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β-Adrenergic receptor/cAMP-mediated signaling and apoptosis of S49 lymphoma cells

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Yan, Lizhen, Volker Herrmann, Jason K. Hofer, and Paul A. Insel. β-Adrenergic receptor/cAMP-mediated signaling and apoptosis of S49 lymphoma cells. *Am J Physiol Cell Physiol* 279: C1665–C1674, 2000.—β-Adrenergic receptor (βAR) activation and/or increases in cAMP regulate growth and proliferation of a variety of cells and, in some cells, promote cell death. In the current studies we addressed the mechanism of this growth reduction by examining βAR-mediated effects in the murine T-lymphoma cell line S49. Wild-type S49 cells, derived from immature thymocytes ($CD4^+/CD8^+$) undergo growth arrest and subsequent death when treated with agents that increase cAMP levels (e.g., βAR agonists, 8-bromo-cAMP, cholera toxin, forskolin). Morphological and biochemical criteria indicate that this cell death is a result of apoptosis. In cyc^- and kin^- S49 cells, which lack $G_s\alpha$ and functional protein kinase A (PKA), respectively, βAR activation of $G_s\alpha$ and cAMP action via PKA are critical steps in this apoptotic pathway. S49 cells that overexpress Bcl-2 are resistant to cAMP-induced apoptosis. We conclude that βAR activation induces apoptosis in immature T lymphocytes via $G_s\alpha$ and PKA, while overexpression of Bcl-2 prevents cell death. βAR/cAMP/PKA-mediated apoptosis may provide a means to control proliferation of immature T cells in vivo.

programmed cell death; protein kinase A; forskolin; $G_s\alpha$

EUKARYOTIC CELLS have evolved a simple but carefully modulated pathway to regulate formation and action of the second messenger cAMP. A large variety of hormones and neurotransmitters bind to plasma membrane receptors and activate heterotrimeric GTP binding (G) proteins, which, through actions of their α- and βγ-subunits, regulate positively (i.e., G_s) or negatively (i.e., G_i) the enzyme adenylyl cyclase (AC). AC catalyzes cAMP formation from ATP, and cAMP, in turn, binds to its intracellular "receptor," the regulatory (R) subunit of cAMP-dependent protein kinase (PKA), thereby dissociating R subunits from the catalytic subunit of PKA. This dissociation allows the catalyst of PKA to promote the phosphorylation of cellular proteins involved in metabolic regulation, growth control, and differentiated functions, such as secretion and muscle contraction. In the current studies, we have focused on the role of cAMP in regulation of cell growth and cell death.

Death of eukaryotic cells occurs either via necrosis or apoptosis, which are functionally, morphologically, biochemically, and mechanistically different processes. In contrast to necrosis, which is a pathological response to adverse conditions (i.e., injury), apoptosis can occur physiologically such that cells are deleted in an orderly and highly regulated manner. Apoptotic cells characteristically undergo cell shrinkage, membrane remodeling/blebbing, phosphatidylserine redistribution to the cell surface, DNA fragmentation and condensation, and formation of apoptotic bodies (3, 16, 21). By contrast, necrotic cells do not exhibit these morphological and biochemical processes. As a tightly regulated physiological process, apoptosis plays a vital role in tissue homeostasis, embryonic development, and the immune response; it can be stimulated by a wide variety of extracellular and intracellular "death stimuli," such as DNA damage, oxidative stress, hormones, cytokines, and drugs.

cAMP is a unique second messenger in that it has an antiapoptotic role in certain cell types (4, 12, 14, 25) and is proapoptotic in others (18, 22–23, 26–27, 30, 32). The reason for these different responses in different cell types is unclear. In the current studies we undertook experiments to examine cell growth and death in response to agents that increase cAMP in the murine T-lymphoma cell line S49. S49 cells were originally isolated from a Balb/c mouse as transplantable T-cell tumors derived from an immature thymocyte ($CD4^+/CD8^+$) (19, 33). Increases in cAMP can kill S49 cells, and based on resistance to this killing, several S49 variants with lesions in the pathway of cAMP formation and action have been generated (33). In the current studies, we assessed whether the death of S49 cells induced by β-adrenergic receptor (βAR) and G_s activation and/or increases in cAMP is due to necrosis or apoptosis. By employing morphological and biochemical criteria, we show that the death induced by βAR activation and/or increases in cAMP is due to apoptosis and not necrosis and that activation of G_s and AC are sufficient to induce S49 cell apoptosis. Furthermore, our data show that PKA is also obligatorily required for βAR- and cAMP-mediated apoptosis and that the apoptosis induced by cAMP signaling

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components can be largely blocked by overexpression of the Bcl-2 protein.

METHODS

Cell culture. Wild-type (WT), kin$^-$ (which lack activation of the catalytic subunit of PKA), cyc$^-$ (which lack expression of G_s), neo (which were selected to be neomycin resistant), and Bcl-2 (which overexpress Bcl-2) S49 cell lines were cultivated at 37°C in a 90% air-10% CO_2 atmosphere in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum, 100 U/ml penicillin, and 100 U/ml streptomycin (17, 19, 33). Cells were maintained in logarithmic growth. For experiments, cells were cultured at 2×10^5 cells/ml, and before drugs were applied, 100 μM 3-isobutyl-1-methylxanthine, a cyclic nucleotide phosphodiesterase inhibitor, was incubated with cells for 30 min. S49-neo and S49-Bcl-2 cell lines were created by stably infecting S49 cells with a recombinant amphotropic retrovirus carrying a G418 antibiotic resistance gene alone or in combination with a Bcl-2 complementary DNA, respectively (17).

Measurement of cell viability. Cell viability was determined by either trypan blue exclusion or a flow cytometric method. Trypan blue was added to cell suspensions (final concentration 0.1%), which were incubated at room temperature for 1 min, and the cells were counted with a hemocytometer. At least 100 cells were counted per sample. The percentage of viable cells was calculated by dividing the number of cells excluding trypan blue by the total number of cells and multiplying by 100. For flow cytometric analysis, cells were pelleted, washed, and resuspended in phosphate-buffered solution (PBS). Cells were examined on a Becton Dickinson FACScan using CELLQuest software (Becton Dickinson Immunocytometry System, San Jose, CA). Individual populations of cells (5,000 cells per experimental sample) were selected by gating with the use of a forward light scattering vs. side light scattering dot plot as a measure of cell viability.

ISEL+ staining. For ISEL+ staining (in situ end labeling analysis for apoptosis), we adapted a previously published procedure (6). Cells were air-dried on Superfrost Plus microscope slides (Fisher Scientific) and then incubated in labeling mix [100 mM potassium cacodylate, 2 mM $CoCl_2$, 0.2 mM dithiothreitol, and 0.5 μM digoxigenin-11-dUTP (Boehringer Mannheim) containing 150 U/ml terminal deoxynucleotide transferase (GIBCO-BRL)]. Slides were then incubated in a humidified chamber for 1 h at 37°C. Incorporated digoxigenin-11-dUTP was detected with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (Boehringer Mannheim). After slides were washed, alkaline phosphatase activity was detected by incubation in substrate solution containing 4-nitroblue tetrazolium chloride (Boehringer Mannheim) and X-Phosphate (Boehringer Mannheim). Color was allowed to develop for 1 h, after which the reaction was stopped by transferring slides into 1× Tris-buffered saline.

Agarose gel DNA electrophoresis. The cells were first washed with PBS, and their DNA was isolated using a Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN). DNA concentration was determined by ultraviolet (UV) absorbance at 260 nm, and the DNA (20 μg/lane) was electrophoresed on 2% agarose gels. The gels were stained with ethidium bromide (0.5 μg/ml) for 15 min thereafter and then visualized and photographed under UV light.

Annexin V binding assay by flow cytometry. Cells were treated for 60 h, and then $0.5–1.0 \times 10^6$ cells were pelleted and washed with Hanks' balanced salt solution (HBSS) plus 1 mM Ca^{2+}. FITC-conjugated annexin V (annexin V-FITC; 0.2 μg; Caltag Laboratories, San Francisco, CA) was added to the cells resuspended in 100 μl of HBSS and incubated at 37°C for 15 min. The cells were washed and resuspended in 1 ml of HBSS and analyzed as described for *Measurement of cell viability*. FITC was detected using a 530/30-nm bandpass filter (FL1 channel).

Analysis of DNA content and cell cycle by flow cytometry. Cells were pelleted, washed with PBS one time, and suspended with the addition of 2 ml of ethanol in 200 μl of PBS. Fixed cells were pelleted at 800 *g* and resuspended in 1 ml of PBS plus 1% bovine serum albumin. Preboiled RNase A (GIBCO) was added to a final concentration of 100 μg/ml RNase A together with propidium iodide (PI; Sigma) to 50 μg/ml. The suspension was then incubated at room temperature for 15 min and subjected to flow cytometric analysis with excitation at 488 nm and emission measured at 560–640 nm (FL2 mode).

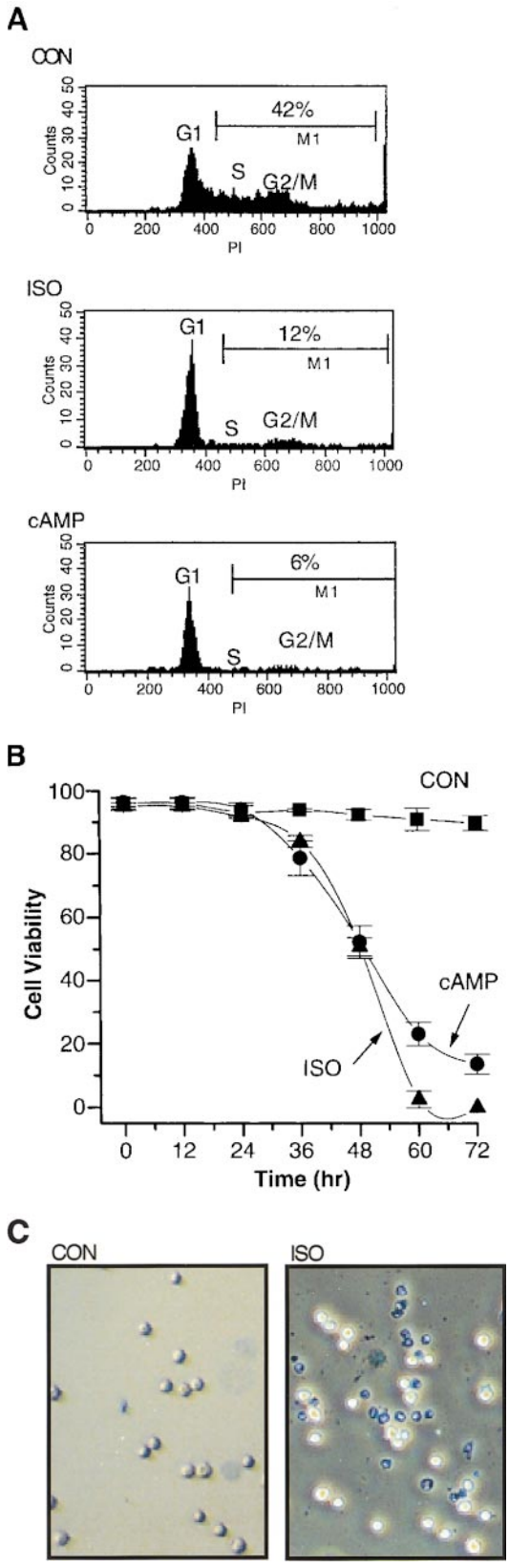
Data analysis. Unless indicated otherwise, all experiments were conducted at least three times and yielded similar results. In general, representative results from a single experiment are presented.

RESULTS

Both isoproterenol and cAMP cause cell arrest at G_1 and decrease the number of viable S49 cells. βAR activation and/or increases in the second messenger cAMP have been shown to arrest the cell cycle progression of S49 lymphoma cells at G_1 and, ultimately, to cause the death of these cells (9, 33). As a starting point for studies with the current batches of cells, we assessed the effects of the βAR agonist isoproterenol (Iso; 100 μM) and the cAMP analog 8-bromo-cAMP (8-BrcAMP; 1 mM) on the cell cycle progression and cell survival of WT S49 cells. For cell cycle analysis, asynchronized S49 cells were stained with PI and then analyzed for DNA content by using the FACScan. We found that 42% of untreated WT S49 cells were in the S and G_2/M phase of the cell cycle (Fig. 1*A*, *top*), but with Iso treatment for 24 h, only 12% of the cells were in S and G_2/M (Fig. 1*A*, *middle*). Treatment with 8-BrcAMP for 24 h led to 6% of the WT S49 cells in S and G_2/M (Fig. 1*A*, *bottom*). In parallel with the cell cycle analysis, we determined the effect of Iso and 8-BrcAMP on the viability of S49 cells by the trypan blue exclusion assay. The results (Fig. 1*B*) showed that WT S49 cells died in a time-dependent fashion: cell viability declined rapidly after 36 h of treatment, with the majority of the S49 cells dead after 72 h. Together, these results confirm that βAR activation and increases in cAMP arrest the cell cycle progression in G_1 and ultimately kill S49 cells.

Iso induces S49 cell death by apoptosis. Because βAR activation and/or increases in cAMP caused the death of S49 cells, we conducted studies to determine whether this cell death is due to either necrosis or apoptosis. We first examined the morphological changes of WT S49 cells treated with Iso. Apoptotic cells are smaller than live cells because of nuclear and cytoplasmic condensation. As shown in Fig. 1*C*, the trypan blue-stained (and therefore dead) cells were smaller than the unstained, live cells (*right*). The observation that cell death is accompanied by cell size

shrinkage suggests that Iso-induced cell death of S49 cells is likely due to apoptosis (16, 17). We found similar results with cAMP-treated S49 cells (data not shown). In subsequent experiments we used 100 uM Iso because of concerns about its oxidation during the lengthy culture period, although lower concentrations (≤100 uM) were able to promote cell death.



During apoptosis cells undergo not only morphological changes but also biochemical changes, such as the translocation of phosphatidylserine from the inner leaflet to the outer leaflet of the plasma membrane (11, 24). Accordingly, we stained S49 cells with annexin V-FITC, which binds to phosphatidylserine, and then subjected the cells to FACScan analysis to measure forward light scattering (FSC) (Fig. 2, *top*) and FITC fluorescence intensity (Fig. 2, *bottom*). FSC intensity is positively correlated with cell size, and cells with low FSC are small and not viable. FSC measurements indicate that 90%, 65%, and 4% of the cells were viable when they were not treated, treated with Iso for 36 h, or treated with Iso for 60 h, respectively (Fig. 2, *top*). These results are consistent with the results suggesting that treatment with Iso causes cell death (Fig. 1, *B* and *C*). The intensity of fluorescence of annexin V-FITC-stained cells is directly proportional to the amount of phosphatidylserine on the outer leaflet of the plasma membrane. The results (Fig. 2) indicate that 10%, 32%, and ≥93% of the S49 cells were annexin V positive when they were untreated, treated with Iso for 36 h, or treated with Iso for 60 h, respectively, providing further evidence that βAR activation causes the death of S49 cells via apoptosis.

One of the earliest recognizable changes denoting an irreversible commitment to cell death is the activation of a calcium-dependent endonuclease and nuclear DNA fragmentation (16). The endonuclease attacks the linker regions between nucleosomes, and internucleosomal cleavage results in the formation of DNA fragments of 180–200 base pairs, thereby generating terminal 3′-hydroxyl ends where the DNA has been nicked. There are several methods for detecting fragmentation of DNA. ISEL+ analysis and agarose gel electrophoresis were used in this study. With the ISEL+ technique, the double-strand DNA breaks produced during apoptosis are identified by attaching labeled nucleotides to free DNA ends; the label is then visualized to identify dying cells by virtue of their nuclear DNA fragmentation (6). As shown in Fig. 3, control cells were ISEL+ negative, but a large portion

Fig. 1. The cAMP analog 8-bromo-cAMP (cAMP) and β-adrenergic receptor agonist isoproterenol (Iso) cause S49 cell cycle arrest at G_1 and subsequent cell death. *A*: cell cycle analysis of S49 cells treated with Iso or cAMP. S49 cells were untreated (control) or treated with either 100 μM Iso or 1 mM cAMP for 24 h, treated with RNase, stained with propidium iodide, and then subjected to FACScan analysis as described in METHODS. The peaks representing cells at G_1 phase (1n DNA content) and G_2/M phase (2n DNA content) are indicated. M1 is the histogram marker used to define the subpopulation of cells in S and G_2/M phase. Con, control. *B*: viability of S49 cells treated with Iso or cAMP. S49 cells were untreated or treated with either 100 μM Iso or 1 mM cAMP for 12, 24, 36, 48, 60, and 72 h. Cell viability was determined by trypan blue exclusion as described in METHODS. *C*: Iso induces S49 cell death by apoptosis: microscopic appearance of S49 cells. S49 cells untreated or treated with 100 μM Iso for 48 h and subsequently stained with trypan blue as described in Fig. 1*B* and photographed.

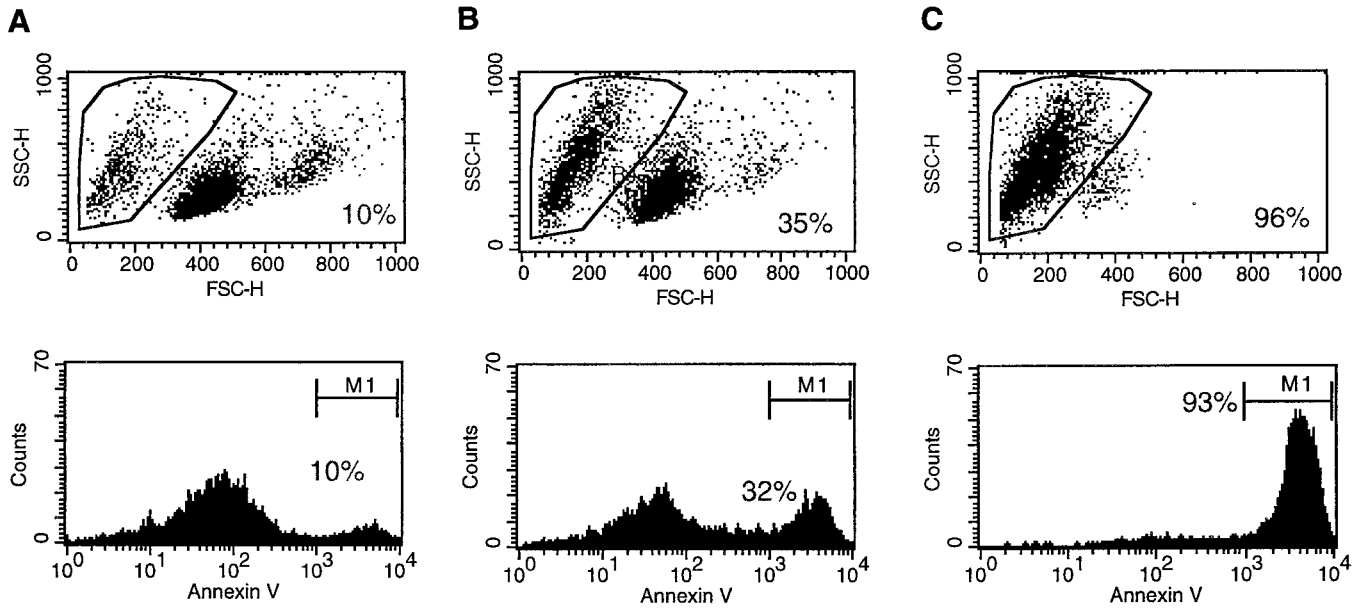


Fig. 2. Iso induces S49 cell death by apoptosis: annexin V staining. S49 wild-type (WT) cells untreated (*A*) or treated with 100 μM Iso for either 36 (*B*) or 60 h (*C*) were stained with FITC-labeled annexin V and then subjected to FACScan analysis. *Top*: plots of forward light scattering (FSC) vs. side light scattering (SSC). The gated areas represent viable cells based on cell size: 90%, 65%, and 4% of cells were viable in untreated cells, cells treated with Iso for 36 h, and cells treated with Iso for 60 h, respectively. *Bottom*: plots of annexin V staining. A total of 5,000 cells was analyzed for each sample. The gated areas represent annexin V-positive cells. The percentage of nonviable or annexin V-FITC-stained cells was estimated as indicated. R_1 is the polygonal region created for defining the subpopulation of dead cells.

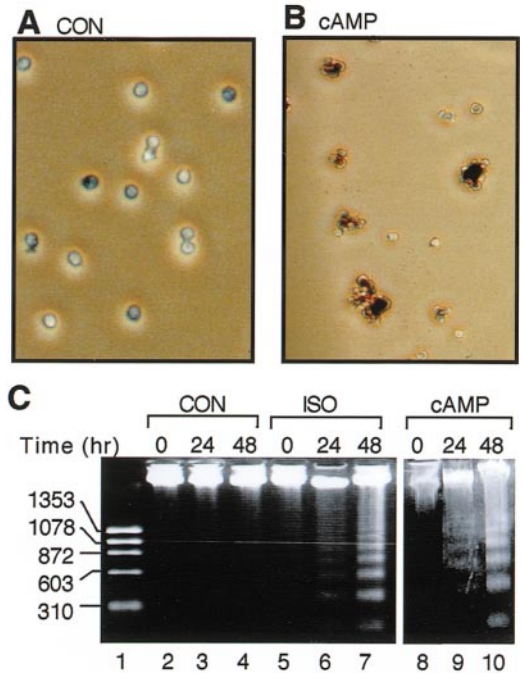


Fig. 3. Iso induces S49 cell death by apoptosis: DNA fragmentation. In situ end labeling (ISEL+) staining of control WT cells incubated without (*A*) and with 1 mM cAMP for 48 h (*B*). *C*: agarose gel electrophoresis of DNA isolated from WT S49 cells untreated or treated with 100 μM Iso or 1 mM cAMP for various amounts of time. The size of DNA molecular mass markers loaded in *lane 1* is indicated (*left*).

of the cells treated with Iso became ISEL+ positive. In parallel, we prepared genomic DNA from S49 cells untreated or treated with Iso or 8-BrcAMP and subjected it to agarose gel electrophoresis. As shown in Fig. 3*C*, genomic DNA was intact in the control (*lanes 2–4*), became slightly fragmented after 24-h treatment with Iso (*lane 6*), and became highly fragmented after 48-h treatment with Iso (*lane 7*). Similar results were observed for 8-BrcAMP (Fig. 3*C*, *lanes 8–10*). Together, the results showing that Iso- and 8-BrcAMP-induced cell death is accompanied by cell size reduction, translocation of phosphatidylserine to the outer leaflet of plasma membrane, and DNA fragmentation provide strong evidence that S49 cells undergo apoptosis upon βAR activation and increase in intracellular cAMP levels.

$G_s\alpha$ is required for Iso-induced S49 cell apoptosis. The observation that both βAR activation and increased intracellular cAMP levels lead to S49 cell apoptosis suggests that the activation of the signal cascade involving βAR, G_s protein, AC, and PKA is proapoptotic in T lymphocytes. To define the role of these various signaling components in S49 cell apoptosis, we used a series of mutant S49 cells with lesions in key signaling components.

We reasoned that *1*) direct activation of G_s should be sufficient to induce S49 cell apoptosis in a βAR-independent fashion, and *2*) $G_s\alpha$ should be required for Iso-mediated apoptosis. We first tested whether direct activation of G_s is sufficient to induce S49 cell apoptosis by using cholera toxin, which ADP ribosylates and

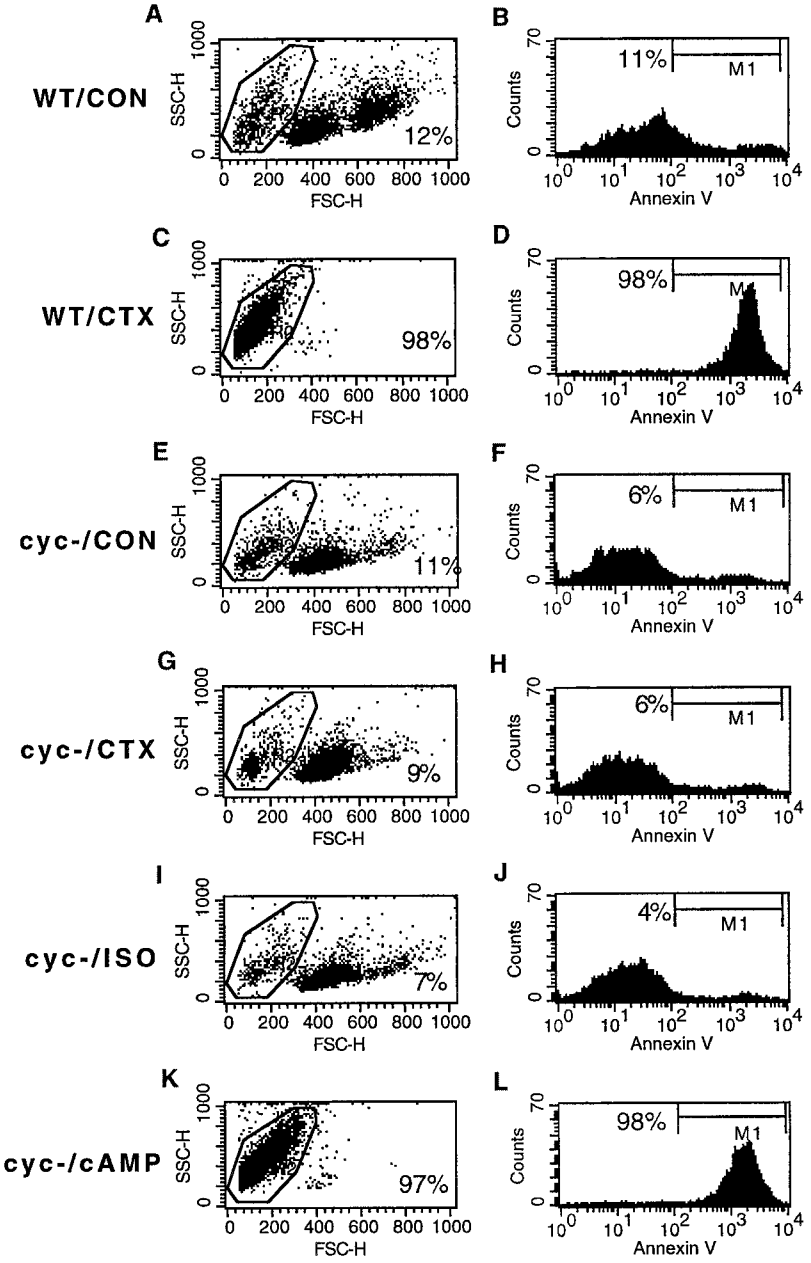


Fig. 4. $G_s\alpha$ is required for Iso-induced cell apoptosis. WT (*A–D*) and cyc$^-$ S49 cells (*E–L*) were untreated or treated with 100 μM Iso, cholera toxin (CTX) (200 ng/ml), or cAMP (1 mM) for 60 h. The cells were stained with annexin V-FITC and subjected to FACScan analysis as in Fig. 2. The percentage of nonviable cells based on cell shrinkage (*A, C, E, G, I* and *K*) and apoptotic cells based on annexin V staining (*B, D, F, H, J* and *L*) in each treatment was estimated as indicated.

irreversibly activates $G_s\alpha$. We assessed cell size and annexin V staining by FSC and counts, respectively, and found that, compared with control cells (Fig. 4, *A* and *B*), S49 cells treated with cholera toxin show evidence of cell death (Fig. 4, *C* and *D*). Furthermore, we found that cholera toxin did not induce death and apoptosis of cyc$^-$ cells (compare Fig. 4, *G* vs. *E* and *H* vs. *F*, respectively), a S49 mutant cell line that lacks $G_s\alpha$ (13, 33). The results with cholera toxin thus show that S49 cell apoptosis can occur by activation of G_s and requires functional $G_s\alpha$. cyc$^-$ S49 cells were also resistant to cell killing by Iso (Fig. 4, *I* and *J*) but remained susceptible to 8-BrcAMP-induced apoptosis (Fig. 4, *K* and *L*), indicating that the lack of Iso- and cholera toxin-induced apoptosis is due to the lack of $G_s\alpha$ and not to some other defect of the apoptotic machinery. Consistent with results from annexin V-FITC staining, no apparent genomic DNA fragmentation was observed in cyc$^-$ cells treated with Iso (Fig. 5, *lane 3* vs. *lane 4*). Thus activation of $G_s\alpha$ appears to be necessary and sufficient for βAR-induced apoptosis of S49 cells.

AC activation is sufficient to induce S49 cell apoptosis. In the above-mentioned studies, we showed that βAR activation as well as $G_s\alpha$ activation can induce S49 cell apoptosis. Given that $G_s\alpha$ activates AC, we tested whether direct activation of AC is sufficient to cause apoptosis of S49 cells by using the diterpene forskolin, an AC activator in these cells (10). We found that forskolin promoted apoptosis of S49 cells, as indicated by FSC (Fig. 6, *C* vs. *A*) and staining with annexin V-FITC (Fig. *6*, *D* vs. *B*). On the basis of these

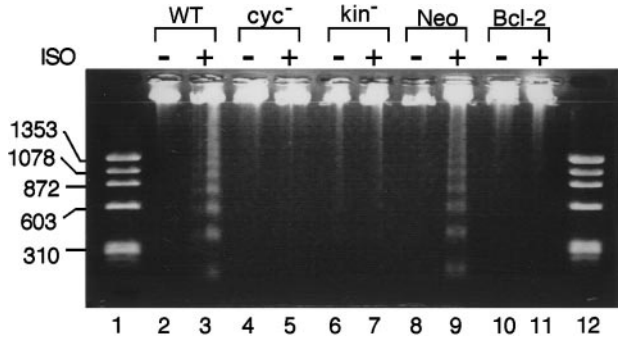


Fig. 5. Effect of Iso treatment on DNA integrity of various S49 cells. WT (*lanes 2* and *3*), cyc$^-$ (*lanes 4* and *5*), kin$^-$ (*lanes 6* and *7*), neo (*lanes 8* and *9*), and Bcl-2 (*lanes 10* and *11*) cells were untreated or treated with 100 μM Iso for 48 h. Genomic DNA was isolated from the cells, separated by electrophoresis on 2% agarose gels (20 μg/lane), stained with ethidium bromide (0.5 μg/ml), and photographed as in Fig. 3*C*. *Lanes 1* and *12*: DNA molecular mass markers.

results, we have concluded that activation of AC is a sufficient upstream signal to promote S49 cell apoptosis.

PKA is required for βAR, G_s, and AC activation-induced S49 cell apoptosis. Because activation of each of the signaling molecules tested thus far (βAR, G_s, and AC) increases cAMP concentration and promotes apoptosis, and because 8-BrcAMP was able to directly induce S49 cell apoptosis, we hypothesized that the proapoptotic effects were all mediated by activation of PKA. To test this hypothesis, we took advantage of the availability of kin$^-$ S49 cells, a mutant S49 cell lacking functional PKA activity (33). First, we determined whether activation of βAR by Iso induces apoptosis of kin$^-$ cells. Unlike results with WT S49 cells (Fig. 2), Iso-treated kin$^-$ cells showed no changes in cell size (Fig. 7, *C* vs. *A*) and were negative for annexin V-FITC staining (Fig. 7, *D* vs. *B*), implying that PKA is required for βAR activation-mediated apoptosis of S49 cells. Consistent with the cell size analysis and annexin V-FITC staining, genomic DNA fragmentation was not observed in kin$^-$ cells treated with Iso (Fig. 5, *lane 5* vs. *lane 6*). In additional studies we found that cholera toxin, forskolin, and 8-BrcAMP were all unable to promote apoptosis in kin$^-$ cells, as assessed by cell size analysis and annexin V-FITC staining (Fig. 7, *E–J*). These results indicate that PKA is required for the apoptotic effect of βAR activation, G_s activation, AC activation, and cAMP itself.

Bcl-2 blocks both Iso- and cAMP-induced S49 cell death. One of the principal mechanisms of apoptosis involves pro- and antiapoptotic proteins of the Bcl-2 family and their ability to alter mitochondrial efflux of cytochrome *c* and activation of caspases (as reviewed in Refs. 2 and 8). Because Bcl-2 is an antiapoptotic protein, we reasoned that overexpression of Bcl-2 might be protective of βAR/G_s/AC/cAMP-promoted apoptosis in these cells. S49 cells expressing the G418 resistance gene alone (neo cells) or with recombinant human Bcl-2 proteins (Bcl-2 cells) were untreated or treated with Iso, cholera toxin, or 8-BrcAMP. On the basis of FSC analysis, Iso treatment dramatically decreased the cell size of neo cells (Fig. 8, *M* vs. *K*) but resulted in only a slight decrease in the cell size of the Bcl-2 cells (Fig. 8, *C* vs. *A*). Similar results were found for Bcl-2 cells treated with cholera toxin (Fig. 8, *E* vs. *A*), forskolin (Fig. 8, *G* vs. *A*), or 8-BrcAMP (Fig. 8, *I* vs. *A*). The treated Bcl-2 cells showed an increase in annexin V-FITC staining (Fig. 8, *D*, *F*, *H*, and *J*, respectively, vs. *B*), but the increase was much less than that in the neo cells treated with Iso (Fig. 8, *N* vs. *L*). Unlike results with the neo cells, we also detected no genomic DNA fragmentation of Bcl-2 overexpressing cells treated with Iso (Fig. 5, *lanes 8–11*). These results indicate that Bcl-2 overexpression in S49 cells largely blocks the apoptotic effect of activation of βAR, G_s, and PKA.

DISCUSSION

The current studies have shown that the βAR agonist Iso and the second messenger cAMP promote the apoptotic death of S49 lymphoma cells, as evidenced by DNA fragmentation and changes in plasma membrane integrity. We have further demonstrated that this apoptosis is mediated by a signaling pathway involving βAR, G_s, AC, and PKA. Studies with cholera toxin (a G_s activator) and forskolin (an AC activator) demonstrated that these agents promote apoptosis of S49 cells. We also have shown that key downstream components are required for the apoptotic effect of particular upstream ones. Thus Iso induces apoptosis in WT, but not in cyc$^-$ or kin$^-$, S49 cells, which lack G_s and PKA, respectively; cholera toxin and forskolin induce apoptosis in WT S49 cells, but not in kin$^-$ cells. Furthermore, we have shown that the lack of an upstream component does not impair the apoptotic effect of a downstream one, in that cAMP is capable of inducing apoptosis in cyc$^-$ as well as WT S49 cells.

cAMP has been shown to promote apoptosis in several other cell types, including myeloid progenitor cells (22), leukemic cells (27, 32), ovarian cancer cells (30), granulosal cells (23), human B-precursor cells (27), and

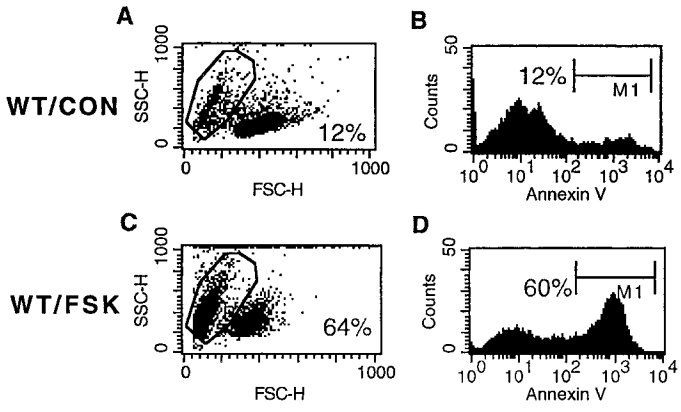


Fig. 6. Adenylyl cyclase activation is sufficient to induce S49 apoptosis. WT S49 cells were untreated or treated with 10 μM forskolin (Fsk) for 60 h. The cells were stained with annexin V-FITC and subjected to FACScan analysis as in Fig. 2. The percentage of nonviable cells based on FSC (*A* and *C*) and apoptotic cells based on annexin V staining (*B* and *D*) in each treatment was estimated as indicated.

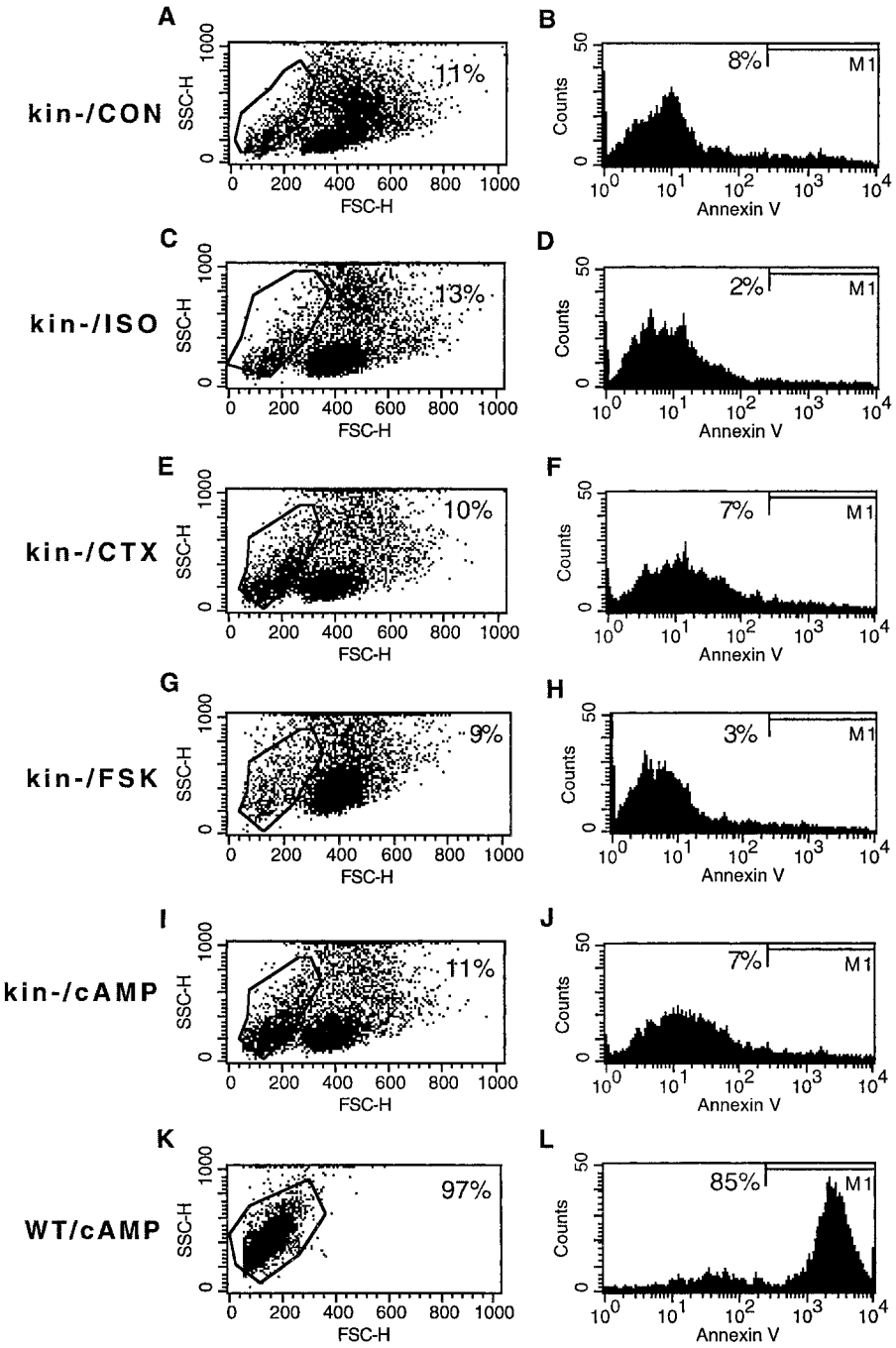


Fig. 7. Protein kinase A (PKA) is essential for S49 cell apoptosis induced by cAMP signaling cascade. S49 kin$^-$ cells (*A–J*) and WT cells (*K* and *L*) were untreated or treated with 100 μM Iso, CTX (200 ng/ml), 10 μM Fsk, or 1 mM cAMP for 60 h. The cells were stained with annexin V-FITC and subjected to FACScan analysis as in Fig. 2. The percentage of nonviable cells based on FSC (*A, C, E, G, I* and *K*) and apoptotic cells based on annexin V staining (*B, D, F, H, J* and *L*) in each treatment was estimated as indicated.

rat cardiac myocytes (18). Previous studies generally used inhibitors or related approaches to conclude that apoptosis in response to cAMP is mediated by PKA activation. Pharmacological inhibitors can be fraught with other problems [e.g., H89, a PKA inhibitor, has been recently shown to block βAR (28)], and thus definitive evidence for a role of PKA in apoptosis has been open to question. Our use of various S49 mutant cells lacking expression or function of distinct cAMP signaling components, including G_s and PKA, provides "genetic" evidence that the apoptotic effects, at least in S49 cells, are mediated largely, if not entirely, via PKA activation.

In contrast to the proapoptotic effects discussed above, cAMP also has been shown to be antiapoptotic in other systems such as murine macrophage-derived RAW 264.7 cells (14), mesencephalic dopaminergic neurons (25), HL-60 cells (20), and newborn rat retina (34). Thus the effect of cAMP/PKA activation on apoptosis is tissue and cell specific. The basis for such specificity is currently unknown. As a serine/threonine kinase, PKA promotes phosphorylation of a wide array of cellular proteins in S49 cells (31) as well as other cell types. A challenge for future research is to identify the substrates whose phosphorylation by PKA promotes or inhibits apoptosis. Our results showing that overex-

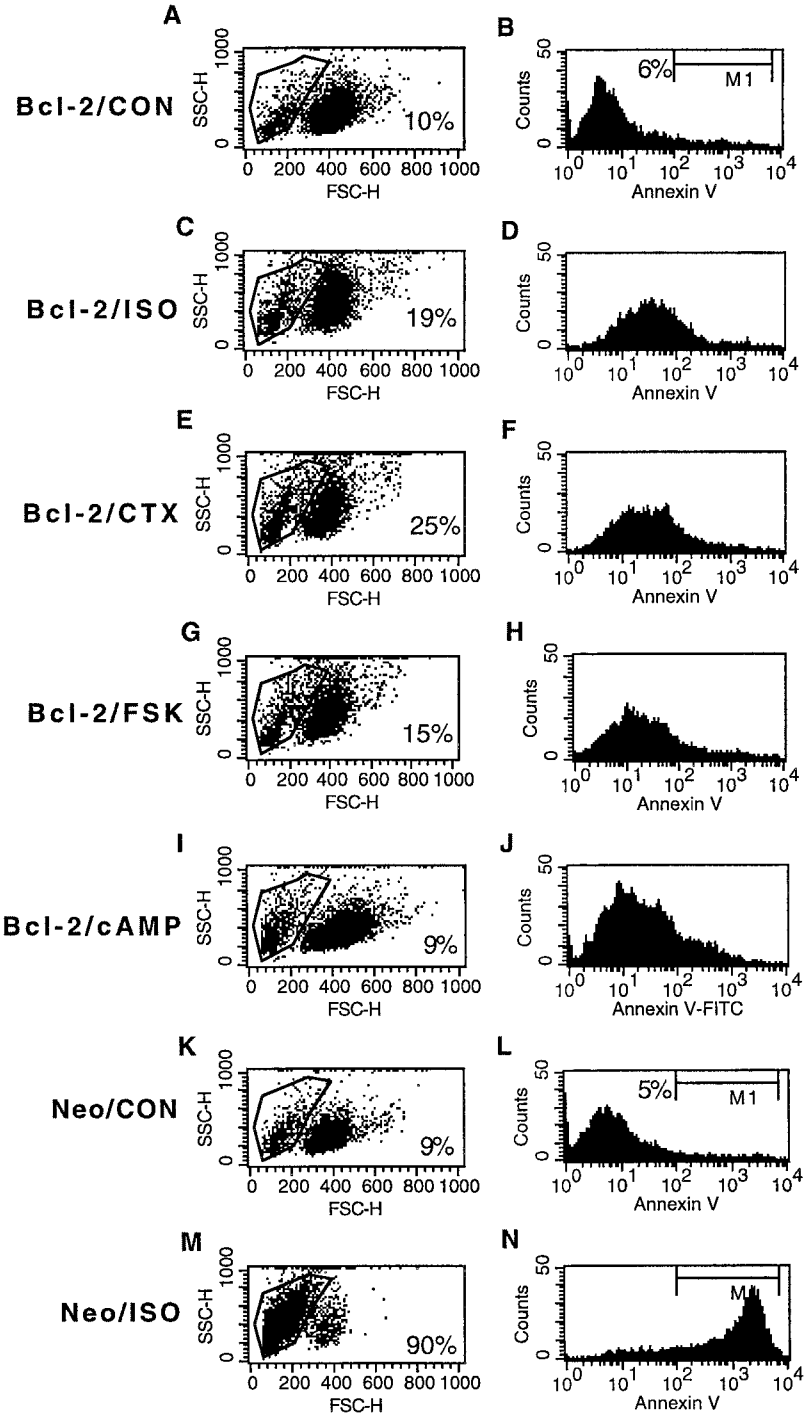


Fig. 8. Bcl-2 overexpression blocks cAMP signaling cascade-induced apoptosis. WT S49 cells were stably transfected with recombinant retroviruses carrying either a G418 resistance gene alone (neo) (*K–N*) or with this gene in combination with Bcl-2 cDNA (*A–J*). The neo (*K–N*) and Bcl-2 cells (*A–J*) were untreated or treated with 100 μM Iso, CTX (200 ng/ml), 10 μM Fsk, or 1 mM cAMP for 60 h. The cells were stained with annexin V-FITC and subjected to FACScan analysis as in Fig. 2. The percentage of nonviable cells based on FSC (*A, C, E, G, I, K* and *M*) and apoptotic cells based on annexin V staining (*B, D, F, H, J, L* and *N*) in each treatment was estimated as indicated.

pression of Bcl-2 prominently inhibits cAMP-induced apoptosis suggest that PKA may either directly or indirectly inhibit the function of Bcl-2, an antiapoptotic molecule, or perhaps other Bcl family members (2, 15). On the other hand, the fact that Bcl-2 overexpressing cells do not undergo apoptosis, but do show a somewhat enhanced expression of annexin V, suggests that PKA may act on other molecules in addition to Bcl-2 in initiating apoptosis. Future studies will need to be undertaken to determine interaction of PKA with Bcl-2 and other Bcl-2 family membranes in S49 cells. It is possible that PKA-mediated phosphorylation alters the function of Bcl-2 family members by affecting protein function, stability, or homo-/heterodimerization (2, 15, 27).

The current data on proapoptotic effects of agents that increase cAMP (in S49 cells) are reminiscent of previous data from examination of cell death in S49 cells by other stimuli. For example, glucocorticoids, cycloheximide, calcium ionophores, and Ca^{2+}-ATPase inhibitors can promote apoptosis in S49 cells, and overexpression of Bcl-2 is able to partially or fully block apoptosis by those agents (5, 7, 17, 29). Such results

suggest that different initiating stimuli share common components in promoting apoptosis of lymphoid cells.

The pro- and antiapoptotic effects of cAMP could have important therapeutic implications. Our data, as well as previous results, show that cAMP promotes the apoptosis of various lymphoid cells, including T-lymphoma cells, B-precursor cells, lymphoblastic leukemia cells, and myeloid progenitor cell lines (see e.g., 22, 27, 32). Such results suggest that increases in cAMP may have a role in the treatment of leukemia and lymphoma. Another setting is human immunovirus infection, in which increases in cAMP and PKA type 1 appear to contribute to immune suppression and apoptosis; this pathway has been proposed as a possible therapeutic target (1). An understanding of the detailed mechanisms by which cAMP regulates apoptosis should thus prove important in determining whether such actions of cAMP and PKA can be useful targets in the treatment of diseases in which one may wish to promote or prevent cAMP-mediated apoptosis.

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