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Type I cAMP-dependent Protein Kinase Delays Apoptosis in Human Neutrophils at a Site Upstream of Caspase-3*

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Current data suggest that apoptosis controls neutrophil numbers in tissues. We analyzed roles for and the sites of action for the cAMP-dependent protein kinases (cAPKs) in apoptosis induced in human neutrophils by *in vitro* storage, cycloheximide (CHX) exposure, and anti-Fas exposure. Treatment with 8-chlorophenylthio-cAMP (8-CPT-cAMP) prolonged the time required for 50% of the cells to exhibit apoptotic morphology (t_{50}) from 16.3 to 41.8 h (*in vitro* culture), from 2.4 to 7.8 h (CHX), and from 4.8 to 6.5 h (anti-Fas). CHX ± 8-CPT-cAMP did not significantly alter resting intracellular calcium levels and H-89, a selective inhibitor of cAPK, had no effect on apoptosis in the absence of the analogue. In contrast, site-selective cAMP analogues that specifically activated the type I cAPK, but not type II cAPK, synergistically attenuated apoptosis. Exposure to 8-CPT-cAMP delayed, in parallel, the activity of caspase-3 (CPP-32β), whereas mitogen-activated protein kinase kinase (MAPKK) inhibitor, PD98059, had no effect on CHX-induced apoptosis ± 8-CPT-cAMP. Together these results indicate that type I cAPK activation is necessary and sufficient to mediate cAMP-induced delay in human neutrophil apoptosis induced by several mechanisms and suggest that one of the major sites of cAPK action is upstream of caspase-3 (CPP-32β) activation.

Human neutrophils are short-lived cells that play important roles in both host defense and acute inflammation in humans (1, 2). Recent data suggest that apoptosis, a physiologic mechanism for cell death, regulates both production and survival of neutrophils, representing a basic biological mechanism for this cell type (3–6). Various inflammatory mediators are known to accelerate or delay apoptosis in neutrophils (7–9), but little about the intracellular signaling pathways that regulate this process (10) is understood. Recent evidence indicates that a family of cysteine proteases, the caspases, are critical components of apoptosis cascades, are primary determinants for the orderly disassembly of structural components, and incapacitate repair and homeostatic mechanisms in cells (10). Roles for caspase proteases in apoptosis have been analyzed in several cell types but have not been investigated in neutrophils.

Intracellular signaling mechanisms typically involve protein kinases as major regulators of cellular processes (11). The cAMP-dependent protein kinases (cAPK),[1] one of the best understood classes of protein kinases, are tetramers composed of two regulatory (R) and two catalytic (C) subunits and are functionally regulated by cAMP (12–14). Binding of cAMP to the R subunit releases the catalytically active C subunit from the R subunit, resulting in protein phosphorylations. This activates downstream signaling and transcription events, which likely account for recognized roles for cAMP in neutrophil chemotaxis (15), neutrophil respiratory burst activity (16), and more recently, apoptosis in neutrophils and other cell types (17–20). The cAPK exists as two major isoforms, type I and type II, with different R subunits, RI and RII subunits, respectively (12–14). The R subunits exhibit two different cAMP binding sites, designated sites A (or site 2) and B (or site 1), which interact with positive cooperativity for binding cyclic nucleotides, activating the C subunit and regulating physiological responses (14, 21). This positive cooperativity provides a physiological amplification mechanism for cAMP-mediated events that occur through cAPK and a means for distinguishing between activation of type I and type II cAPK by using cAMP analogues that bind differently to sites A and B on type I and type II cAPK.

In the following studies, we utilized three different models for apoptosis, *i.e. in vitro* storage, cycloheximide exposure, and anti-Fas exposure, to examine the roles for cAMP and cAPK and potential sites of action in this process. Apoptosis was quantified using morphologic changes and annexin V-FITC binding as markers. cAPK activity was manipulated by cAMP analogue exposure, inhibition using selective inhibitors, and synergistic activation with type-specific cAMP analogue pairs. Caspase substrates (22, 23) and inhibitors of MAPKK (24) were utilized to define sites of convergence between cAPK and apoptosis pathways. The data show that stimulation of cAPK activity delays apoptosis in all three model systems and that activation of type I cAPK is responsible for the observed effects by delaying the activation of the cysteine protease, caspase-3.

MATERIALS AND METHODS

Isolation of Neutrophils—Heparinized (~1 unit/ml) venous blood was obtained from healthy adult volunteers of either sex. Neutrophils were purified by sedimentation through a Hypaque-Ficoll step gradient, dex-

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[1] The abbreviations used are: cAPK, cAMP-dependent protein kinase; R, regulatory; C, catalytic; CHX, cycloheximide; t_{50}, time required for 50% of neutrophils to undergo morphologic apoptosis; CPP-32β, caspase-3; MAPKK, mitogen-activated protein kinase kinase; PD98059, MAPKK inhibitor; HBSSw, Hanks balanced salt solution with Ca^{2+} and Mg^{2+}; HBSSw/o, HBSS without Ca^{2+} or Mg^{2+}; 8-CPT-cAMP, 8-chlorophenylthio-cAMP; 8AHA-cAMP, 8-(6-aminohexyl)amino-cAMP; N6B-cAMP, N^6-benzoyl-cAMP; N6MB-cAMP, N^6-monobutyryl-cAMP; ICE, interleukin-1β-converting enzyme; AFC, 7-amino-4-trifluoromethyl coumarin; YVAD-AFC, tyrosine-valine-alanine-aspartate-AFC; DEVD-AFC, aspartate-glutamate-valine-aspartate-AFC; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; FITC, fluorescein isothiocyanate.

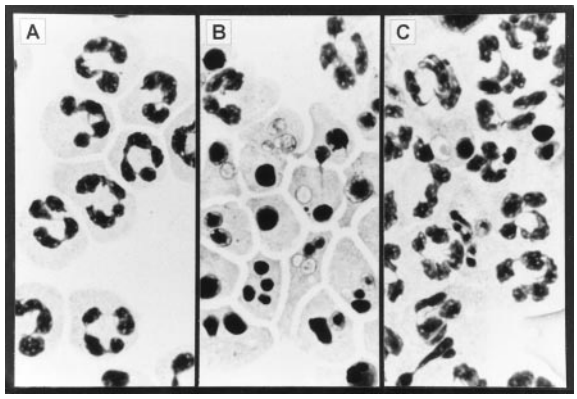

FIG. 1. **Photomicrographs of freshly purified human neutrophils, demonstrating the morphologic changes of apoptosis.** *A*, freshly purified neutrophils show segmented nuclei with lobes attached by chromatin threads, heterogeneous staining of nuclear chromatin, and pale cytoplasm. *B*, cycloheximide-exposed (6 h) human neutrophils exhibit separation or fragmentation of nuclear lobes, homogeneous nuclear chromatin staining, loss of chromatin from nuclear fragments, cellular fragmentation, shrinkage, and cytoplasmic basophilia. *C*, neutrophils exposed for 6 h to cycloheximide/8-CPT-cAMP (500 μM) show occasional cells with apoptotic morphology.

tran sedimentation, and hypotonic lysis as described previously (25). The resulting preparations were >95% neutrophils by morphology and greater than 98% viable (trypan blue exclusion).

For some experiments, purified neutrophils were resuspended in 50% autologous plasma that was harvested from above the Hypaque-Ficoll gradient after the initial purification step. This plasma was sedimented (500 × *g*, 10 min, 4 °C) to remove cellular elements and passed through a 0.45-μm filter to remove contaminating platelets before use.

Apoptosis Models—Three different methods for apoptosis induction were employed. For *in vitro* storage of neutrophils, 1 ml aliquots of 5×10^6 freshly purified cells (RPMI 1640 medium, 10% fetal bovine serum) were placed in 1.5 ml unsealed polypropylene microcentrifuge tubes and held at 37 °C, 5% humidified CO_2 for 0, 2, 4, 6, 8, 24, and 48 h. For cycloheximide exposure of neutrophils, 1 ml aliquots (5×10^6 cells in either RPMI 1640 medium, 10% fetal bovine serum, or 50% autologous plasma) were supplemented with 1 mM cycloheximide (CHX) and stored in unsealed 1.5-ml polypropylene microcentrifuge tubes at 37 °C, 5% humidified CO_2 for 0, 2, 4, 6, and 8 h. CHX-exposed cells in autologous plasma were used in initial studies of cAMP effects and in cAMP analogue synergism studies. CHX-exposed cells in RPMI 1640, 10% fetal bovine serum were used in experiments to estimate t_{50} values. A high concentration of CHX (1 mM) was used to shorten the time for maximal apoptosis to 6–8 h. No necrosis was observed as determined by trypan blue staining. For anti-Fas exposure of neutrophils, 1-ml aliquots containing 5×10^6 cells in RPMI 1640, 10% fetal bovine serum were supplemented with 100 or 500 ng/ml murine monoclonal IgM anti-Fas (26) (CH-11, MBL, Nagoya, Japan) and stored at 37 °C, 5% humidified CO_2 for 0, 2, 4, 6 and 8 h.

Assessment of Apoptosis—Two methods were used for quantitative assessment of neutrophil apoptosis. Morphologic apoptosis was estimated as follows. 50-μl aliquots of cells from each apoptosis model were diluted 1:3 with Hanks' balanced salt solution with Ca^{2+} and Mg^{2+} (HBSSw), and 75 μl of cell suspension was applied to a glass slide using a Cytospin III (Shandon Southern; Sewickley, PA). After air drying, applied cells were stained with modified Wright-Giemsa stain (Diff-Quik; Baxter Scientific, Miami, FL), dried, and examined microscopically. A minimum of 200 cells/slide were examined and graded as apoptotic/non-apoptotic. In human neutrophils, morphologic changes of apoptosis are striking (Fig. 1) and include separation of nuclear lobes, replacement of heterogeneous nuclear chromatin staining with dense homogeneous nuclear chromatin staining, loss of nuclear chromatin, cellular and nuclear fragmentation, and basophilic cytoplasmic staining. For these studies, the morphologic criteria for neutrophil apoptosis were 1) one or more densely stained nuclear fragments/cell, 2) absence of chromatin within nuclear fragments, and/or 3) a cellular fragment containing a densely stained nuclear fragment.

Annexin V-FITC binding was used as the second quantitative apoptosis marker. Annexin V exhibits calcium-dependent binding to phosphatidylserine (27). Although phosphatidylserine is typically restricted to the inner leaflet of the cell membrane in normal cells and is therefore inaccessible to annexin V in solution, apoptotic cells express phosphatidylserine in their outer membrane leaflet (27), resulting in ready binding of annexin V to their surfaces. Annexin V-FITC labeling was performed as recommended by the manufacturer (Alexis Corp.; San Diego, CA). Neutrophils (5×10^5) from each experimental condition were sedimented, resuspended in 100 μl of binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) containing 1 mg/ml annexin V-FITC and held at room temperature for 10 min in the dark. Labeled cells were washed once in the same buffer, and a wet preparation of cells was examined with an Olympus BH-2 epifluorescence photomicroscope. After minimizing background phase-contrast illumination to allow identification of all cells in a field, epifluorescence illumination was added, and a minimum of 350 cells were examined on each slide and graded as annexin V positive (bright green-yellow fluorescence) or negative (weak or no fluorescence).

Chromatin fragmentation was examined as a qualitative marker for apoptosis in CHX-exposed neutrophils only (28). Aliquots (2.5×10^6) of neutrophils from different conditions were resuspended in 400 μl of lysis buffer (0.2% Triton X-100, 10 mM Tris, 1 mM EDTA) and after vigorous mixing, were allowed to sit on ice for 20 min. After the addition of 5 M NaCl (100 μl) and isopropanol (500 μl), samples were held at −20 °C for 60 min and sedimented at 14,000 rpm for 10 min, and the pellet was retrieved and dried. After resuspension of the pellet in 20 μl of TBE (90 mM Tris-borate, 25 mM disodium EDTA, pH 8.3), 4 μl of 30% Ficoll were added, and the sample was boiled for 5 min. Electrophoresis was carried out in a 1.5% agarose gel at 30 V for 18–20 h. After electrophoresis, the agarose gel was stained with ethidium bromide and photographed under ultraviolet illumination.

T_{50} Determinations—The time required for 50% of neutrophils to undergo morphologic apoptosis (t_{50}) was determined by plotting the percentage of apoptotic forms observed at each time point and defining the time at which 50% of the cells exhibited apoptotic forms by linear regression of data points as indicated in the legend to Fig. 2. The t_{50} value for *in vitro* storage was determined by linear regression between the 24 and 48 h time point and represents an estimation. Unless otherwise noted, all t_{50} determinations are based on the observation of cells suspended in RPMI 1640 medium, 10% fetal bovine serum, with appropriate additions.

Cyclic AMP Analogue and PD98059 Exposures—To examine the effects of cAMP on apoptosis induction, exposure to 8-CPT-cAMP, a membrane-permeant, relatively phosphodiesterase-resistant cAMP analogue, was used alone at varying concentrations and for varying durations. To determine if MAPKK was involved in the cAMP effect, incubations were carried out in the presence or absence of 100 μM PD98059 (Calbiochem), a mitogen-activated protein kinase kinase (MAPKK)-specific inhibitor (24). Neutrophils were exposed to 8-CPT-cAMP for 10 min before apoptotic stimuli were added, and remained exposed to 8-CPT-cAMP throughout these experiments. When PD98059 was present, preincubation was for 1.5 h, and the inhibitor remained throughout the experiment.

Cyclic AMP analogue synergism experiments utilized pairs of different cAMP analogues to determine which cAPK isotype (14, 21, 29) was responsible for effects on apoptosis induction. Cyclic AMP analogues bind selectively to the two cAMP binding sites (site A and site B) on the R subunit of cAPK and can mimic the positively cooperative binding of cAMP that normally occurs at these sites and leads to activation of cAPK (14, 21, 29, 30). This positive cooperativity of binding is reflected in a positive cooperativity of cAPK activation and cAPK-mediated cellular responses. Depending on their structures, cAMP analogues at low concentrations selectively bind to either site A or site B. By using specific pairs of cAMP analogues (one that binds to site A only and the other that binds to site B only), it is possible to mimic the cooperative binding of cAMP and initiate cAPK-specific cell responses that, in the presence of both analogues, are greater than the sum of the responses to the individual analogues alone. Low concentrations of analogues are required to maintain the selectivity of binding at the respective sites. High concentrations of a single analogue result in binding to both sites A and B and the potential for cooperative binding to R and activation of cAPK, and synergism of cell responses by two separate analogues are lost. Furthermore, it is possible to use specific cAMP analogue pairs that bind differently to sites A and B on the type I *versus* the type II cAPK to differentiate responses that are mediated by each cAPK isotype. Consequently, specific pairs of analogues were used to determine the involvement of cAPK type I or type II in cAMP-mediated effects.

Neutrophils stored *in vitro*, exposed to CHX, or exposed to anti-Fas were combined with varying concentrations of appropriate site-selective/cAPK isotype-selective cAMP analogues as follows. The 8-CPT-

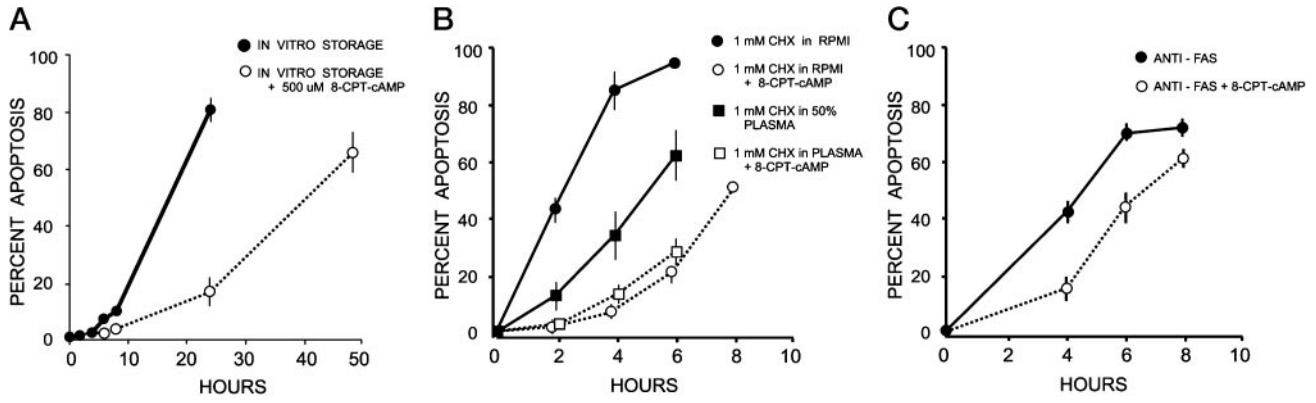

FIG. 2. **Effects of time and 8-CPT-cAMP on morphologic apoptosis in human neutrophils stored *in vitro* (*A*), exposed to 1 mM cycloheximide (*B*), and exposed to 500 ng/ml murine monoclonal anti-Fas (*C*).** 8-CPT-cAMP (500 μM) was added to cells 10 min before the initiation of *in vitro* culture, CHX, or anti-Fas exposure. The cAMP analogue remained throughout the experiment. Data shown are mean ± S.E. for 4–6 experiments.

cAMP/8AHA-cAMP pair (8CPT/8AHA) and the N6B-cAMP/8AHA pair (N6B/8AHA) were used as type I cAPK-specific pairs. The 8-CPT-cAMP/N6B-cAMP pair (8CPT/N6B) and the 8-CPT-cAMP/N6MB-cAMP pair (8CPT/N6MB) were used as type II cAPK-specific pairs as described previously (14, 21, 29, 30). In all experiments, the priming analogue concentration was adjusted to achieve 5–10% maximum effect, and the concentration of the other analogue in the pair was varied. The effects of the priming analogue A alone and the second analogue B alone were arithmetically summed to estimate the predicted effect of the A + B mixture. This predicted response was compared with the experimentally observed response to the "A + B" mixture. If synergy was present, the observed A + B response was greater than the predicted A + B response. If no synergy was present, the observed A + B response and the predicted A + B response were equal.

Measurements of the Intracellular Free Calcium Concentration—Purified neutrophils were adjusted to 5×10^6/ml in Hanks' balanced salt solution without Ca^{2+} or Mg^{2+} (HBSSw/o) and loaded with 2.5 μM Fura2AM (Molecular Probes; Eugene, OR) for 45 min, 37 °C, 5% CO_2 in dark. After loading, cells were sedimented, washed twice in 15-ml volumes of 4 °C HBSSw/o, readjusted to 10^7 cells/ml in 50% autologous plasma ± 1 mM CHX, ± 500 μM 8-CPT-cAMP in aliquots, and incubated at 37 °C, 5% CO_2 in the dark for the times specified. After incubation, cells were sedimented, resuspended in HBSSw at 6×10^6/ml, and examined in an LS50B spectrofluorometer (Perkin-Elmer) with excitation wavelengths of 340 and 380 nm and emission wavelength of 510 nm.

In the spectrofluorometer, the Fura2 fluorescence of each cell aliquot was measured with cells alone, cells + 0.1% Triton X-100, and cells + 0.1% Triton X-100 + 20 mM EGTA to measure basal cell signal, maximum ratio of Fura2 fluorescence, and minimum ratio of Fura2 fluorescence, respectively. The recorded fluorescence levels were then used to calculate the resting intracellular calcium concentration as described (33). The fluorescence intensity of loaded cells was noted to decrease progressively over the 210 min of incubation in 50% plasma (fresh, 67 ± 4 fluorescence units, $n = 5$; 210 min, 18 ± 2 fluorescence units, $n = 5$), which precluded loading cells at time 0 and following intracellular calcium levels over time. Therefore, cells were first incubated for 4 h then loaded with Fura2AM and examined for their intracellular free calcium levels.

cAPK Inhibition Experiments—The selective cAPK inhibitor H-89 (Sigma) was prepared as a 10 mM stock in water and used at final concentrations of 5 and 25 μM.

Quantitation of Interleukin-1β-converting Enzyme (ICE)-specific and CPP-32β-specific Activity—Neutrophils (5×10^6) were lysed in 100 μl of 10 mM potassium phosphate, 1 mM EDTA buffer containing 0.5% Triton X-100 supplemented with 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 mM dithiothreitol for 15 min and spun at 14,000 rpm for 20 min. The lysate (100 μg) was diluted to 1 ml with ICE buffer (50 mM HEPES, 10% sucrose, 0.1% Chaps, pH 7.5) containing 50 μM YVAD-AFC (tyrosine-valine-alanine-aspartate-7-amino-4-trifluoromethyl coumarin) or DEVD-AFC (aspartate-glutamate-valine-aspartate-AFC) (22, 23) and 10 mM freshly prepared dithiothreitol. After various incubation times at 37 °C, 500 μl of reaction mixture was diluted with 1.5 ml of ICE buffer and fluorescence (excitation 400 nm and emission 505 nm) was read at 1- and 2-h time points. The release of the fluorochrome was linear with time and protein concentration used. Blanks in the absence of cell lysates were also carried out to determine background fluorescence. Standards containing 0–1500 nM AFC were utilized to determine the amount of fluorochrome released. Specificity for caspase-3 activity, as opposed to nonspecific hydrolysis of DEVD-AFC was demonstrated by the inhibitor DEVD-CHO (DEVD aldehyde). Very low levels of nonspecific activity were present, and the effects of the inhibitor were concentration-dependent.

Statistical Analysis—Results are reported as mean ± S.E. values, with the number of different experiments indicated as the value for n. Summary means are compared by Student's t test, and when natural pairing occurs within an experiment, the paired sample t test is used. Statistical significance is declared at $p < 0.05$.

RESULTS

The Effects of 8-CPT-cAMP on Apoptosis Induction in Three Different Models Using Three Different Indicators of Apoptosis—The results shown in Table I compare two quantitative markers of apoptosis, morphology and annexin V-FITC binding, in three different models of apoptosis. *In vitro* culture, CHX exposure, and anti-Fas exposure induced extensive apoptosis in all three models, as indicated by both markers. At the indicated time points, both markers demonstrated that 500 μM 8-CPT-cAMP inhibited apoptosis in all three models. Annexin V-FITC was a more sensitive marker for the effects of cAMP than morphology, especially for the CHX model. This might be expected for an early marker of apoptosis such as annexin V-FITC *versus* a later marker of apoptosis such as morphology (31).

To demonstrate the temporal characteristics of apoptosis for each model in the presence and absence of cAMP, Fig. 2 shows time courses for apoptosis in the presence and absence of 8-CPT-cAMP in all three models using morphology as the apoptosis marker. Table II indicates the time required for 50% of the cells to exhibit apoptotic morphology (t_{50}) as determined from the time courses in Fig. 2. Compared with *in vitro* culture alone, induction of apoptosis by CHX and anti-Fas binding in the absence of cAMP analogue decreased the t_{50} values by 6.8- and 3.4-fold, respectively. In the presence of 500 μM 8-CPT-cAMP, apoptosis was delayed in all three models in a time-dependent (Fig. 2, *A–C*) and a concentration-dependent (not shown) manner. Cyclic AMP analogue treatment increased the t_{50} values (Table II) 2.6-, 3.3-, and 1.4-fold for *in vitro* culture, CHX-exposure, and anti-Fas-exposure, respectively. When DNA fragmentation was used as a qualitative marker of apoptosis, the intensity of DNA laddering in the presence of cAMP analogue was less at all time points tested in the CHX model (Fig. 3). Because of the qualitative nature of this indicator, DNA fragmentation was not tested in the other models.

The time courses for apoptosis suggest that the cAMP-mediated delay in apoptosis occurs in the initial phases of the

TABLE I

Two quantitative markers demonstrate that 8-CPT-cAMP inhibits apoptosis induced by different stimuli

Isolated neutrophils were incubated in the presence or absence of 8-CPT-cAMP, and apoptosis was induced by *in vitro* culture, CHX exposure, or anti-Fas exposure. At the indicated times, cells were analyzed for apoptotic morphology and annexin V-FITC binding as described under "Materials and Methods." The numbers represent the mean and S.E. of the percentage of cells exhibiting the indicated markers from six experiments. Statistical significances of the effect of 8-CPT-cAMP are indicated.

Conditions	Morphology	Annexin V-FITC
	% of apoptosis	*% of apoptosis*
In vitro culture (24 h)	75.5 ± 6	81.4 ± 4.2
In vitro culture + 500 μM 8CPT-cAMP (24 h)	14.5 ± 2[a]	17.6 ± 5[a]
CHX, 1 mM (6 h)	93.9 ± 1.7	94.3 ± 1.7
CHX + 500 μM 8CPT-cAMP (6 h)	24 ± 5.6[a]	42.3 ± 1.7[a]
Anti-Fas 500 ng/ml (6 h)	60.5 ± 4.4	71.2 ± 2.8
Anti-Fas + 500 μM 8CPT-cAMP (6 h)	33.5 ± 4.8[b]	45.6 ± 5.3[b]

[a] $p < 0.001$.
[b] $p < 0.01$.

TABLE II

T_{50} values for the appearance of apoptotic morphology in the presence and absence of 8-CPT-cAMP for three different apoptosis models

Apoptosis was induced by *in vitro* culture, CHX exposure (1 mM) and anti-Fas exposure (500 ng/ml) in the presence and absence of 8-CPT-cAMP (500 μM). At various times, cells were analyzed for apoptotic morphology as described under "Materials and Methods." The T_{50} values indicate the mean and S.E. ($n = 5$) of the time required for 50% of the cells to exhibit apoptotic morphology. The statistical significance of the effect of 8-CPT-cAMP is indicated.

Conditions	T_{50} based on morphology	
	No addition	500 μM 8CPT-cAMP
In vitro culture	16.3 ± 1.3	41.8 ± 2.9[a]
CHX-1 mM	2.4 ± 0.2	7.8 ± 0.3[a]
Anti-Fas 500 ng/ml	4.8 ± 0.5	6.5 ± 0.3[b]

[a] $p < 0.0001$.
[b] $p < 0.05$.

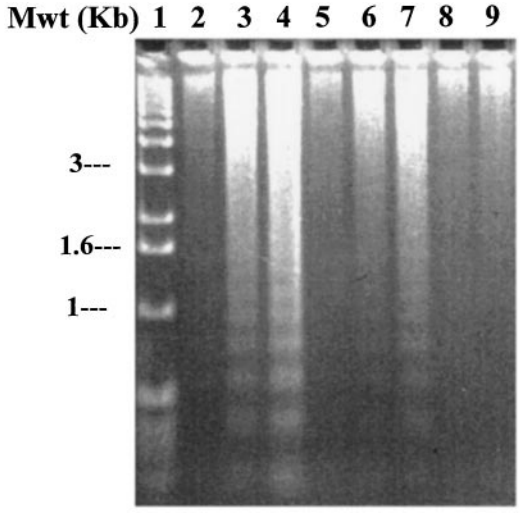

FIG. 3. **Chromatin fragmentation in human neutrophils exposed to 1 mM cycloheximide.** Agarose gel electrophoresis patterns observed with molecular size markers (*Mwt*) (*lane 1*), neutrophils exposed to 1 mM CHX for 2 h (*lane 2*), 4 h (*lane 3*), and 6 h (*lane 4*), neutrophils exposed to 1 mM CHX + 500 μM 8-CPT-cAMP for 2 h (*lane 5*), 4 h (*lane 6*), and 6 h (*lane 7*), control neutrophils at 6 h (*lane 8*), and control neutrophils + 500 μM 8-CPT-cAMP at 6 h (*lane 9*).

process (Fig. 2). In the presence of 8-CPT-cAMP, a decreased rate of apoptosis is evident at early phases of the time course, but later, the rates of apoptosis are similar in cAMP analogue-treated and -untreated cells. It could be argued that this is due to a decrease in the concentration of the analogue due to hydrolysis during the incubation. However, this seems unlikely because 8-CPT-cAMP is relatively resistant to cAMP phosphodiesterases (32), and the same effect is observed in the *in vitro* culture model, which is incubated for extended periods of time.

In the CHX model, neutrophils were incubated in RPMI or autologous plasma with or without 8-CPT-cAMP (Fig. 2*B*). In the absence of cAMP analogue, apoptosis was greater in the presence RPMI than in the presence of autologous plasma. The presence or absence of autologous plasma had no effect on the cAMP-mediated delay in apoptosis. Fas-induced apoptosis was not observed in the presence of autologous plasma (not shown). These results suggest that autologous plasma may contain anti-apoptotic factors. No further studies were carried out to define these putative factors.

Effects of Basal cAPK Inhibition on Spontaneous Neutrophil Apoptosis—The inhibitory effects of cAMP analogue exposure on morphologic apoptosis and annexin V-FITC binding in all apoptosis models suggested a role for the cAPK using both markers. To examine whether basal (unstimulated) cAPK activity suppressed morphologic apoptosis in neutrophils, H-89, a selective inhibitor of cAPK (33), was used. In the absence of cAMP analogues, H-89 exposure had no significant effect on the percentage of apoptotic forms after 8 h of *in vitro* cultivation (control, 15.3% ± 2.3; 25 μM H-89, 9 ± 1%), 6 h of CHX exposure (control, 74.8 ± 2.5; 25 μM H-89, 66 ± 5.0%), or 6 h of anti-Fas exposure (control, 73.7 ± 5.4; 25 μM H-89, 70.1 ± 9.9). These results suggest that basal cAPK activity does not play a role in suppressing neutrophil apoptosis.

The Effect of CHX Exposure on Intracellular Calcium Concentrations in the Presence and Absence of 8-CPT-cAMP—Because changes in intracellular free calcium concentrations are reported to influence apoptosis in neutrophils (34), the effects of CHX and 8-CPT-cAMP exposure on Fura2-based estimates of intracellular calcium concentrations were examined. Intracellular free calcium levels in control cells rose spontaneously from 30 ± 4 nM at 5 min to 65 ± 12 nM at 210 min ($n = 5$), and these were not affected by 1 mM CHX exposure (27 ± 2 nM and 81 ± 22 nM respectively, $n = 5$), 500 μM 8-CPT-cAMP exposure (33 ± 2 nM and 56 ± 11 nM, respectively, $n = 5$), or CHX + 8-CPT-cAMP (29 ± 2 nM and 62 ± 12 nM, respectively, $n = 5$). Because no effect on intracellular free calcium concentration was observed in the CHX apoptosis model, this approach was not applied to the *in vitro* storage and anti-Fas exposure models.

Determination of the cAPK Isoform Mediating the cAMP Analogue Effects—Exposure to cAMP analogues results in stimulated rather than basal cAPK activity. Therefore, the lack of effects from H-89 exposure did not eliminate a role for stimulated cAPK in suppression of neutrophil apoptosis. To determine if cAPK activation played a role and to determine which cAPK isotype was responsible for the observed cAMP analogue effects, pairs of site-selective cAMP analogues specific for either the type I or type II cAPKs were used in each model system (see "Materials and Methods").

A series of cAMP analogue concentration-response experiments were first carried out to define analogue concentrations that delayed relatively low levels of apoptosis, which is required by the method (21) and to determine the order of potency of the analogues. As expected, all cAMP analogues tested effectively delayed the expression of apoptosis using morphology. The order of potency for the analogues was 8-CPT-cAMP > N6B-cAMP = N6MB-cAMP > 8-AHA-cAMP.

Table III illustrates experiments for each of the three models

TABLE III
Site-selective cAMP analogue pairs specific for type I, but not type II, demonstrate synergism for delay in apoptosis induced by in vitro culture

Isolated human neutrophils were maintained in culture for 24 h (A), incubated in the presence of 1 mM CHX for 6 h (B), incubated in the presence of anti-Fas (500 ng/ml) for 4 h (C), in the presence and absence of various site-selective cAMP analogues alone (A or B) or in pairs (A + B) at the indicated concentrations as described under "Material and Methods." Pairs of cAMP analogues that are specific for type I are 8-CPT-cAMP (site A-selective) and 8-AHA-cAMP (site B-selective). Pairs of cAMP analogues that are specific for type II are 8-CPT-cAMP (site B-selective) and N6B-cAMP (site A-selective). The values are the mean and S.E. ($n = 4$) of the differences in the percentage of cells with apoptotic morphology in the presence of the cAMP analogue *versus* control. The effects of each analogue alone are indicated in the column headed "A or B." The columns headed "Predicted (A + B)" indicate the values that are predicted for the effects in the presence of two cAMP analogues if the effects were additive. The column headed "Observed (A + B)" indicates the effects observed when the analogues are incubated together. Type I-specific pairs exhibited synergism indicated by "Observed (A + B)" effects greater than "Predicted (A + B)" effects. Type II-specific pairs exhibited additive effect or the absence of synergism as indicated by "Observed (A + B)" effects approximately equal to "Predicted (A + B)" effects. * indicates $p < 0.05$ by paired t test.

Analogue A	Analogue B	Difference in percent apoptosis *versus* control		
		A or B	Predicted (A + B)	Observed (A + B)
μM	μM			
A				
Type I pair				
8CPT	8AHA			
25		6.3 ± 4		
	667	8.5 ± 5.5	14.3 ± 5.9	28.5 ± 2.0*
	333	0.5 ± 1.3	6.8 ± 3.3	22.1 ± 2.5*
Type II pair				
8CPT	N6B			
25		6.3 ± 4		
	333	38.7 ± 3.8	45.0 ± 5.8	38.8 ± 4.7
	100	26.8 ± 3.2	33.1 ± 3.1	31.4 ± 2.3
B				
Type I pair				
8CPT	8AHA			
75		3.7 ± 3.2		
	1000	14.4 ± 2.1	18.1 ± 5.0	37.0 ± 4.0*
	667	9.3 ± 2.3	12.9 ± 4.5	29.8 ± 3.9*
	333	7.5 ± 3.7	11.2 ± 4.9	21.1 ± 4.5
Type II pair				
8CPT	N6B			
75		3.7 ± 3.2		
	667	59.9 ± 8.0	63.6 ± 10.0	50.9 ± 7.5
	333	24.3 ± 3.0	30.0 ± 3.9	38.0 ± 4.3
	100	14.4 ± 2.0	18.0 ± 4.3	13.8 ± 2.5
C				
Type I pair				
8CPT	8AHA			
100		0.9 ± 0.4		
	1000	0.2 ± 0.2	0.7 ± 0.4	11.2 ± 3.2*
	667	3.3 ± 2.6	2.9 ± 2.9	12.3 ± 2*
Type II pair				
8CPT	N6MB			
100		0.9 ± 0.4		
	667	10.2 ± 3.7	8.6 ± 1.8	10.6 ± 2.3
	333	15 ± 3.8	13.9 ± 2.8	14.3 ± 3.7

of apoptosis that utilize pairs of site-selective cAMP analogues that are specific for the cooperative activation of either type I or type II cAPK. In all three models, the observed (A + B) effects of the combination of all type I cAMP analogue pairs was significantly greater than the predicted (A + B) effects. This indicates that the combination of two type I, site-selective analogues cooperatively activates the type I cAPK, which results in a synergistic delay of apoptosis. In contrast, for all type II analogue pairs, the observed (A + B) effects of the combination of cAMP analogues was similar to the predicted (A + B) effects. This indicates that cooperative activation of the type II cAPK by the two type II site-selective analogues does not delay apoptosis. In all three models of apoptosis induction and in all experiments, synergism for the cAMP-induced delay of apoptosis was present when type I-specific cAMP analogues were used but not when type II-specific cAMP analogues were used. These data suggest that stimulation of the type I cAPK underlies cAMP analogue-mediated suppression of apoptosis. This is consistent with the finding that type I cAPK is the predominant (80–85%) isoform in human peripheral neutrophils as determined by DEAE-Sepharose chromatography (data not shown).

Determination of the Site of cAPK in the Delay of Apoptosis—To determine the site(s) for cAPK-mediated delay in apoptosis, we examined whether activation of cAPK by 8-CPT-cAMP in intact neutrophils affected caspase-3 (CPP-32β) or caspase-1 (ICE) activity *in vitro* or affected apoptotic morphology in the presence of MAPKK inhibition in intact neutrophils.

To determine if cAPK acted upstream or downstream of caspase-3 (CPP-32β), we incubated the fluorochrome peptide DEVD-AFC, a caspase-3 (CPP-32β)-specific substrate (22, 23), with extracts from cells that were induced to undergo apoptosis for various times with CHX (Fig. 4*A*) in the presence and absence of 8-CPT-cAMP. Extracts from cells incubated for 2, 4, or 6 h in the absence of CHX and 8-CPT-cAMP exhibited very low levels of fluorescence, like that indicated at time 0 in Fig. 4. In the presence of CHX alone, caspase-3 activity increased between 0 and 4 h in a time-dependent manner and then decreased between 4 and 6 h. This decrease in caspase activity might be expected in a cell population when 80–100% of the cells exhibit apoptotic morphology (see Fig. 2). In the presence of 8-CPT-cAMP, there was a decrease in caspase-3 activity compared with CHX exposure alone at the 2- and the 4-h time point, and caspase-3 activity continued to increase. However, after 6 h in the presence of cAMP analogue, caspase activity did not reach the maximum seen in the absence of cAMP analogue. Consequently, in the presence of 8-CPT-cAMP, the time course for caspase-3 activity was shifted to the right. The delay in the specific caspase activity was parallel to the delay in the appearance of apoptotic morphology (Fig. 2) and annexin V-FITC binding (not shown) when cAPK was activated in intact neutrophils in the CHX model. Results shown in Fig. 4*B* compare the effects of 8-CPT-cAMP at single time points on caspase-mediated proteolysis of DEVD-AFC in apoptosis induced by *in vitro* culture, CHX-exposure, and anti-Fas exposure. In each model, the activity of caspase-3 was decreased significantly in the presence of the cAMP analogue. These results suggest that cAPK delays apoptosis by acting at a site upstream of caspase-3 in all three models. This suggests that a site or sites is common to all three mechanisms of apoptosis tested in these studies.

In experiments parallel to those shown in Fig. 4, extracts from cells previously incubated in the presence and absence of 8-CPT-cAMP were incubated with a caspase-1 (ICE)-specific fluorochrome substrate, YVAD-AFC (not shown). No caspase-1 (ICE) activity was observed in the presence or absence of cAMP analogue. In agreement with the results of Martins *et al.* (23), these experiments suggest that caspase-1 activity is not required for apoptosis and that cAPK has no effect on caspase-1 activity in any of the three models tested here.

To determine a potential role for MAPKK in apoptosis, isolated human neutrophils were preincubated for 1.5 h with or without 100 μM PD98059 (24) and exposed to CHX in the presence or absence of 500 μM 8-CPT-cAMP (not shown). In the absence or presence of CHX, PD98059 had no effect on neutrophil apoptotic morphology after 2, 4, or 6 h of incubation. As shown in Fig. 2*B*, 8-CPT-cAMP significantly inhibited the appearance of apoptotic morphology, and this was not altered by

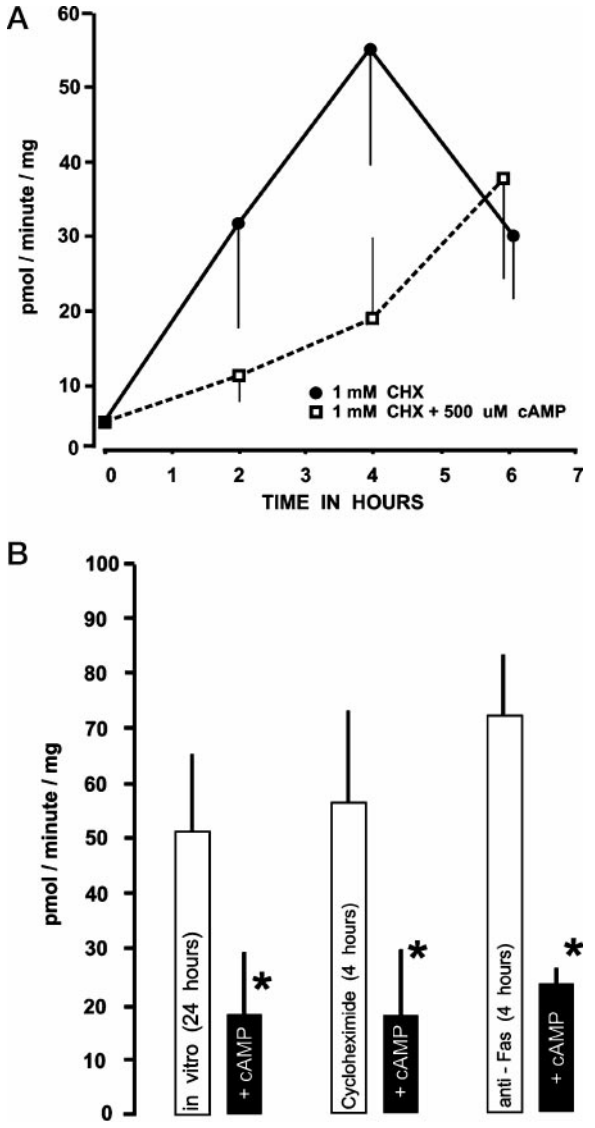

FIG. 4. **Effect of 8-CPT-cAMP on caspase-3 (CPP32β) activity.** Isolated neutrophils were incubated in the presence or absence of 500 μM 8-CPT-cAMP, and apoptosis was induced by *in vitro* culture, CHX exposure, or anti-Fas exposure. At the indicated times, cells were lysed, and DEVD-AFC specific activity was determined as described under "Materials and Methods." *A*, time course of the effect of 500 μM 8CPT-cAMP in the presence and absence of CHX on caspase-3 activity as determined by cleavage of DEVD-AFC was measured at 2, 4, and 6 h time points. The *figure* represent the mean ± S.E. of four different donors. *B*, the effect of 500 μM 8CPT-cAMP on caspase-3 activity in whole cell extracts of cells maintained *in vitro* for 24 h ($n = 4$), exposed to CHX(1 mM) for 4 h ($n = 4$), or anti-Fas (500 ng/ml) for 4 h ($n = 4$).

PD98059. These data indicate that MAPKK is not involved in CHX-induced apoptosis, that the action of cAPK is independent of MAPKK, or that cAPK acts downstream of MAPKK activation. * indicates $p > 0.05$ by paired t test.

DISCUSSION

Neutrophils are terminally differentiated cells that survive a limited time in the peripheral blood and tissues. Because of their central roles in host defense and acute inflammation, the numbers of cells available and the duration of their survival are significant to survival of the host. The signals and mechanisms that control production and survival of these cells are incompletely understood, but both appear to involve apoptosis, the physiologic form of cellular death that is internally controlled by the cell. We sought to examine the roles of second messenger, Ca^{2+}-based, and cAMP-based signaling in human neutrophil apoptosis.

Since cAMP was the first second messenger discovered (35) and is important for regulation of vital functions (*e.g.* proliferation, differentiation, gene transcription) in essentially all organisms, we focused our studies on cAMP and the cAPK in neutrophils. Cyclic AMP regulates apoptosis in several cell types, inhibiting or stimulating the process depending on the cell type and stage of differentiation (17–20). The studies reported here clearly demonstrate that cAMP delays and does not inhibit apoptosis in a time- and concentration-dependent manner in human neutrophils. Furthermore, activation of cAPK is required for this effect, as H-89, a potent and selective inhibitor of cAPK (33), neither induced apoptosis itself nor delayed apoptosis resulting from *in vitro* storage, cycloheximide, or anti-Fas exposure. This suggests that basal levels of cAPK activity do not play significant roles in neutrophil apoptosis. Use of site-selective cAMP analogues allowed clear demonstration that the type I cAPK, but not the type II, mediates the delay in apoptosis. This is consistent with the observation that the type I cAPK is the predominant isoform in human neutrophils, comprising about 85% of total cAPK. The complete lack of synergism when type II pairs were used indicates that the type II cAPK is either unnecessary, not sufficient, or localized (36) in a compartment that is not exposed to substrates needed for delaying apoptosis. It could be argued that the levels of type II cAPK are below the levels of sensitivity for this method. However, this is unlikely because very low levels of cAMP and cAPK activation are required for cAPK-mediated events, and synergism is a result of the cooperative activation of cAPK, which serves as a physiologically relevant means to amplify cAMP signals.

Other studies have demonstrated that elevation of cAMP by forskolin prostaglandin E_2-mimics, and inhibitors of cAMP phosphodiesterases inhibits apoptosis in neutrophils (17). It is possible that elevation of endogenous cAMP levels by agonists such as adrenergic agents, prostaglandins, or other agents in tissues may serve to delay apoptosis and prolong the life of neutrophils *in vivo* under certain circumstances. Generally, elevation of cAMP levels is transient, and prolonged elevation of cAMP is subject to feedback mechanisms that interrupt cAPK signal in various ways. The studies presented here demonstrate that even in the presence of sustained elevation of cAMP levels using relatively stable cAMP analogues, apoptosis is only delayed and not inhibited as reported by others (17). However, for neutrophils, which have relatively short life spans, this delay in apoptosis would be expected to be significant. Activation of type I cAPK is also associated with attenuation of the respiratory burst,[2] suggesting that cAPK prolongs the life of neutrophils with some attenuated functions. This may serve to protect the host against neutrophil-mediated inflammation.

Apoptosis is a complex cellular process that mediates a transition from life to death in most cell types. The process involves diverse structural and biochemical changes, three of which we used as markers in these studies. Many investigators have used morphologic changes to identify apoptotic cells. For neutrophils in single cell suspension, this marker is definitive and quantitative but is not likely to be an early event in the apoptosis process. In comparison, annexin V-FITC binding identifies membrane asymmetry due to transposition of phosphatidylserine from the inner to the outer leaflet of the surface membrane (31). Development of this asymmetry may be an

[2] L. K. Parvathenani, E. S. Buescher, E. Chacon-Cruz, and S. J. Beebe, unpublished data.

early marker for apoptosis, which allows selective recognition and clearance of the apoptotic cell from its normal environment. In close agreement with apoptotic morphology, annexin V-FITC binding also clearly defined the cAMP-mediated delay in apoptosis and was a more sensitive marker for apoptosis and the effects of cAPK. Chromatin fragmentation was used as a third, qualitative marker for apoptosis. This fragmentation results from cleavage of chromatin into approximately 200-base pair fragments by a Ca^{2+}-dependent endonuclease (28). This marker confirmed that the morphologic and annexin V-FITC binding changes observed in cycloheximide-exposed cells were associated with chromatin fragmentation, but because this marker is not quantitative, it was not used to examine cAMP effects or apoptosis induced by either *in vitro* storage of anti-Fas exposure. Thus, we used two functionally distinct quantitative markers to demonstrate apoptosis and the effects on cAMP on apoptosis and found consistency between them. These studies leave little doubt that cAPK are important regulators for delaying apoptosis in neutrophils.

Because apoptosis can result from different stimuli, we used three different models of the process in these studies. The precise mechanisms underlying storage-induced and cycloheximide-induced apoptosis are not known, although stimulation of the S6 kinase and signaling via the mitogen-activated protein kinase pathway may be involved in the CHX model (37, 38). However, anti-Fas exposure has been and continues to be intensively studied, because apoptosis in this situation results from receptor activation at the cell surface and now has a relatively well defined pathway. Cyclic AMP and type I cAPK delayed apoptosis in each of these diverse models, raising questions about the mechanisms of action and the site(s) of action for cAPK in each model. Previous studies suggested that a delay in apoptosis was accompanied by a transient Ca^{2+} spike (34). In the results presented here, neither the induction of apoptosis by CHX nor the delay of apoptosis by cAMP analogues was associated with changes in intracellular Ca^{2+} levels.

We investigated the possibility that by analyzing the well defined actions of the cAPK pathway and determining the sites for cAPK action in human neutrophils, the regulatory sites in one or more pathways for apoptosis could be identified. Cyclic AMP and cAPK could modulate each of the models used in this study at different sites, some shared and some unique or could act at a single site common to all pathways. Several experimental observations suggest that cAPK acts at different sites in each of the models. Although cAMP inhibition is present in all models, the sensitivity to cAMP differs across models: cAMP prolonged neutrophil survival 3.3-, 2.6-, and 1.4-fold in the cycloheximide exposure, *in vitro* storage, and anti-Fas exposure models, respectively. If cAPK acted at a common site in each model, it might reasonably be expected that inhibition would be quantitatively similar. However, it is possible that the intensity of the apoptosis signal is different in each model, and despite a common site of action, cAPK is more effective in some models than in others. A second observation against a common point of action for cAPK in all models is that inhibition of disparate processes (annexin V-FITC binding and morphologic apoptosis) resulted from cAMP exposure. The possibility that surface membrane changes and morphologic changes occur as temporally distinct events also suggests that cAPK has multiple sites of action, as might be expected for a multisubstrate kinase (39). Nevertheless, it could be argued that each of the markers used here occur as a result of a common event, such as caspase-3 (CPP-32β) activation.

Alternatively, it is possible that the same site of cAPK action is common to all models, with the different pathways initiated in each model converging to a common path that involves cAPK. Because cAPK generally acts at rate-limiting steps, it is likely that the site(s) will be an important regulatory locus for control of the process. Studies with the caspase substrates suggest that cAPK acts upstream of caspase-3. It is highly likely that the activation of caspase-3 may be a point of no return in the apoptosis pathway (10). Although sites downstream of caspase-3 activation may modulate apoptosis, it is expected that they are less effective than sites upstream of protease activation. Cyclic AMP/cAPK action upstream of a putative committed step for apoptosis is consistent with cAPK action at a rate-dependent or process-dependent point in apoptosis pathways.

Activation of the mitogen-activated protein kinase pathway has been shown to delay apoptosis in a number of cell types (40). We thus used PD98059 to block the activation of MAPKK before the addition of CHX or cAMP and observed the effects of these agents on apoptotic morphology. PD98059 has been shown to readily cross the cell membrane and to be a highly selective inhibitor of MAPKK1 (24). PD98059 had no effect on the cAPK-mediated delay in apoptosis in the CHX model. This suggests that MAPKK activation is not involved in this model of apoptosis, or if it is involved, cAPK may be acting at a site(s) downstream of or independent of MAPKK to delay CHX-induced apoptosis. Taken together, these results suggests that cAPK acts at a relatively downstream site but upstream of a critical determinant of apoptosis that may be common to all apoptosis pathways.

In summary, our data demonstrate for the first time that through activation of type I cAPK, cAMP delays, but does not prevent, apoptosis in human neutrophils due to *in vitro* storage, cycloheximide exposure, and anti-Fas exposure. A quantitative and temporal analysis of apoptosis in the presence and absence of cAMP analogues using different models, distinct markers, kinase inhibitors, and caspase substrates suggest that the type I cAPK acts at multiple sites that could be either unique or common to the models used in this study. However, a major site of cAPK action is before caspase activation, which may be a common and committed step in all models of apoptosis (10). Cyclic AMP and cAPK may represent a potential therapeutic target for prolonging the life span of myeloid cells *in vivo* and *in vitro*.

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Type I cAMP-dependent Protein Kinase Delays Apoptosis in Human Neutrophils at a Site Upstream of Caspase-3

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