10 mM) almost completely arrested the growth of various cells in culture including PC12 cells [9]; flow cytometric analysis suggested that they stay apparently at the G_0/G_1 phase of the cell division cycle (unpublished observation). The treatment with bafilomycin A_1 decreased the number of viable PC12 cells time- and dose-dependently, as in serum-free medium (Fig. 1A,B). However, the viability of the cells did not decrease by treatment with NH_4Cl up to 10 mM (Fig. 1A). In serum-free medium, PC12 cells die as a consequence of apoptosis [20]. Therefore, we next examined the nuclear morphology of the cells by staining them with Hoechst 33258 (for nuclear condensation) (Fig. 2). Unlike the cells treated with solvent (DMSO) (Fig. 2A), the nuclear morphology of the cells treated with bafilomycin A_1 (Fig. 2B) showed bright chromatin condensation indicative of apoptosis as seen in cells cultured in the absence of serum (24 h) (Fig. 2D and [20]): many small bright dots indicative of apoptotic body formation were obvious in PC12 cells growth-arrested with bafilomycin A_1 in later stages. On the other hand, cells treated with 10 mM NH_4Cl did not show any sign of apoptotic nuclear morphology at any time point tested (Fig. 2C).

3.2. Time course and dose response of induction of chromatin condensation by bafilomycin A_1

Fig. 3 shows the time course and dose response of the induction of chromatin condensation by bafilomycin A_1 com-

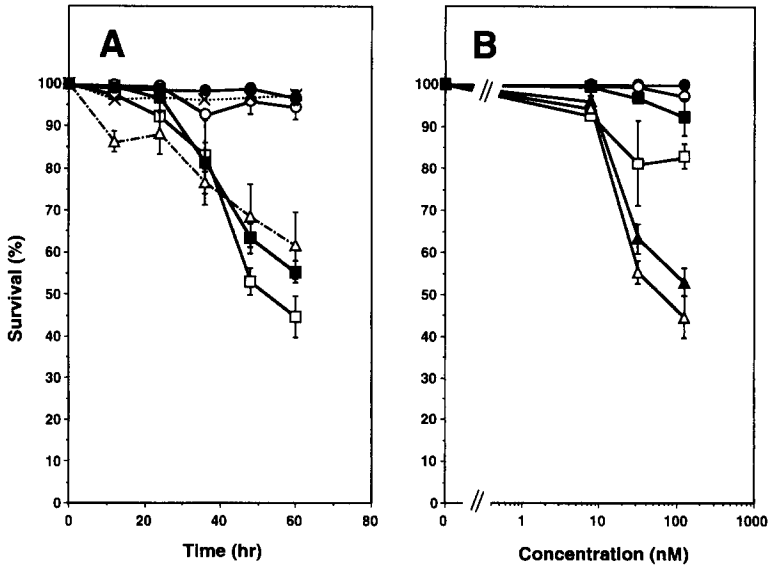

Fig. 1. Time course and dose response of survival of bafilomycin A_1-treated PC12 cells. Cells were scored for percent survival as described in Section 2 after incubation for the indicated periods at different concentrations of bafilomycin A_1. A: Time course. (●) control (complete medium); (○) 7.8 nM bafilomycin A_1; (■) 31 nM bafilomycin A_1; (□) 125 nM bafilomycin A_1; (△) serum-free medium; (×) 10 mM NH_4Cl. B: Dose response. (●) 0 h; (○) 12 h; (■) 24 h; (□) 36 h; (▲) 48 h; (△) 60 h.

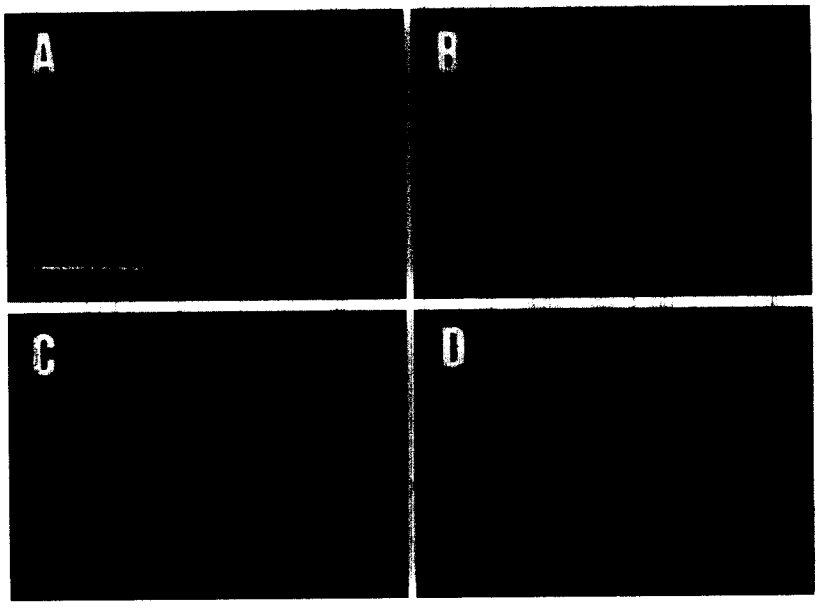

Fig. 2. Morphological features of PC12 cells stained with Hoechst 33258. Cells were stained with Hoechst 33258 and observed under epifluorescence microscopy after incubation in the following conditions: (A) 1% DMSO, 36 h; (B) bafilomycin A_1 (125 nM) in 1% DMSO, 36 h; (C) NH_4Cl (10 mM), 36 h; (D) serum-free medium, 24 h. The length of the bar corresponds to 50 μm. The cells in B and D show bright fluorescence of apoptotic chromatin condensation with occasional small structures resembling apoptotic bodies.

pared with those for the induction of NOG [7]. As shown in Fig. 3A, the morphological change of NOG induction preceded that of chromatin condensation by 10–12 h and appeared maximal at about 36 h at a concentration of 125 nM. The induction of apoptosis required higher concentrations of bafilomycin A_1 than the induction of NOG, and apoptotic change was maximally induced at the concentrations (≥100 nM) of bafilomycin A_1 that were about 3 times higher than those required for NOG (30–100 nM). NH_4Cl does not show any activity of NOG at any concentration [7].

3.3. Induction of chromatin condensation by bafilomycin A_1 requires de novo synthesis of protein and is inhibited by ATA

Fig. 4A shows the effect of cycloheximide (CHX) on the chromatin condensation induced by bafilomycin A_1. About 50% of the chromatin condensation induced by bafilomycin A_1 was inhibited completely by CHX at concentrations higher than 125 μg/ml. However, the same concentration of CHX did not inhibit chromatin condensation induced in cells cultured in serum-free medium, which also confirmed published findings [20]. Fig. 4B shows the effect of ATA, an inhibitor of endonuclease [21], on the bafilomycin A_1 (125 nM)-induced chromatin condensation. ATA (up to 400 nM) similarly inhibited the bafilomycin A_1-induced apoptotic chromatin condensation. ATA also inhibited dose-dependently the chromatin condensation induced in serum-free medium, which also confirmed literature reports [22].

3.4. DNA fragmentation of PC12 cells treated with bafilomycin A_1

Fig. 5 shows the fragmentation of DNA obtained from cells treated with bafilomycin A_1. Bafilomycin A_1 induced DNA fragmentation time- and dose-dependently, with maximum fragmentation attained at 48 h with 500 nM bafilomycin A_1

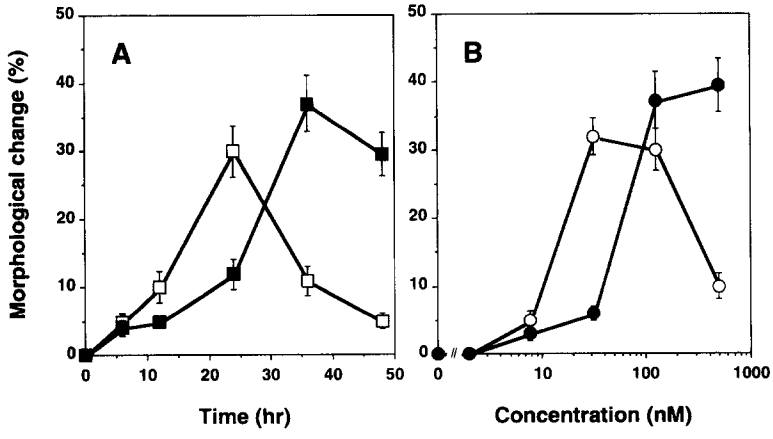

Fig. 3. Time course and dose response of bafilomycin A_1-induced morphological changes of PC12 cells. Cells were scored for NOG and chromatin condensation as described in Section 2 after incubation with bafilomycin A_1. A: Time course. (□) NOG at 30 nM; (■) chromatin condensation at 125 nM. B: Dose response at 36 h. (○) NOG; (●) chromatin condensation. Means ± S.D. of triplicates are plotted.

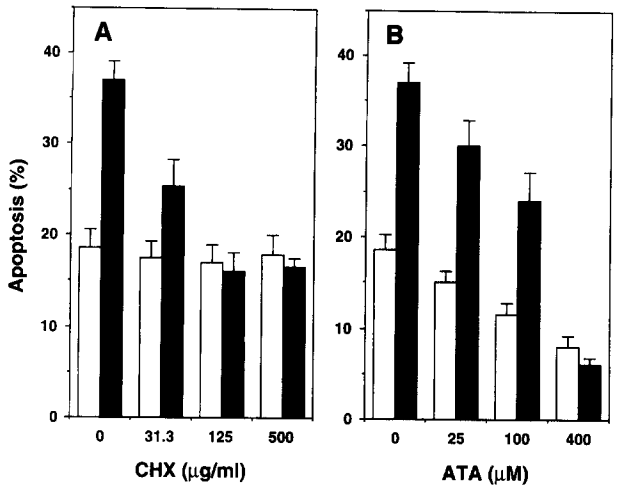

Fig. 4. Effects of cycloheximide (CHX) and aurintricarboxylic acid (ATA) on the induction of apoptosis in PC12 cells. Cells were scored for chromatin condensation as described in Section 2 after incubation in medium containing 125 nM bafilomycin A_1 for 36 h (closed bar) or in serum-free medium for 24 h (open bar) with or without CHX (A) and ATA (B).

in parallel with chromatin condensation, but NH_4Cl up to 10 mM did not (data not shown).

3.5. *Effect of NH_4Cl on the bafilomycin A_1-induced apoptosis of PC12 cells*

Like NH_4Cl (10 mM) (and various weak bases) [18,23], bafilomycin A_1 (100 nM) increases vacuolar pH to neutrality [7,24] and inhibits cell growth completely [9,25]. However, only bafilomycin A_1 induced apoptotic chromatin condensation in PC12 cells (Fig. 2B), suggesting that vacuolar alkalinization has nothing to do with, or is at least insufficient for, the induction of apoptosis. What causes these divergent effects of NH_4Cl and bafilomycin A_1? Unlike NH_4Cl, bafilomycin A_1 might decrease cytosolic pH through inhibition of plasma membrane V-ATPase, if any [26]. Therefore, the divergent effects of these drugs might have originated from their differential effect on cytosolic pH. In fact, 125 nM bafilomycin A_1 sometimes decreased the cytosolic pH of PC12 cells, but only slightly (< 0.1 pH unit) and transiently (< 10 min), if at all, in complete medium and resulted in little decrease in cytosolic pH in the long run (Fig. 6C). Furthermore, the cytosolic pH in the presence or absence of bafilomycin A_1 was raised close to medium pH by 10 mM NH_4Cl (Fig. 6C), but neither the induction of cell death (Fig. 6A) nor that of chromatin condensation (Fig. 6B) in the bafilomycin A_1-treated cells was affected by 10 mM NH_4Cl. Similar observations were made with chloroquine instead of NH_4Cl (data not shown). These findings suggest that bafilomycin A_1-induced apoptosis in PC12 cells is triggered neither by simple vacuolar alkalinization nor by cytosolic acidification.

4. Discussion

We examined the nature of cell growth inhibition by bafilomycin A_1 as compared with that of NH_4Cl. Although both bafilomycin A_1 and NH_4Cl arrested cell growth [9] (probably at the G_0/G_1 phase of the cell cycle), only bafilomycin A_1 induced chromatin condensation (morphological marker of apoptosis [15]) eventually leading to cell death. (Higher concentrations ($\geq$40 mM) of NH_4Cl induced apoptotic changes with peculiar characteristics (unpublished observation).) The chromatin condensation induced by bafilomycin A_1 was not due to its general degenerative effect, because similar chromatin condensation was not induced by other metabolic inhibitors tested including potassium cyanide (data not shown). Furthermore, the chromatin condensation was inhibited by CHX, suggesting its dependence on active de novo synthesis of protein, which is another important criterion for apoptosis: gene-regulated active cell death [27]. However, protein synthesis is not necessarily involved in every type of apoptosis: CHX has no effect on the apoptosis induced in serum-free medium, like staurosporine-induced apoptosis [28] (Fig. 5 and [18]). The bafilomycin A_1-induced apoptosis was also inhibited by ATA, an inhibitor of endonucleases [21], suggesting the participation in this apoptosis of endonuclease which will eventually lead to DNA fragmentation [22]. In fact, ladder formation of DNA (a biochemical marker of apoptosis [16]) was clearly observed in cells treated with bafilomycin A_1 (Fig. 5 and [13]).

Recently, evidence has accumulated that acidification of cytosolic pH is associated with, and sometimes even essential for, some types of apoptosis [29–32], among which the participation of V-ATPase has been suggested to exclude protons from the cytosol. Therefore, the decrease of cytosolic pH, rather than the increase in vacuolar pH, might be the trigger for the apoptotic change induced by bafilomycin A_1. In fact, vacuolar pH was raised not only by bafilomycin A_1 [7,9,10] (pH increased to 5.9 and 6.6 from 5.0 at 100 and 500 nM in PC12 cells [7]) but also by NH_4Cl (pH increased to $\geq$6.4 at 10 mM [18,23]), while the cytosolic pH was raised by NH_4Cl but decreased by bafilomycin A_1 [26]. However, as shown in this study, the cytosolic pH of PC12 cells remained normal in complete medium, probably through Na^+/H^+-antiporter activity, although cytosolic pH sometimes showed a small and transient decrease, and, after 24 h, the apoptotic cell population with acidic cytosol appeared in flow cytometry probably

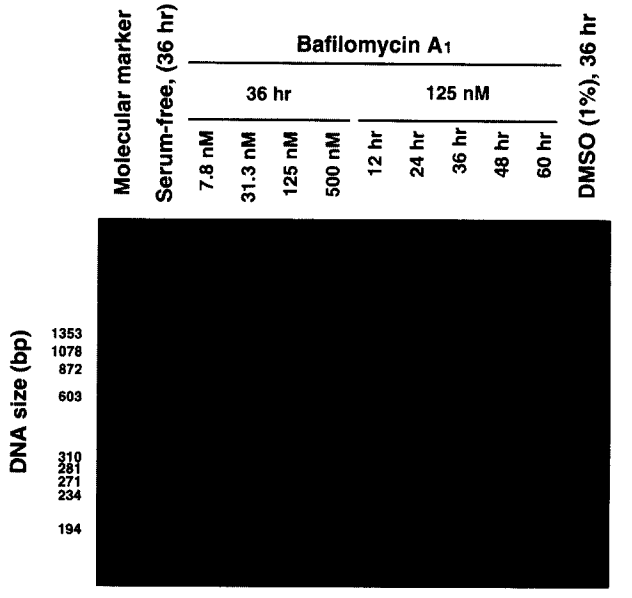

Fig. 5. DNA ladder formation of PC12 cells treated with bafilomycin A_1. The DNA extracted from cells after incubation in the following conditions was electrophoresed on 2% agarose gel and stained with 0.5 μg/ml of ethidium bromide for 10 min.

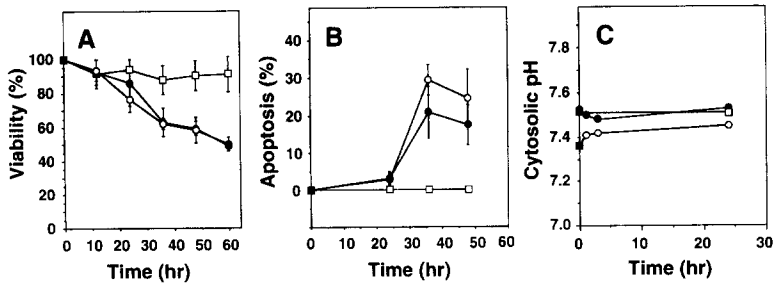

Fig. 6. Effect of NH_4Cl on the cell viability, apoptosis and cytosolic pH of bafilomycin A_1-treated PC12 cells. Cells were either scored for cell viability (A) or for chromatin condensation (B) or loaded with BCECF to estimate cytosolic pH (C) as described in Section 2, at the end of incubation under the indicated conditions. (■) complete medium; (○) 125 nM bafilomycin A_1; (□) 10 mM NH_4Cl; (●) 125 nM bafilomycin A_1–10 mM NH_4Cl.

secondary to apoptotic change (unpublished observation). Furthermore, the cytosolic pH of PC12 cells was raised by 10 mM NH_4Cl regardless of the presence or absence of bafilomycin A_1. However, bafilomycin A_1-induced apoptosis was not affected by the increase of cytosolic pH by 10 mM NH_4Cl (Fig. 6). These findings suggest that bafilomycin A_1-induced apoptosis in PC12 cells does not require cytosolic acidification. Essentially the same conclusion was reached in a study of bafilomycin A_1-induced apoptosis in HL60 leukemic myeloid precursor cells (Kurashima et al., manuscript in preparation).

Both bafilomycin A_1 and NH_4Cl increase the pH of vacuolar organelles and arrest cells at a specific stage of the cell cycle, probably by depriving cells of necessary nutrients such as ferric ions [11,33] and/or growth factors like FGF [34,35]. After a period of arrest at the G_0/G_1 stage, cells cannot perform normal DNA synthesis. According to the 'death by default hypothesis' [36] or 'G_1-arrest hypothesis' [37,38], it will trigger the cells into the apoptotic death cascade. However, only bafilomycin A_1-treated cells entered the apoptotic process. Furthermore, NH_4Cl did not interfere with the apoptotic process induced by bafilomycin A_1. These results suggest that vacuolar alkalinization alone is, at least, insufficient for the induction of apoptosis. The divergence of these effects between bafilomycin A_1 and NH_4Cl in the induction of apoptosis is quite similar to that in the induction of NOG: NH_4Cl neither induced NOG nor suppressed NOG induced by bafilomycin A_1 [7]. As will be shown separately, bafilomycin A_1-induced NOG requires participation of a protein phosphorylation-dephosphorylation cycle of signal transduction (Waritani et al., and Noto et al., manuscripts in preparation). Therefore, there may be serious differences in the signal transduction cascade between bafilomycin A_1-treated and NH_4Cl-treated cells, and these require further study.

Programmed cell death may also play a pivotal role in embryogenesis, immune system maturation, and tissue homeostasis [26,39]. From these points of view, the correlation between apoptosis and cell differentiation should also be examined extensively. Bafilomycins, like retinoic acid (on human embryonal carcinoma cells) [40], induce cell differentiation and apoptosis at the same time. Bafilomycin A_1-induced apoptosis is suppressed by other neuritogenic agents and accelerated by agents that inhibit cell differentiation (manuscript in preparation). Furthermore, cell differentiation and apoptosis are both important to cancer therapy, since cancer cells can be selectively eliminated by artificially triggering either their differentiation or their death through apoptosis: transplanted invasive human pancreatic cancer cells which express a high level of V-ATPase subunit proteins [41] have been selectively induced to die of apoptosis by bafilomycin A_1 in the host nude mice [42].

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