Effect of pH, ionic charge, and osmolality on cytochrome c-mediated caspase-3 activity

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Segal, Mark S., and Elaine Beem. Effect of pH, ionic charge, and osmolality on cytochrome c-mediated caspase-3 activity. Am J Physiol Cell Physiol 281: C1196–C1204, 2001.—Cytochrome c-mediated activation of caspase-3 is the final common pathway for most signals that induce apoptosis. Before release of cytochrome c from mitochondria, K+ and Cl− efflux and intracellular acidification must occur. We have utilized an in vitro assay to examine the role of pH, cations, anions, and uncharged molecules on the process of cytochrome c-mediated activation of procaspase-3. In this cell-free system, a pH above 7.4 severely suppressed the activation of procaspase-3 but not the activity of caspase-3. KCl, NaCl, and other salts all inhibited caspase activation, but uncharged molecules did not. Comparison of the inhibitory capacity of various salts suggests that the crucial element in causing suppression is the cation. The inhibition of alkaline pH could be overcome by increasing concentrations of cytochrome c, whereas the inhibition of ionic charge could not, suggesting that pH and salts affect the activation of caspase-3 by different mechanisms.

Apoptosis; cell death; potassium

THE PROCESS of preordained cell death is known as apoptosis or programmed cell death. This process, distinct from necrosis, is characterized by a series of cellular events, such as cell shrinkage, phosphatidylserine externalization (14), membrane blebbing (29), and DNA fragmentation (35). Some of these changes are a direct result of enzymes, collectively termed caspasas, which are constitutively produced, highly conserved, aspartic acid-specific cysteine proteases (10). Caspases, normally present in cells in the form of inactive zymogens, are activated by autoproteolysis or cleavage by other caspases. A number of independent pathways have been implicated in the activation of different caspases with a variety of triggering mechanisms, involving an assortment of accessory proteins.

Of the many stimuli that induce apoptosis, the majority of them act by releasing cytochrome c from the inner mitochondrial matrix (6, 8, 16, 17, 20, 21, 23). Cytosolic cytochrome c is a critical factor contributing to the ultimate formation of a large, ~700-kDa, heterogeneous protein assembly termed the apoptosome, consisting of oligomers of Apaf-1, cytochrome c, and procaspase-9 (7, 9, 37). Formation of the apoptosome requires dATP/ATP (23) and is critical for the activation of caspase-9 (28).

Intracellular acidification (22) and ion efflux (2, 5, 25) must precede cytochrome c release from mitochondria. The requirement for K+ efflux in apoptosis was partly explained by the demonstration that KCl inhibited both the nuclease activity from apoptotic thymocytes and the activation of caspase-3 in vitro (18). However, the mechanism of ionic inhibition is unknown. The identification of whether the cation or anion is responsible for the inhibition of caspase-3 activation may provide insight into the mechanism of apoptosome formation.

To investigate the role that ionic charge and pH play in cytochrome c-mediated apoptosis, we used an in vitro assay in which cytosol was dependent on addition of cytochrome c for activation of caspase-3. With this assay, we demonstrate that cytochrome c-mediated activation of caspase-3 is inhibited by alkaline pH and that the inhibition by high pH can be overcome with an excess of cytochrome c. By comparing the ability of a variety of salts to inhibit this activity, we demonstrate that cations appear to exert a stronger influence than anions on the activation of caspase-3. Although both cations and alkalinity inhibit caspase activation, we demonstrate that they inhibit by a different mechanism.

MATERIALS AND METHODS

Materials and supplies. All tissue culture reagents were obtained from Life Technologies (Rockville, MD); all other reagents are from Sigma (St. Louis, MO) unless otherwise indicated.

Cell culture. The human embryonic kidney fibroblast cell line 293T Fas (a kind gift of Dr. Jürg Tschopp, Institut de Biochimie, University of Lausanne, Switzerland) was cultured at 37°C in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin, in a humidified atmosphere with 5% (vol/vol) CO2.

Preparation of the cytosol. Cytosol preparation was performed at 4°C. Cells scraped from eight 150-mm tissue culture plates were resuspended in phosphate-buffered saline...
(137 mM NaCl, 2.6 mM KCl, 10 mM Na2HPO4, and 1.7 mM K2HPO4, pH 7.4), pelleted at 300 g, washed twice, and resuspended in 1 ml of extraction buffer (50 mM HEPES, 50 mM NaCl, 2 mM MgCl2, and 5 mM EGTA). After addition of the protease inhibitors leupeptin, pepstatin, and phenylmethylsulfonyl fluoride at 1 μg/ml, the suspension was homogenized by 10 passages through a 25-gauge needle. The homogenate was centrifuged at 20,000 g for 5 min at 4°C, and the supernatant was collected and centrifuged at 100,000 g for 1 h in a 70.1 Ti rotor in a Beckman L8-70M ultracentrifuge (Beckman Instruments, Fullerton, CA) before being filtered through a 0.45-μm filter (Fisher Scientific, Atlanta, GA). The protein concentration of a 1:50 dilution of homogenate (to decrease HEPES interference) was measured using the Lowry procedure (24). The protein concentration of the cytosol was in the range of 2–3 mg/ml. Cytosol was stored in aliquots at −80°C until use.

**Assay of caspase activity.** Components of a routine assay, 2.5 μg of lysate protein, 0.25 μg of rat heart cytochrome c, an ATP regeneration system (1 mM ATP, 8 mM phosphocreatinine, 1.5 mM creatine kinase, and 5 mg/ml creatine kinase), and Z-DEVD-R110 substrate (Z-DEVD-R110; Molecular Probes, Eugene, OR), were combined on ice and brought up to a final volume of 12 μl in a buffer consisting of 10 mM PIPES, 10 mM dithiothreitol (experiments involving ZnCl2 used 20 mM β-mercaptoethanol instead of dithiothreitol to avoid Zn2+ binding), and 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), pH 7.1. Kinetic measurements of caspase activity were recorded using a Bio-Tek FL600 Microplate Fluorescent Reader (Bio-Tek Instruments, Winooski, VT). The cleavage of the rhodamine substrate was determined with the use of a 485-nm excitation filter and a 530-nm emission filter at a temperature of 25°C. The pH of the reaction mixtures was adjusted for individual assays within a range between 6.8 and 7.8 by addition of NaOH and was measured with a needle pH electrode (MI408 Microelectrodes, Bedford NH) attached to an AB150 Accumet pH meter.

Rate of caspase-3 cleavage was determined by calculating the slope at the last readings at which the substrate was in excess and then converting the relative fluorescence units (RFU) per minute to nanomoles of DEVD substrate cleaved per minute per milligram of cytosol.

**Measurement of inhibitory activity.** Inhibition of caspase activity of different salts and sugars was expressed in terms of the molarity of the compound required to reduce the activity to 50% of the control. The RFU values of all reactions were compared at the last time point at which the positive control reaction was still increasing linearly. At this time point, the RFU values were plotted vs. concentration and the Marquardt-Levenberg algorithm (26) was used to derive the best fit for each curve. This equation was then solved to calculate the necessary molarity required to inhibit the reaction to half the maximum RFU value. This molarity was used to assign a relative inhibitory potency for each reagent.

**Measurement of Na+/K+ concentration and osmole.** The osmolality of reaction mixtures was measured on a 5500 Vapor Pressure Osmometer (Wescor, Logan, UT). Samples (10 μl) of reaction mixture were applied to filter disks and vaporized in the osmometer, and the osmolality in osmoles was recorded. The total free K+ and free Na+ concentration in the cytosol was determined with a K+ and Na+ microelectrode (Microelectrodes, Bedford NH) attached to an AB150 Accumet pH meter.

**Immuno depletion of caspase-3.** Cytosol was incubated at 4°C rotating with either 2 μl of mouse monoclonal antibody, at 1 mg/ml, to caspase-3 (Pharmergen, San Diego, CA) or nonspecific mouse monoclonal antibody (IgG2a) at 1 mg/ml (Sigma). Antigen antibody complexes were precipitated by addition of a 5% (vol/vol) slurry of protein A agarose beads (Repligen, Needham, MA) in PBS with 1 mg/ml bovine serum albumin. Depleted cytosol was separated from beads by centrifugation at 5,000 g for 5 min and used in the caspase activity assays.

**Immunoblot analysis of cytosols.** A fixed quantity of cytosol was activated as above in the presence of KCl or ZnCl2 or at a pH of 7.8. The activated cytosol was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane as previously described (12). The blot was then probed with an anti-caspase-9 antibody that recognizes the activated form with greater affinity than the pro form (Oncogene, San Diego, CA) or with an anti-caspase-3 antibody that recognizes procaspase-3 preferentially over active caspase-3 (Chemicon, Temecula, CA). After being washed in PBS/0.2% Tween 20, the blot was overlaid with horseradish peroxidase-conjugated secondary antibody, and the immunoreactive polypeptides were detected by enhanced chemiluminescence (Pierce, Rockford, IL) and imaged using the Fluorchem Imaging System and software (Alpha Innotech, San Leandro, CA).

**Statistical analysis.** Statistical significance for the effects of salts and pH on activation of caspase-3 activity was determined by analysis of variance and Student’s t-test (34).

**RESULTS**

**Cytochrome c-induced Z-DEVD-R110 cleavage is concentration dependent and specifically due to caspase-3 activity.** Cytosol used for in vitro assays was shown previously to provide most of the components required for caspase-3 activation (13). The requirement for addition of cytochrome c to the cytosol varies with the method of cell homogenization. When cells were lysed by shear force, as described in MATERIALS AND METHODS, caspase-3 activation was measurable only after addition of cytochrome c (Fig. 1A). This finding is in accordance with previously published observations (18, 23), although other protocols of cytosol preparation have reported sufficient residual cytochrome c in lysate to obviate the need for supplementation (9). Increasing concentrations of cytochrome c led to an increase in the rate of substrate cleavage to a maximum value of 8 nmol·min⁻¹·mg cytosolic protein⁻¹ (Fig. 1B), although this maximum rate varied slightly with each cytosol preparation. The addition of a caspase-3 inhibitor, DEVD-fmk, or a caspase-9 inhibitor, Z-LEHD-fmk, prevented the cytochrome c-induced caspase activity (Table 1). The fluorescence was specifically due to caspase-3 activity, since cytosol immunodepleted of cytochrome c had levels of substrate cleavage no greater than cytosol with no cytochrome c added (Fig. 1C).

Activation of caspase-3 activity by cytochrome c is thought to occur by activating cleavage of caspase-9 in the apoptosome Aspaf-1/procaspase-9/cytochrome c (dATP/ADP) complex (37). Consistent with this model, addition of cytochrome c to the cytosol produced...
cleavage of both procaspase-9 and procaspase-3 as analyzed by immunoblot (Fig. 2, lanes 1 and 2).

Increasing pH diminishes cytochrome c-mediated activation of caspase-3. Intracellular acidification is associated with apoptosis (15) and is necessary for cytochrome c release (22). To test the influence of pH on cytochrome c-mediated activation of caspase-3 within cytosol, we performed the in vitro apoptosis assay at different physiological pH values. From pH 7.0 to 7.6, the activity of caspase-3 was inversely related to pH (Fig. 3A). At a pH of 7.6, the caspase-3 activity was below that seen in the no cytochrome c control. At the nonphysiological pH of 8.2, cleavage of substrate was not detected.

Studies performed using purified caspase-3 showed no more than a 10% difference in enzymatic activity at pH values between 6.8 and 7.8 (30), suggesting that the alkaline pH effect was due to inhibition of procaspase-3 activation rather than inhibition of active caspase-3. To confirm this possibility, we activated caspase-3 by adding cytochrome c before altering pH, allowing cleavage of all substrate (30 min into the assay). At 30 min, the pH was either kept at pH 7.0 or raised to pH 7.8 by the addition of NaOH. Additional fluorescent substrate was added, and the rate of substrate cleavage was

Table 1. Inhibition of caspase-3 activation by inhibitors for caspase-3 and caspase-9

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>%Caspase-3 Inhibition</th>
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<tr>
<td>DEVD-fmk, µM</td>
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<tr>
<td>10</td>
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</tr>
<tr>
<td>1</td>
<td>66.5</td>
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<td>33.3</td>
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<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LEVD-fmk, µM</td>
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<td>80.1</td>
</tr>
<tr>
<td>1</td>
<td>50.7</td>
</tr>
<tr>
<td>0.1</td>
<td>11.7</td>
</tr>
<tr>
<td>0.01</td>
<td>4.4</td>
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<tr>
<td>0.001</td>
<td>5.3</td>
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Values are %caspase-3 inhibition after 50 min of incubation with the caspase-3 inhibitor DEVD-fmk or the caspase-9 inhibitor LEVD-fmk.

Fig. 1. Caspase-3 is specifically activated by cytochrome c in a concentration-dependent manner. A: caspase-3 was activated in 12-µl reaction mixtures consisting of 293T Fas cell lysate (2.5 µg protein), DEVD-rhodamine substrate, dATP, and increasing concentrations of rat cytochrome c. Concentrations of cytochrome c from 0 (○), 5 (□), 10 (▲), 20 (■), and 40 µg/ml (●) were added to the reaction mixtures, and substrate cleavage was monitored by fluorescence emission at a wavelength of 530 nm. Results are means ± SE of triplicate samples and are representative of 3 different experiments. Statistical analysis was performed on the maximal rate of cleavage determined at each concentration of cytochrome c while substrate was still in excess.

Fig. 2. Caspase-3 is specifically activated by cytochrome c in a concentration-dependent manner. A: caspase-3 was activated in 12-µl reaction mixtures consisting of 293T Fas cell lysate (2.5 µg protein), DEVD-rhodamine substrate, dATP, and increasing concentrations of rat cytochrome c. Concentrations of cytochrome c from 0 (○), 5 (□), 10 (▲), 20 (■), and 40 µg/ml (●) were added to the reaction mixtures, and substrate cleavage was monitored by fluorescence emission at a wavelength of 530 nm. Results are means ± SE of triplicate samples and are representative of 3 different experiments. Statistical analysis was performed on the maximal rate of cleavage determined at each concentration of cytochrome c while substrate was still in excess. *P < 0.05 vs. 0 µg/ml cytochrome c. RFU, relative fluorescence units. B: the maximum rate of cleavage obtained at each concentration of cytochrome c was obtained by linear regression and is expressed as nanomoles of DEVD-R110 substrate-min⁻¹-mg cytosolic protein⁻¹. Results are means ± SE of duplicate samples and are representative of 2 different experiments. C: enzymatic specificity was tested by immunodepletion of cytosol. Aliquots (50 µl) of 293T Fas cytosol were incubated at 4°C in the presence of 2 µg of monoclonal antibody directed against caspase-3 or nonspecific mouse IgG2a. Immune complexes were absorbed from the cytosol by the addition of protein A agarose beads and were precipitated by centrifugation. Caspase-3-depleted cytosol (●), IgG2a-treated cytosol (▲), or untreated cytosol (○) was tested for cytochrome c-mediated activity. Nondepleted cytosol without cytochrome c (○) is also shown.
Fig. 2. Cations and alkaline conditions inhibit cleavage of procaspase-9 and procaspase-3. An equal quantity of inactivated cytosol (lane 1), cytosol activated by the addition of cytochrome c (lane 2), cytosol activated by the addition of cytochrome c at a pH of 7.8 (lane 3), or cytosol in the presence of 140 mM KCl (lane 4) or 2.8 mM ZnCl2 (lane 5) was separated on SDS-polyacrylamide gels and transferred to nitrocellulose membrane. The membranes were then probed with an anti-caspase-9 antibody (with a higher affinity for the activated form than the pro enzyme) or an anti-caspase-3 antibody (with higher affinity for the pro form) and developed with chemiluminescence. Experiment is representative of 4 different experiments. Left: molecular mass markers.

monitored. As shown in Fig. 3B, the rate of cleavage of active caspase-3 within the cytosol was not significantly affected by increasing the pH to 7.8. Thus the profound inhibition of alkaline pH on caspase-3 activity cannot be explained by an effect on active caspase.

As discussed in our Introduction, caspase-9 activation through apoptosome formation is thought to be required for activation of caspase-3. In principle, alkaline pH could inhibit caspase-3 activation by inhibiting either caspase-9 activity or procaspase-9 activation. We found that varying pH 6.8 to 7.8 had no significant effect on the enzymatic activity of recombinant caspase-9 and procaspase-3. An equal quantity of inactivated cytosol and determined osmolality of 225 mosmol, with a free K+ concentration of 45 mM (as determined by K+- or Na+-selective microelectrodes). Because 2.5 μl of cytosol were used in a final volume of 12 μl, the cytosol contributed a cation concentration of 12 mM to the assay. As shown in Fig. 4A, KCl inhibited cytochrome c-mediated activation of caspase-3 in a concentration-dependent manner, with 50% inhibition relative to the cytochrome c-activated control at a KCl concentration of 20 mM (Table 2). As shown in Fig. 2, lane 4, KCl addition to cytosol completely inhibited cleavage (activation) of procaspase-9 and procaspase-3.

To examine the ion specificity for inhibition of caspase-3 activation, we tested the ability of salts other than KCl to inhibit caspase-3 activation at varying salt concentrations. The time point chosen for comparison among the assays was the last time point at which fluorescence was still linearly increasing. The fluorescence (in RFU) of each reaction at this time point was plotted against salt concentration, and nonlinear regression was used to determine the molarity required to yield an RFU value 50% of that of the control reaction (+cytochrome c, no salt). A representative experiment for NaCl is shown in Fig. 4B. Table 3 lists the individual salts added to the caspase-3 reaction mixtures, along with measured osmolality and calculated concentrations of anions and cations. The measured osmolality of NaCl, KCl, MgCl2, and CaCl2 was close to that predicted from the molarity of the solutions, since these salts dissociate almost completely.

Several chloride salts tested varied widely in their ability to inhibit caspase-3 activation. KCl showed 50% inhibition at 20 mM, whereas the 50% inhibitory concentrations for NaCl, CaCl2, and MgCl2 were 10.6, 57.4, and 24.5 mM, respectively (Table 2). The free Cl− concentration at the 50% inhibitory concentration varied over 10-fold among the different salts tested (Table 2). For KCl, NaCl, CaCl2, and MgCl2, the free Cl− concentrations at 50% inhibition were calculated to be 18.9, 9.9, 108.6, and 44.8 mM, respectively. These results strongly suggest that the cation rather than the chloride anion was responsible for the inhibition of caspase-3 activation. A large difference in IC50 was observed among the cations tested, with the rank order Na+ > Mg2+ ≈ K+ > choline > Ca2+. There was no consistent difference in the ability of monovalent or divalent cations to inhibit activation. Zn2+ was a more potent agent than NaCl, but as shown below, it inhibits activated caspase-3 activity, and therefore its effect on caspase-3 activation could not be examined.

Majority of cations do not inhibit active caspase-3. To determine whether the cations have any effect on activated caspase-3, salts were added to the reaction mixtures after the caspase-3 was activated by the addition of cytochrome c and were allowed to reach maximum measurable activity. With the notable exception of Zn2+, the addition of salts at concentrations that clearly inhibited the activation of caspase-3 had limited or no effect on the cleavage rate of preactivated caspase-3.

As reviewed in the Introduction, recent studies have demonstrated that a lowering of cytosolic K+ activity is required for apoptosis, but the ion specificity and target sites for the effect of KCl have not been identified. We therefore tested the effect of KCl on caspase-3 activation in vitro. The 293T Fas cell cytosol, before addition of any inhibitory salts, had a measured osmolality of 225 mosmol, with a free K+ concentration of 13 mM and a free Na+ concentration...
caspase-3 (Fig. 5). ZnCl₂ at a concentration of 5 mM markedly inhibited active caspase-3.

To determine whether low concentrations of ZnCl₂ inhibited activation of caspase-9 and caspase-3 or just inhibited the active enzymes, cytosol activated in the presence of low concentrations of ZnCl₂ was analyzed by immunoblot analysis (Fig. 2, lane 5). The results suggest that Zn²⁺ inhibits cleavage of procaspase-9 and caspase-9 enzymatic activity.

Inhibitory effect of KCl on activation of caspase-3 cannot be overcome by the addition of cytochrome c. As shown above, excess cytochrome c suppressed pH inhibition. To determine whether additional cytochrome c could overcome the inhibitory effect of salt on activation of caspase-3, we added up to four times the normal cytochrome c concentration to the reaction assay. In marked contrast to findings with inhibition by high pH, increasing the cytochrome c concentration did not overcome the suppression of caspase-3 activity by KCl (Fig. 6). This finding indicates that ionic charge is inhibiting caspase-3 activation by a mechanism different from that of pH.

Increasing osmolality does not inhibit caspase-3 activation. Cellular osmolality is determined by and equal to the extracellular environment of the cell. In the majority of circumstances, it is ~295 mosmol with ~150 mosmol contributed by monovalent cations. To determine whether overall osmolality affected cytochrome c-mediated caspase-3 activation, we added uncharged sugars to the assay. Increasing the final osmolality to >300 mosmol, by adding sucrose or sorbitol, did not inhibit caspase-3 activity (Table 2). These results suggest that charged molecules are required to inhibit cytochrome c-mediated caspase-3 activity.

DISCUSSION

Caspase-3 involvement in the process of apoptosis has received considerable attention in recent years because it is the downstream effector caspase responsible for activating dismantling enzymes and cleaving...
substrate was still in excess. The rate of cleavage was determined at each concentration of NaCl while substrate was still in excess. *
P < 0.05 vs. 0 mM NaCl with cytochrome c.
Cytochrome c serves as one of the throttles controlling the decision about this inhibition because they dissociate to the same extent (Table 3). In addition, if the anion were involved, divalent cations associated with two Cl\(^{-}\) ions per molecule should inhibit at the same free anion concentration results in inhibition of cell shrinkage in apoptosis. We designed assays to determine which charged particle is inhibiting the cytochrome c-mediated activation of caspase-3.

Previously, it had been demonstrated that purified, active caspase-3 has only slight changes in activity between the pH of 6.7 and 7.8. This finding is in contrast to our results demonstrating that the activation of caspase-3 is markedly affected in the pH range of 6.8–7.8. At a pH >7.6, no caspase-3 activity was detected above the negative control value. Our results for the effect of pH on caspase-3 activity correlate closely with those published recently (27). To exclude the possibility that the presence of other cytosolic proteins led to the inhibition of caspase-3 enzymatic activity at alkaline pH values, we activated caspase-3 before raising the pH and determining the enzymatic rate. The enzymatic rate of previously activated caspase-3 was not significantly affected, even if the pH value was increased to 7.8. The distinction between the alkaline effect on the activation and not the activity of caspase-3 suggested that alkaline pH had an effect on a step preceding activation of the enzyme, possibly the assembly of the apoptosome, or activation of caspase-9 activity. However, the enzymatic rate of recombinant active caspase-9 was not affected by pH between a pH of 7.0 and 7.8 (data not shown), and cleavage of both procaspase-9 and procaspase-3 was inhibited by alkaline conditions (Fig. 2). Additional studies are needed to determine whether pH is affecting apoptosome formation.

Our studies, consistent with previous reports, demonstrate that normal intracellular concentrations of KCl inhibit caspase-3 activation (Fig. 4) (19). All salts, monovalent as well as divalent, also inhibited caspase-3 activation (Table 2).

Maeno et al. (25) reported that blockade of Cl\(^{-}\) or K\(^{+}\) channels results in inhibition of cell shrinkage in apoptosis. We designed assays to determine which charged particle is inhibiting the cytochrome c-mediated activation of caspase-3. Our studies suggest a 50\% inhibition in caspase activity at a concentration of 20 mM for KCl and 10.6 mM for NaCl (Table 2). If the anion were involved in the inhibition of caspase-3 activation, both NaCl and KCl would be equally effective in bringing about this inhibition because they dissociate to the same extent (Table 3). In addition, if the anion were involved, divalent cations associated with two Cl\(^{-}\) atoms per molecule should inhibit at the same free anion for apoptosis to advance. Therefore, identifying what impels a cell toward that irrevocable decision is crucial. This study provides information concerning the influence of the pH, ionic charge, and osmolality of the cytosol on cytochrome c-mediated activation of caspase-3.

We used an in vitro system in which the addition of cytochrome c to cytosol produced caspase-3 activation. This assay is specific for activation of caspase-3 as demonstrated by inhibition of the activity with DEVD-fmk (a caspase-3 inhibitor) and by elimination of the activity by immunodepletion of caspase-3 before the assay. The rate of caspase-3 cleavage increased with increasing concentrations of cytochrome c to a maximum of \(~8\) nmol substrate\(\cdot\)min\(^{-1}\)\cdot mg cytosolic protein\(^{-1}\).

Structural and cell cycle proteins (10). Caspase-3 activity is detectable after the cell passes through a crucial commitment phase of apoptosis, during which time the apoptosome, composed of oligomerized Apaf-1/cytochrome c/procaspase-9/(dATP/ATP), is assembled, providing the necessary components to advance a self-destructive cascade and ensuring cell death. The assembly of the apoptosome apparently serves as one of the throttles controlling the decision.
The cation concentration was calculated from the percent dissociation of the salt (determined by the osmolality reading). For example, if a 140 mM solution of NaCl dissociated 100%, it would have an osmolality of 280 mosmol. Because the osmolality was measured in at 261 mosmol, 19 mM NaCl did not dissociate, and thus there is only a 121 mM concentration of free cation (140 mM – 19 mM). The anion concentration at the indicated molarity was calculated from the percent dissociation of the salt (determined by the osmolality reading) relative to the expected osmolality for the given molarity. NA, not applicable. *P < 0.05 vs. NaCl.

<table>
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<th>Reagent</th>
<th>Molarity of Stock Solution, mM</th>
<th>Measured Osmolality of Stock Solution, mosmol</th>
<th>Calculated Cation Concentration of Stock Solution, mM</th>
<th>Calculated Anion Concentration of Stock Solution, mM</th>
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<tr>
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</tr>
<tr>
<td>Sucrose</td>
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<td>325</td>
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</table>

Values for calculated molarity to inhibit caspase-3 by 50% are means ± SE of final molarity of component in the reaction mix. The calculated cation concentration added at inhibitory molarity is the concentration of cation added to the assay at the concentration required to give 50% inhibition [as calculated from the cation concentration of the stock solution (see Table 3)]. The calculated anion concentration added at inhibitory molarity is the concentration of anion added to the assay at the concentration required to give 50% inhibition [as calculated from the anion concentration of the stock solution (see Table 3)]. NA, not applicable. *P < 0.05 vs. NaCl.
motic solutes. If the latter were the case, our data suggest that these osmoses would need to be uncharged. This is known to occur in cells when they are exposed to a hypertonic environment (3, 31, 32). Further investigations are needed to distinguish these possibilities.

Previously it was reported that KCl inhibits the nuclease activity required for the DNA fragmentation observed during apoptosis (18). In a series of experiments in which salts were added to preactivated caspase-3 within the cytosol, 140 mM NaCl or KCl had little effect on caspase-3 enzymatic activity. This finding demonstrates that ionic charge is not inhibitory at all steps along the apoptotic pathway.

We found that the inhibition of alkalinity could be overcome with excess cytochrome c. In the standard assay, $2.1 \times 10^{-11}$ moles of cytochrome c were added to 2.5 $\mu$g of cytosolic protein in 12 $\mu$l, yielding a final concentration of 20 $\mu$g/ml. This concentration is 100 times the concentration of cytochrome c per milligram of cytosol found in rat cardiomyocytes (1). This finding implies that only a fraction of the cytochrome c added to the cytosol in our assay is functional, that a component needed to accelerate caspase activation at lower cytochrome c concentrations may be missing from the in vitro assay, or that in vivo caspase-3 activation occurs at a much slower rate than in our in vitro assay. Our data suggest that the lower the concentration of cytochrome c, the greater the effect of pH change.

The ability of alkalinity and cations to inhibit cytochrome c-mediated activation of caspase-3 is not specific for human embryonic kidney cell cytosol. When the assay is performed with cytosol derived from human umbilical vein endothelial cells, Jurkat cells, and insect cell cytosol derived from SF9 cells, similar results were obtained (data not shown). Thus the effect of pH and cations appears to be a general phenomenon of cytochrome c activation of caspase-3.

Although our studies found that both alkaline pH and elevated KCl inhibit caspase-3 activation, these conditions appear to act in different ways. An increase in the concentration of cytochrome c was able to overcome the inhibition by alkaline pH, whereas excess cytochrome c was unable to overcome the inhibition by KCl. Thus, unlike the modulatory effect of pH, if the cell is above a threshold KCl concentration, cytochrome c will not be able to mediate caspase-3 activation (Fig. 4A), and apoptosis will not proceed. This was confirmed by immunoblot analysis. While KCl completely eliminated cleavage of procaspase-9 and procaspase-3 (Fig. 2, lane 4), a pH of 7.8 decreased but did not eliminate cleavage of procaspase-9 while abrogating the cleavage of procaspase-3 (Fig. 2, lane 3). A reasonable hypothesis is that pH affects the cytochrome c/Apaf-1 oligomerization step. Cationic strength may inhibit either the interaction of caspase-9 with the apoptosome or the activation of caspase-9. The caspase recruitment domain of Apaf-1 consists of an acidic patch (D27, E39, and E40, and E41) that interacts with a basic patch on caspase-9 (11, 36). Cations may interact with the acidic patch of Apaf-1 and prevent caspase-9 recruitment into the apoptosome. This hypothesis would predict that by adding additional procaspase-9 to cytosol, the inhibitory effects of ionic charge might be overcome as well. Our results provide a means to dissect out distinct steps in caspase-3 activation that will allow these possibilities to be examined in future studies.

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