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Calcium-dependent DNA fragmentation in human synovial cells exposed to cold shock

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Exposure of confluent human synovial McCoy's cells to near-freezing temperatures followed by rewarming at 37°C resulted in endonuclease activation and cell death characteristic of a suicide process known as apoptosis. Both DNA fragmentation and cell killing were dependent on a sustained increase in the cytosolic Ca^{2+} concentration. Sensitivity to cold shock-induced endonuclease activation was critically dependent on the cell cycle (proliferative) status and limited to confluent cells, whereas cells in the logarithmic growth phase were completely resistant. However, DNA fragmentation was promoted in the proliferating McCoy's cells pretreated with H-7 or sphingosine, inhibitors of protein kinase C. In addition, phorbol ester, known to activate PKC, inhibited DNA fragmentation in the confluent cells. Our findings indicate that cold shock-induced DNA fragmentation in McCoy's cells is dependent on a sustained Ca^{2+} increase, and sensitivity to the process appears to be regulated by the status of protein kinase C.

Apoptosis; Endonuclease; Calmodulin; Protein kinase C

1. INTRODUCTION

Apoptosis, or programmed cell death (PCD), is a process of selective cell deletion characterized by several early morphological changes, including plasma and nuclear membrane blebbing, organelle relocalization, and chromatin condensation [1]. The latter phenomenon has been linked to the activation of an endogenous nuclear endonuclease that cleaves host chromatin into oligonucleosome-length DNA fragments [2], and this event may directly precipitate cell death [3]. Endonuclease activation has therefore served as a reliable marker for apoptosis.

Our recent work has been concerned with characterizing the biochemical mechanisms underlying apoptosis in immature thymocytes. We have shown that a sustained increase in the cytosolic Ca^{2+} concentration mediates both endonuclease activation and cell death in response to a number of relevant stimuli [3-6]. Calmodulin antagonists block DNA fragmentation [4], suggesting a critical role for calmodulin in the process. Agents that stimulate protein kinase C (PKC) block endonuclease activation, whereas PKC inhibitors promote DNA fragmentation [6,7]. Because Ca^{2+} increases and PKC activation are critically involved in lymphoid cell proliferation [8], we have proposed that thymocyte apoptosis results from 'unbalanced' proliferation signalling, when a sustained Ca^{2+} increase occurs in the absence of sufficient PKC activation.

Liepins and Younghusband [9] reported that exposure of P815 mastocytoma cells to cold (but not freezing) temperatures for 1-2 h followed by rewarming to 37°C resulted in endonuclease activation. Similar findings in Chinese hamster V79 fibroblasts were recently presented by Soloff et al. [10], who suggested that the response was due to the induction of apoptosis. In the present study we examined the roles of Ca^{2+} and PKC in the DNA fragmentation and cell killing observed in human synovial McCoy's cells exposed to cold shock. Our data indicate that both are mediated by a sustained Ca^{2+} increase, and PKC activation appears to inhibit the response. Combined with our earlier observations in thymocytes, our data suggest that these biochemical mechanisms are generally involved in the endonuclease activation associated with programmed cell death.

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Abbreviations: PCD, programmed cell death; PKC, protein kinase C; Compound R24571 calmidizolium, (1-[bis(4-chlorophenyl)methyl]-3-[2-(2,4-dichlorophenyl)-2-[(2,4-dichlorophenyl)-methoxy]-ethyl]-1-H-imidazolium chloride, calmidazolium; quin-2, 2-[(2-bis(carboxymethylamino-5-methylphenoxy)methyl]-6-methoxy-8-bis(carboxymethyl)aminoquinoline; quin-2 AM, tetraacetoxymethylester of quin-2; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TPA, 12-*o*-tetradecanoylphorbol-13-acetate; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine

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2. MATERIALS AND METHODS

Human synovial McCoy's cells (kindly provided by Dr O. Cantoni, University of Urbino, Italy) were grown at 37°C in a humidified 5% CO_2, 95% air atmosphere as monolayers in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics (penicillin and streptomycin). Cells were seeded at 1×10^6 per tissue culture dish (90 mm diameter). Prior to the experiments the medium was changed to the RPMI-1640 medium without serum, and the dishes were exposed for various time periods to cold (2°C). Cells were then rewarmed by changing to 37°C RPMI-1640 medium supplemented with serum. Cytotoxicity was measured by following the release of lactate dehydrogenase (LDH) into the incubation medium, and protein content was assessed by the method of Lowry et al. [11].

DNA fragmentation was determined essentially as described previously [2–7]. Cells (approximately 5×10^6) were scraped from culture dishes, isolated by centrifugation for 10 min at $450 \times g$, resuspended in 1 ml of a lysis buffer containing 5 mM Tris-HCl, 20 mM EDTA, 0.5% Triton X-100, pH 8.0, and allowed to lyse for 15 min on ice. Samples were then centrifuged at $27\,000 \times g$ to separate high-molecular-weight chromatin (pellet) from cleavage products (supernatant). Pellets were resuspended in 1 ml of a buffer containing 10 mM Tris-HCl and 1 mM EDTA, pH 8.0. Pellets and supernatants were assayed for DNA content using the diphenylamine reagent [12]. All assays were done in triplicate.

For Ca^{2+} measurements, McCoy's cells were scraped from culture dishes, resuspended in fresh serum-free RPMI-1640 medium at a concentration of 2×10^6 cells/ml, and incubated for 30 min with 5 μM quin-2 AM under gentle, continuous stirring at 37°C. Cells were washed extensively in serum-free medium to remove extracellular quin-2. Cytosolic Ca^{2+} concentrations were determined in cells exposed to cold shock and rewarming as described previously [4], using a Perkin-Elmer LS 5B spectrophotofluorometer. Leakage of hydrolyzed quin-2 was estimated by the changes in fluorescence intensity obtained after addition of 10 mM EGTA to unpermeabilized cells and was accounted for in the calculation of the final free Ca^{2+} concentration. Total cellular Ca^{2+} released by treatment with the Ca^{2+} ionophore A23187 was determined using arsenazo III, as described previously [13].

3. RESULTS AND DISCUSSION

Previous work has shown that exposure of different cell types to cold but non-freezing temperatures results in DNA fragmentation following rewarming at 37°C [9,10]. We also detected DNA fragmentation in McCoy's cells that required exposure to cold for at least 60 min (fig.1A); following rewarming DNA fragmentation was extremely rapid. Longer periods of exposure resulted in proportionate increases in DNA fragmentation levels. Adherent cell lines grow exponentially until contact inhibition arrests their growth at confluence. We found that sensitivity to cold shock-induced DNA fragmentation increased with time following trypsinization, dilution, and replating (fig.1B), until cells reached confluence (about 72 h; G. Bellomo, unpublished), as observed previously [10].

We have recently shown that a sustained increase in the cytosolic Ca^{2+} concentration stimulates DNA fragmentation in thymocytes [3–6]. We therefore asked whether a similar mechanism was involved in cold shock-stimulated endonuclease activation. Cold shock resulted in a sustained Ca^{2+} increase in the McCoy's cells (fig.2). Loading cells with high concentrations of

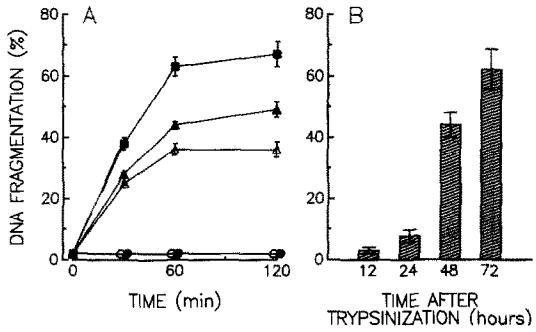

Fig.1. Chromatin cleavage in human synovial McCoy's cells exposed to cold shock. (A) Confluent cells (72 h) were exposed to cold (2°C) for (○) 0, (●) 30, (△) 60, (▲)90 or (■) 120 min, rewarmed to 37°C, and DNA fragmentation was determined after the indicated times as described in section 2. (B) Confluent cells (72 h) were trypsinized and replated in fresh growth medium. After the indicated times had elapsed, cells were exposed to cold for 90 min, rewarmed to 37°C for 60 min, and DNA fragmentation was determined as described in section 2. Mean ± SD; $n = 3$.

Ca^{2+}-sensitive dye, quin-2 AM, to buffer changes in the cytosolic Ca^{2+} level [4], blocked both DNA fragmentation (table 1) and cell killing (fig.3). Incubation of cells in a Ca^{2+}-depleted medium only partially inhibited DNA fragmentation, suggesting that the immediate origin of the Ca^{2+} increase during rewarming was primarily intracellular. Supporting this assumption, total intracellular Ca^{2+} content increased during the period of exposure to cold and returned to basal levels following rewarming (fig.2B), suggesting that Ca^{2+} release from intracellular stores had occurred.

Our previous work has suggested that the Ca^{2+}-binding regulatory protein, calmodulin, mediates endonuclease activation in thymocytes [4] and isolated rat liver nuclei [14]. The calmodulin antagonists, calmidizolium and flunarizine, blocked DNA fragmentation in McCoy's cells exposed to cold shock (table 1), suggesting that the mechanism was calmodulin-dependent and providing further support for the assumption that endonuclease activation was mediated by calcium.

Sensitivity to cold shock appears to be dependent on proliferative status, and cells that are in the logarithmic growth phase do not undergo DNA fragmentation ([10], fig.1). One biochemical phenomenon associated with proliferation is PKC activation [8], which inhibits endonuclease activation in immature thymocytes [6,7]. To test the possibility that PKC activity in the McCoy's cells at the exponential phase of growth contributed to their resistance to cold shock-induced DNA fragmentation, we tested the effects of two widely used PKC inhibitors, H-7 and sphingosine. The inhibitors promoted DNA fragmentation in the proliferating McCoy's cells exposed to cold shock (table 2); the agents did not induce DNA fragmentation in cells that had not been exposed to cold shock (not shown). The effects of H-7 and

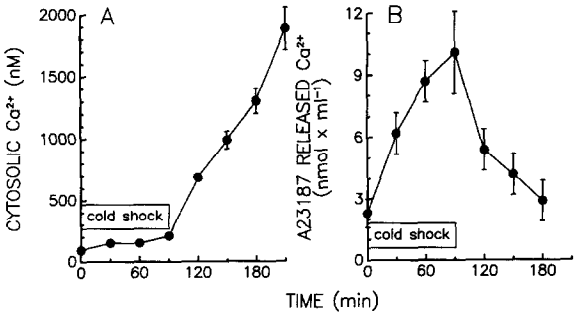

Fig.2. Intracellular calcium changes in McCoy's cells exposed to cold shock. (A) Cytosolic Ca^{2+} changes were monitored in confluent cells exposed to cold shock and following rewarming, as described in section 2. In control cells, the Ca^{2+} concentration was 91 ± 8 nM. (B) Intracellular Ca^{2+} pool sizes were assessed, using the Ca^{2+} ionophore A23187, at the indicated times during cold shock and rewarming, as described in section 2. Mean ± SD; $n = 3$.

sphingosine were associated with potentiation of the Ca^{2+} increases in the cells (table 2).

Phorbol esters are potent inducers of cell proliferation, and their effects have been linked to stimulation of PKC [8]. In previous work we have shown that phorbol esters inhibit PCD in thymocytes [6,7]. The phorbol ester TPA also inhibited DNA fragmentation in the confluent McCoy's cells exposed to cold shock (table 2). The effect was linked to an inhibition of the cold shock-induced Ca^{2+} increase.

Exposure of mammalian cells to low temperatures followed by incubation at 37°C results in cell death. It has been proposed that cell killing occurs via the induction of an endogenous suicide process known as apoptosis [10]. Our recent work has been concerned with identifying the biochemical mechanisms underlying

Table 1

Effects of various agents on cold shock-induced DNA fragmentation in McCoy's cells

Conditions	DNA fragmentation (%)	Inhibition (%)
Control	1.7 ± 0.4	
Cold shock, control medium	45.6 ± 4.8	
Ca^{2+}-free medium	34.3 ± 3.2	25
+ quin-2	15.7 ± 2.5	65
+ flunarizine	4.2 ± 0.8	91
+ calmidizolium	4.2 ± 1.1	91

McCoy's cells grown to confluence (72 h) were exposed to cold shock for 90 min in serum-free medium followed by incubation for 120 min at 37°C in a serum-supplemented medium. After cold shock cells were incubated at 37°C in a Ca^{2+}-free medium supplemented with 1 mM EGTA where indicated. For quin-2 loading, cells were preincubated with 50 μM quin-2 AM for 30 min, washed twice in phosphate-buffered saline and subsequently exposed to cold shock as previously described. This resulted in an intracellular hydrolyzed quin-2 content of approximately 850 μM, calculated in the presence of digitonin and a saturating concentration of $CaCl_2$ from a quin-2 fluorescence intensity standard curve. Calmidizolium (R-24751) (10 μM, final concentration), OAG (2 μM) and flunarizine (5 μM) were present in the incubation mediums during cold shock and subsequent incubation at 37°C. DNA fragmentation was assayed as described in section 2.

Table 2

Effects of phorbol ester and PKC inhibitors on sensitivity of McCoy's cells to cold shock

Conditions	Cytosolic Ca^{2+} (nM)	DNA fragmentation (%)
Control	91 ± 8	0.8 ± 0.2
Cold shock, 24 h cells	211 ± 45	4 ± 0.7
+ 50 μM H-7	358 ± 39	19 ± 4
+ 50 μM sphingosine	388 ± 33	21 ± 3
Cold shock, 72 h cells	641 ± 88	59 ± 6
+ 1 μM TPA	270 ± 41	30 ± 5

Cells were incubated for 2 h with inhibitors prior to exposure to cold shock for 90 min, as described in section 2. Cytosolic Ca^{2+} concentrations were determined 20 min and DNA fragmentation 60 min after rewarming. Mean ± SD; $n = 3$.

apoptosis in immature thymocytes, and we have shown that both endonuclease activation and cell death are mediated by a sustained increase in the cytosolic Ca^{2+} concentration. In this report we have shown that cold shock-induced cell death in McCoy's cells occurs via a similar biochemical mechanism. Furthermore, calmodulin antagonists blocked DNA fragmentation, as was observed previously in thymocytes and isolated rat liver nuclei, supporting a critical role for Ca^{2+} in the response.

Sensitivity to the induction of apoptosis appears to be dependent on cellular proliferative status. Sellins and Cohen [15] showed that γ-irradiation induces apoptosis in resting T and B lymphocytes, whereas mitogen-activated T and B lymphoblasts are resistant. Soloff et al. observed DNA fragmentation in confluent but not proliferating V79 fibroblasts [10]. Our own observations indicate that the sensitivity of McCoy's cells to cold shock increases with time following replating (fig.1B). These observations suggest that activated and/or proliferating cells are generally resistant to endonuclease activation, although they provide no mechanistic explanation for the effects. We have recently shown that agents which stimulate PKC block DNA fragmentation in thymocytes [6,7], and PKC activation

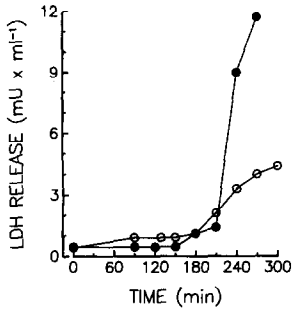

Fig.3. Cell killing is prevented by buffering the cytosolic Ca^{2+} concentration in McCoy's cells exposed to cold shock. Cells were incubated in the absence (●) or presence (○) of 50 μM quin-2 AM prior to exposure to cold shock, and lactate dehydrogenase was measured in the incubation medium at the indicated times after rewarming. Results of one experiment typical of four replicates.

is a general requirement for cell proliferation [8]. As PKC inhibitors promote DNA fragmentation in proliferating McCoy's cells, PKC may be involved in their resistance to cold shock. Conversely, the confluent McCoy's cells were resistant to cold shock following pretreatment with phorbol esters. These data support our previous observations and strongly suggest that the differential sensitivity of cells to induction of apoptosis at different points in the cell cycle is linked to the activation status of PKC.

At present we do not know whether cold shock is ever relevant in vivo. However, cold shock-induced cell death appears to occur via apoptosis, a process that is involved in normal cell turnover, hormone-induced tissue atrophy, and effector cell-mediated target cell cytolysis [1,16]. In addition, exciting new evidence suggests that induction of apoptosis by monoclonal antibodies may be an effective means of stimulating tumor regression [17]. Thus, cold shock may be useful as a model system to further characterize the biochemical mechanisms involved in this physiological mode of cell death.

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