Enhancement of Ricin Cytotoxicity in Chinese Hamster Ovary Cells by Depletion of Intracellular K^+: Evidence for an Na^+/H^+ Exchange System in Chinese Hamster Ovary Cells

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ABSTRACT Depletion of intracellular K^+ has been reported to result in an arrest of the formation of coated pits in human fibroblasts (Larkin, J. M., M. S. Brown, J. L. Goldstein, and R. G. W. Anderson, 1983, *Cell*, 33:273–285). We have studied the effects of K^+ depletion on the cytotoxicities of ricin, *Pseudomonas* exotoxin A, and diphtheria toxin in Chinese hamster ovary (CHO) cells. The cytotoxicities of ricin and *Pseudomonas* toxin were enhanced in K^+-depleted CHO cells whereas the cytotoxicity of diphtheria toxin was reduced by K^+ depletion. The effects of NH_4Cl on the cytotoxicities of ricin, *Pseudomonas* toxin, and diphtheria toxin were found to be similar to those of K^+ depletion, and there were no additive or synergistic effects on ricin cytotoxicity by NH_4Cl in K^+-depleted medium.

The enhancement of ricin cytotoxicity by K^+ depletion could be completely reversed by the addition of K^+, Rb^+, and partially by the addition of Cs^+, before the ricin treatment, whereas Li^+ was ineffective. These protective effects of K^+ or Rb^+ requires a functional Na^+/K^+ ATPase. CHO cells grown in K^+-depleted media were found to contain 6.3-fold increase in intracellular Na^+ level, concomitant with a 10-fold reduction in intracellular K^+ level. The enhanced cytotoxicity of ricin in K^+-free medium and the increased uptake of Na^+ could be abolished by amiloride or amiloride analogues, which are known to be potent inhibitors of the Na^+/H^+ antiport system. Our results suggest that a depletion of intracellular K^+ results in an influx of Na^+, which is accompanied by the extrusion of H^+. Consequently, there is an alkalinization of the cytosol and the ricin-containing endosomes. As a result, ricin is more efficiently released from the endosomes in K^+-depleted cells. Results from the studies of the binding, internalization, and degradation of ^{125}I-ricin, and the kinetics of inhibition of protein synthesis by ricin in K^+-depleted cells are consistent with this working hypothesis.

It is generally assumed that the entry of plant and bacterial toxins such as ricin, *Pseudomonas* exotoxin A, and diphtheria toxin into mammalian cells involves a sequence of events called receptor-mediated endocytosis (1). After binding of the toxins to the cell surface receptors and the transport of the toxin-receptor complex to endocytic vesicles inside the cell, the toxin molecules remain in the vesicles until they are released into the cytosol to reach their intracellular targets, i.e., the ribosomes or elongation factors. The nature of the release mechanism that allows the translocation of hydrophilic polypeptides across the vesicle membrane remains obscure. For the release of diphtheria toxin from the endocytic vesicles, acidification of the endosomes by an ATP-dependent proton pump appears to be required (2, 3). Acidification of endosomes may result in a dissociation of the ligand from the receptor, may allow the ligand to assume a conformation compatible with transmembrane translocation of the ligand, or both. The requirement of this acidification for the release of diphtheria toxin into the cytosol is suggested by the protection of diphtheria toxin cytotoxicity by NH_4Cl (4–6). Acidification does not appear to be required for the release of ricin from the endocytic vesicles. On the contrary, alkaline pH is optimal for ricin intoxication (7). This apparent difference in pH optima of intoxication of mammalian cells by ricin and

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diphtheria toxin is further evidenced by the fact that Chinese hamster ovary (CHO)[1] mutant cell lines defective in acidification of endosomes are resistant to diphtheria toxin but hypersensitive to ricin (8). The mechanism of alkalinization required for ricin intoxication remains unknown.

An amiloride-sensitive Na^+/H^+ exchange system has been identified in the plasma membrane of a wide variety of animal cells (9–21). This system can potentially function to extrude intracellular protons generated by cellular metabolism using the inwardly directed Na^+ gradient as the driving force. Another important feature of the Na^+/H^+ exchange system is its rapid activation by a variety of mitogenic polypeptides, such as epidermal growth factor, platelet-derived growth factor and serum, and the activation of this Na^+/H^+ exchange system has been shown to result in cytoplasmic alkalinization (22–25). The influx of Na^+ through amiloride-sensitive Na^+/H^+ exchanger and the ensuing intracellular alkalinization have been proposed as a possible messenger of growth factor actions (23, 26–29). Recent reports showed that this exchange system is also activated by exposure of cells to hypertonic medium (13, 30, 31), osmotic shrinking (32, 33), incubation in the presence of ouabain (20), and loading of the cells with acid (23, 29, 32). It was shown recently that depletion of intracellular K^+ arrests the formation of coated pits in human fibroblasts (34). In this study, we examined the effect of K^+ depletion on the cytotoxicities of ricin, *Pseudomonas* exotoxin A, and diphtheria toxin in CHO cells. Our results indicate that the depletion of intracellular K^+ enhances Na^+ uptake in CHO cells through an amiloride-sensitive Na^+/H^+ exchange mechanism that enhances the cytotoxicity of ricin and *Pseudomonas* exotoxin A but protects CHO cells from diphtheria toxin. Amiloride and amiloride analogues, known to be potent inhibitors of the Na^+/H^+ exchange system (29, 35, 36), prevent the enhancement of ricin cytotoxicity of CHO cells in K^+-depleted medium. Our results suggest that depletion of intracellular K^+ activates the Na^+/H^+ exchange system, which results in cytoplasmic alkalinization. Consequently, the release of ricin from endosomes is enhanced by the alkalinization of the cytosol and that of the endosomes in K^+-depleted cells.

MATERIALS AND METHODS

Materials: The following chemicals were purchased from Sigma Chemical Co. (St. Louis, MO): ouabain, nigericin, rubidium chloride, lithium chloride, D-galactose, and lactoperoxidase. Cesium chloride was obtained from Var-Lac-Oid Chemical Co. (Elizabeth, NJ). Ricin and diphtheria toxin were purchased from Miles Laboratories, Inc. (Elkhart, IN) and Connaught Laboratories (Willowdale, Ontario), respectively. *Pseudomonas aeruginosa* extotoxin A was a gift from Dr. S. Leppla (U. S. Army Medical Research Institute of Infectious Diseases, Frederick, MD). Amiloride analogues were synthesized as previously described (37–49). Fetal bovine serum was purchased from Dutchland, Inc. (Denver, PA). Powdered α-minimal essential medium (α-MEM) and α-MEM suspension culture medium were obtained from Flow Laboratories, Inc. (McLean, VA). [^{3}H]Leucine (110 Ci/mmol) was purchased from ICN Chemicals and Radioisotope Division. Na^{125}I (14.5 mCi/μg iodine) was obtained from Amersham Corp. (Arlington Heights, IL).

Cell Culture: A CHO cell line auxotrophic for proline (CHO *pro*) was a gift of Dr. L. Siminovitch (Toronto). Cells were grown as monolayer in α-MEM supplemented with 10% fetal bovine serum and 10 μg each of adenosine, guanosine, cytidine, and thymidine per milliliter of medium, or as suspension culture in α-MEM supplemented with 10% fetal bovine serum, 40 μg/ml proline, and 10 μg/ml each of adenosine, guanosine, cytidine, and thymidine. Penicillin (100 μg/ml) and streptomycin (100 μg/ml) were added to the cell culture medium. Cells were incubated in a humidified 5% CO_2 atmosphere at 37°C.

Measurement of Cytotoxicity in K^+-depleted Medium: CHO *pro* cells (3.5×10^5 cells) were plated on six-well Linbro plates (Linbro Div., Flow Laboratories, Inc.). The next day, the monolayer culture was washed with 2 ml buffer A (50 mM Na HEPES buffer at pH 7.2 containing 100 mM NaCl), incubated in 2 ml hypotonic medium (α-MEM/H_2O = 1:1, vol/vol) for 5 min, and then incubated in isotonic K^+-free medium for 30 min at 37°C. After the 30-min incubation in K^+-free medium, the cells were exposed to varying concentrations of ricin (for 60 min), *Pseudomonas* toxin (for 90 min), or diphtheria toxin (for 90 min), respectively. Unbound toxins were removed by washing with phosphate-buffered saline (PBS) three times; in the case of ricin, the washing solution also contained 0.1 M galactose. The cells were then incubated in α-MEM containing 1% fetal bovine serum for 20 h at 37°C, washed, and incubated in serum-free, leucine-free α-MEM with [^{3}H]leucine (0.5 μCi/ml, 4.1 nM) for 2 h at 37°C. The monolayer culture was fixed with two washings of 3% (wt/vol) perchloric acid–0.5% (wt/vol) phosphotungstic acid, washed with PBS, and dissolved in 1 ml of 0.5 N NaOH. An 0.1-ml aliquot of the dissolved cells was transferred to a scintillation vial, neutralized with 0.1 N HCl, mixed wih 4 ml of Ready-Solv EP scintillation solution (Beckman Instruments, Inc., Fullerton, CA), and counted in a Beckman LS7500 liquid scintillation counter. The effects of toxins on the incorporation of [^{3}H]leucine into perchloric acid–phosphotungstic acid insoluble material were used as measurements of the cytotoxicities of these toxins.

Measurement of Intracellular Na^+ and K^+ Concentration: CHO *pro* cells (4×10^6) were plated in 100-mm dishes 24 h before the experiment. Cells from two plates were sufficient for one determination of intracellular Na^+ or K^+ concentration. After 24 h, the monolayer culture was washed twice with 10 ml buffer A (50 mM Na HEPES buffer at pH 7.2 containing 100 mM NaCl), incubated in 6 ml hypotonic medium (α-MEM/H_2O = 1:1, vol/vol) for 5 min after one wash with hypotonic medium. The cells were then incubated in isotonic α-MEM with or without potassium (plus or minus 40 μM dimethylamiloride) for 2 h at 37°C. After 2 h the medium was removed and the cells were quickly rinsed by sequentially dipping each dish in four beakers containing 500 ml of cold 0.1 M $MgCl_2$ in 10 mM Tris-HCl, pH 7.5. Each dish was completely rinsed within 10 s after the removal of the medium. The residual liquid was completely drained, and the cell monolayer was dissolved in either 2 ml of 0.1 N KOH for Na^+ measurement or 2 ml of 0.1 N NaOH for K^+ measurement with rocking for 30 min. The dissolved cells were then transferred to a 15-ml plastic tube, and the pH was adjusted to 6–7 with 6 N HCl. A heavy precipitate was removed by centrifugation at 2,500 rpm (Sorvall RC 3B; Sorvall Biomedical Div., DuPont Instruments, Wilmington, DE) for 10 min and the supernatant was filtered through a 0.22-μm filter. The filtrate was diluted with an equal volume of a 15 mM LiCl solution, and Na^+ and K^+ concentrations were measured with a Beckman Klina Flame Emission Photometer (model 6521), bypassing the dilutor. Control experiments were carried out in the absence of cells to determine background Na^+ and K^+ concentrations.

Measurements of Binding, Internalization, and Degradation of ^{125}I-Ricin: Ricin was radiolabeled with ^{125}I by a lactoperoxidase-catalyzed reaction described previously (41). Measurements of the binding, internalization, and degradation of ^{125}I-ricin were carried out with CHO cells grown in spinner culture. CHO *pro* cells in suspension culture were washed twice with buffer A, incubated first in hypotonic media (α-MEM/H_2O = 1:1, vol/vol) for 5 min, and then for an additional 60 min at 37°C in isotonic α-MEM with or without K^+ ion. These cells were then incubated at 0 or 37°C with ^{125}I-ricin (2×10^5 cpm/1×10^6 cells per ml) for 60 min. An aliquot of cell-bound ^{125}I-ricin was measured and the remaining cells were washed with 0.1 M galactose to determine internalized ^{125}I-ricin. For the analysis of ^{125}I-ricin degradation, CHO cells containing internalized ^{125}I-ricin were suspended in α-MEM with or without K^+ ion, and incubated at 37°C in plastic tubes precoated with bovine serum albumin (1×10^6 cells/0.5 ml per tube). At varying intervals, cells in duplicate tubes were centrifuged and the 5% trichloroacetic acid–soluble counts in the supernatant were measured.

RESULTS

Effects of K^+ Depletion, and Addition of NH_4Cl or Nigericin on the Cytotoxicities of Ricin, Pseudomonas Toxin, and Diphtheria Toxin in CHO Cells

The ID_{50} (concentration of toxins resulting in a 50% inhibition of [^{3}H]leucine incorporation into cellular proteins) of

[1] *Abbreviations used in this paper:* CHO, Chinese hamster ovary; α-MEM, α-minimum essential medium.

ricin was reduced 15-fold when CHO cells were preincubated in K^+-free medium for 30 min at 37°C (Fig. 1*A*, 4.5 ng/ml in K^+-depleted medium vs. 70 ng/ml in regular α-MEM). Likewise, the ID_{50} of *Pseudomonas* toxin was reduced sixfold by K^+ depletion (Fig. 1*B*, 54 vs. 330 ng/ml). In contrast, the ID_{50} of diphtheria toxin was increased sixfold (from 0.33 Lf/ml in normal medium to 1.9 Lf/ml in K^+-depleted medium, Fig. 1*C*).

It has been shown previously that nigericin (or monensin) or NH_4Cl enhances the cytotoxicity of ricin in CHO cells (42). As can be seen in Fig. 1*A*, the extent of enhancement of ricin cytotoxicity by NH_4Cl in CHO cells is similar to that obtained with K^+ depletion. To ascertain whether the effect of K^+ depletion and that of NH_4Cl are additive, synergistic, or indifferent, we compared the cytotoxicity of ricin in CHO cells in K^+-depleted medium with the added NH_4Cl. As shown in Fig. 1*A*, treatment of CHO cells with NH_4Cl in K^+-depleted medium did not result in any additive or synergistic effect, as compared with treatment with K^+ depletion or NH_4Cl alone. These results suggest that NH_4Cl treatment and K^+ depletion may affect ricin cytotoxicity by a common mechanism.

Treatment of CHO cells with a low concentration of nigericin (10^{-8} M) markedly enhanced the cytotoxicity of ricin (ID_{50} of 0.04 vs. 70 ng/ml), the cytotoxicity of *Pseudomonas* toxin (23 vs. 330 ng/ml) and, to a minimal extent, that of diphtheria toxin (0.19 vs. 0.33 Lf/ml). Treatment of CHO cells with NH_4Cl slightly enhanced the cytotoxicity of *Pseudomonas* toxin but markedly protected CHO cells from the cytotoxicity of diphtheria toxin (Fig. 1, *B* and *C*). These results taken together indicate that (*a*) nigericin affects the cytotoxicities of these toxins at a step of intoxication process different from that affected by K^+ depletion and NH_4Cl, and (*b*) the effects of nigericin, NH_4Cl and K^+ depletion on the cytotoxicities of ricin and *Pseudomonas* toxin differ from those on the cytotoxicity of diphtheria toxin.

Effects of Various Monovalent Cations on the Enhancement of Ricin Cytotoxicity by K^+ Depletion

The enhancement of ricin cytotoxicity by K^+ depletion in CHO cells was found to be reversible. Addition of KCl at a final concentration of 200 μM did not alter the ID_{50} of ricin in K^+-depleted medium, whereas the addition of 1 mM KCl (one-fifth the KCl concentration present in normal growth medium) completely reversed the ID_{50} to the control value (Fig. 2). RbCl at 1 mM was as effective as 1 mM KCl, CsCl at 1 mM was partially effective, and LiCl at 1 mM was totally ineffective in reversing the enhancement of ricin cytotoxicity by K^+ depletion (Fig. 3). The efficacies of these monovalent cations in reversing the effect of K^+ depletion on ricin cytotoxicity appear to be correlated with their specificities as substrates for the transport system mediated by the Na^+/K^+ ATPase (43). Rb^+ ion is known to be transported as efficiently as K^+ ion by the Na^+/K^+ ATPase, Cs^+ ion less efficiently, whereas Li^+ ion is transported by the Na^+/H^+ antiport system (44). The results shown in Fig. 4 further support this interpretation. The restoration of ricin cytotoxicity to the control values in K^+ depleted medium by 1 mM KCl or 1 mM RbCl was inhibited by 100 μM ouabain, a specific inhibitor of Na^+/K^+ ATPase (Fig. 4*A*). The partial restoration of ricin cytotoxicity by 1 mM CsCl was also inhibited by 100 μM ouabain (Fig. 4*B*). These results suggest that a depletion of intracellular K^+ ion results in an increased cytotoxicity of ricin and *Pseudomonas* toxin, and a decreased cytotoxicity of diphtheria toxin in CHO cells.

Prevention of the Enhancement of Ricin Cytotoxicity in K^+-depleted Medium by Amiloride and Amiloride Analogue

An amiloride-sensitive Na^+/H^+ exchange system was re-

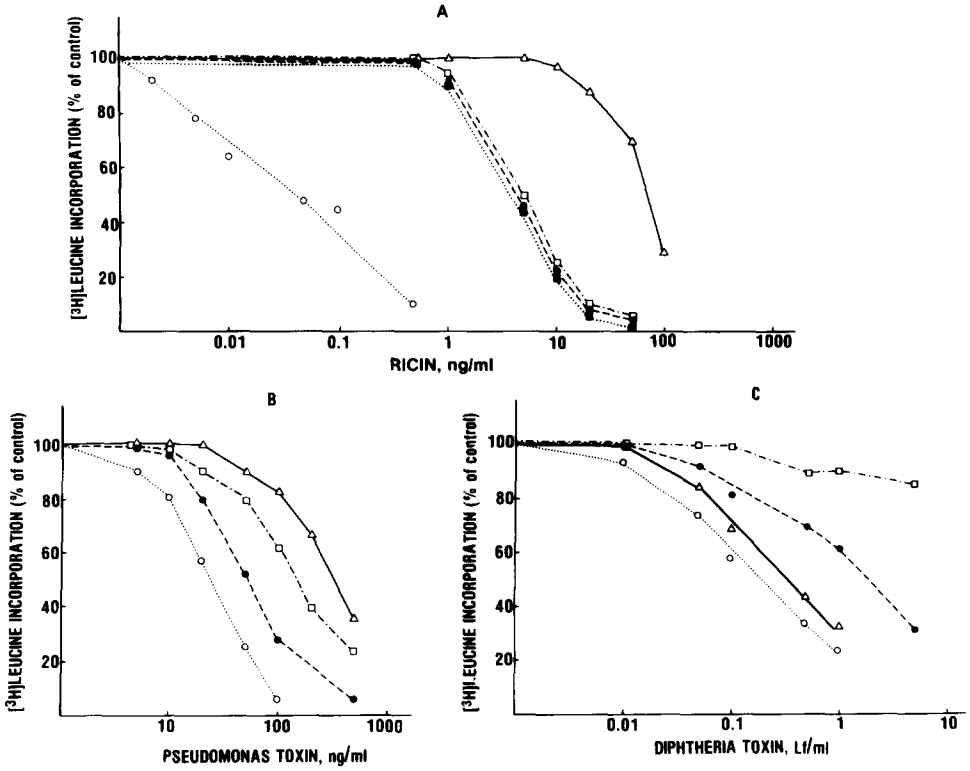


FIGURE 1 Effect of K^+-free medium, NH_4Cl, and nigericin on the cytotoxicities of ricin, *Pseudomonas* toxin, and diphtheria toxin in CHO *pro* cells. Cells (3.5×10^5) were seeded into 35-mm dishes 24 h before the experiment. The cells were subjected to a 5-min hypotonic shock, then incubated for 30 min in isotonic α-MEM with or without potassium, NH_4Cl, or nigericin. The cells were then exposed to different concentrations of ricin (for 60 min), *Pseudomonas* toxin (for 90 min), and diphtheria toxin (for 90 min). After 20 h further incubation at 37°C in α-MEM containing 1% fetal calf serum, the cells were washed and incubated in serum-free, leucine-free α-MEM, and labeled with [^{3}H]leucine (0.5 μCi/ml) for 2 h at 37°C. △, normal medium; ●, K^+-free medium; □, normal medium + 10 mM NH_4Cl; ■, K^+-free medium + 10 mM NH_4Cl; O, normal medium + 10^{-8} M nigericin.

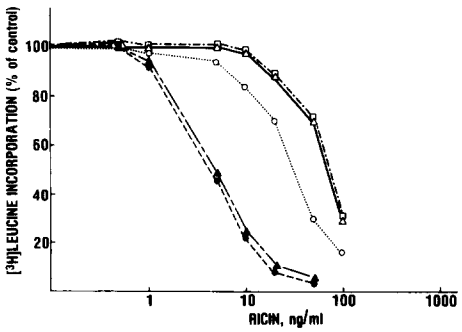


FIGURE 2 Effect of addition of different concentrations of KCl on the cytotoxicity of ricin in K^+-free medium. The CHO *pro* cells were subjected to the following sequential incubations at 37°C: a 5-min hypotonic shock, followed by a 30-min incubation in isotonic α-MEM without potassium; a 60-min incubation in K^+-free α-MEM in the presence of different concentrations of KCl. The cells were then exposed to different concentrations of ricin for 60 min. After 20 h further incubation, the inhibition of protein synthesis was measured as described in the legend to the Fig. 1. Δ, normal medium; ●, K^+-free medium; ▲, K^+-free medium + 200 μM KCl; O, K^+-free medium + 500 μM KCl; □, K^+-free medium + 1 mM KCl.

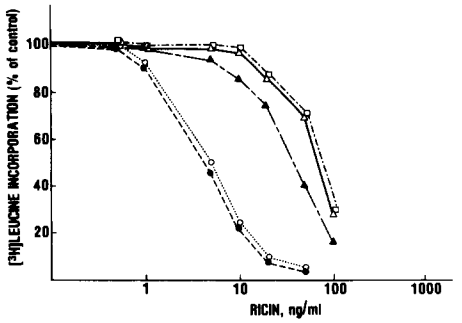


FIGURE 3 Prevention of the enhanced ricin cytotoxicity in K^+-free medium by monovalent cations. The CHO *pro* cells were subjected to the following sequential incubation at 37°C: a 5-min hypotonic shock, followed by a 30-min incubation in isotonic K^+-free medium; a 60-min incubation in K^+-free medium containing 1 mM of one of the following cations: KCl or RbCl (□); CsCl (▲); LiCl (O). The cells were then exposed to different concentrations of ricin for 60 min. After 20 h further incubation, the inhibition of protein synthesis was measured as described in the legend to Fig. 1. Δ, normal medium; ●, K^+-free medium.

cently identified in the plasma membrane of a wide variety of animal cells (9–21). To ascertain whether depletion of intracellular K^+ activates the amiloride-sensitive Na^+/H^+ exchange system, which may be involved in the enhancement of ricin cytotoxicity in CHO cells by K^+ depletion, we measured both the intracellular levels of Na^+ and K^+ ions, and the cytotoxicity of ricin in CHO cells grown in K^+-depleted medium containing various concentrations of amiloride and five amiloride analogues that are known to be potent inhibitors of the Na^+/H^+ exchange system (29, 35, 36). As shown in Fig. 5, exposure of cells to a K^+-free medium results in a 6.3-fold increase in the intracellular level of Na^+ ion which can be inhibited by 5-*N*,*N*-dimethylamiloride; dimethylamiloride (40 μM) inhibits the elevation of Na^+ level by 72% but does not affect the decrease in intracellular K^+ level (10-fold). Fig. 5 also shows that dimethylamiloride (40 μM) prevents the enhancement of ricin cytotoxicity in K^+-free medium by 74.2%. These results strongly suggest that depletion of intracellular K^+ enhances the uptake of Na^+ through a Na^+/H^+ exchange system.

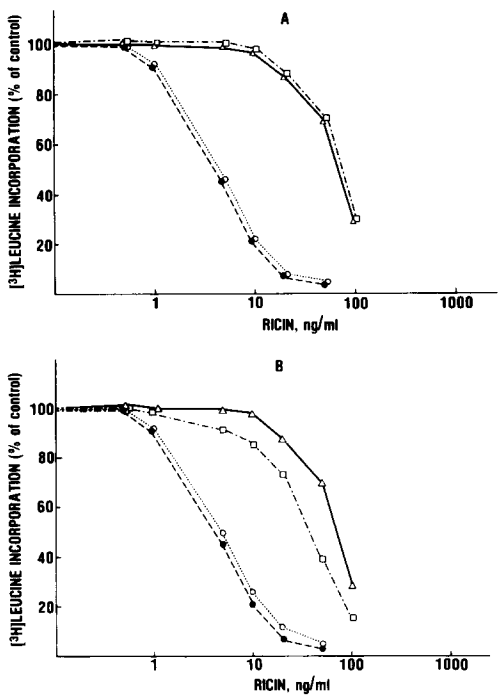


FIGURE 4 Effect of ouabain on the prevention of the enhanced ricin cytotoxicity in K^+-free medium by monovalent cations. The CHO *pro* cells were subjected to the following sequential incubations at 37°C: a 5-min hypotonic shock, followed by a 30-min incubation in the isotonic K^+-free medium with or without 100 μM ouabain; a 60-min incubation in K^+-free α-MEM containing the same ouabain concentration plus 1 mM of one of the cations. The cells were then exposed to different concentrations of ricin for 60 min. After 20 h further incubation, the inhibition of protein synthesis was measured as described in the legend to Fig. 1. (A) Δ, normal medium; ●, K^+-free medium; □, K^+-free medium + 1 mM KCl or RbCl; O, K^+-free medium + 100 μM ouabain + 1 mM KCl or RbCl. (*B*) Δ, normal medium; ●, K^+-free medium; □, K^+-free medium + 1 mM CsCl; O, K^+-free medium + 100 μM ouabain + 1 mM CsCl.

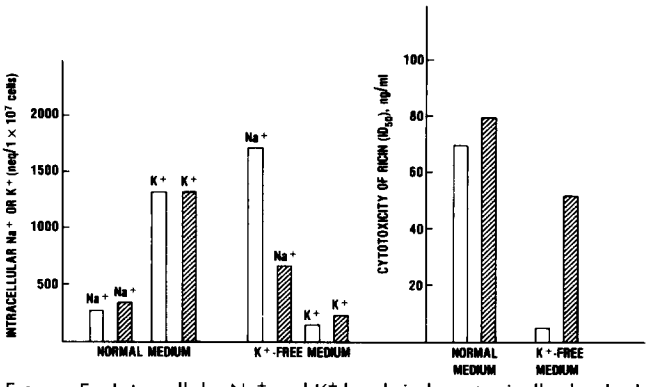


FIGURE 5 Intracellular Na^+ and K^+ levels in hypotonically shocked CHO *pro* cells incubated in K^+-free medium or normal medium. Cells (4×10^6) were seeded into 100-mm dishes 24 h before the experiment. The cells were subjected to a 5-min hypotonic shock, then a 2-h incubation in isotonic α-MEM with or without potassium (plus or minus 40 μM dimethylamiloride). After 2 h the levels of intracellular Na^+ and K^+ was measured as described in Materials and Methods. The cytotoxicity of ricin was measured as described in the legend to the Fig. 1. Hatched bars indicate the results in the presence of 40 μM dimethylamiloride.

As can be seen in Fig. 6 the enhancement of ricin cytotox-

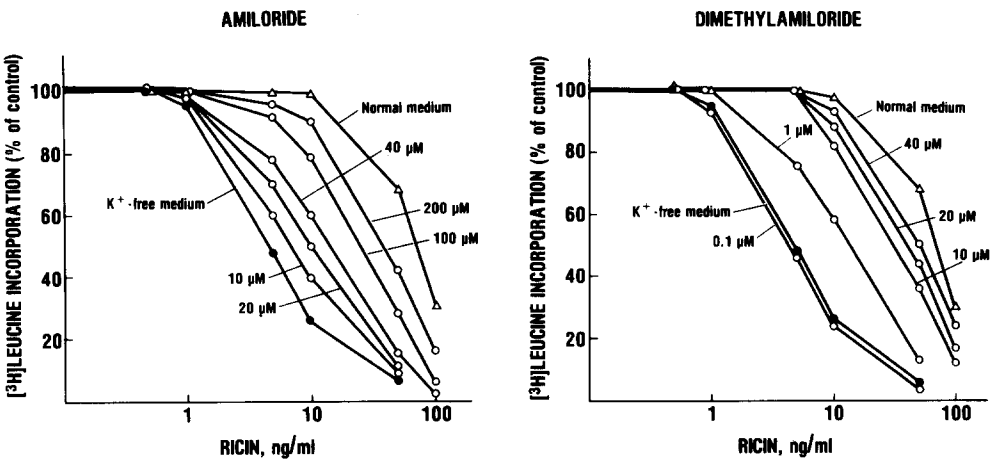
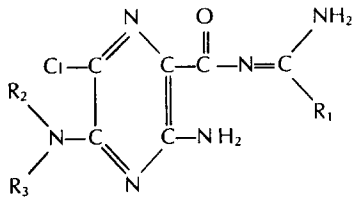


FIGURE 6 Inhibition by amiloride and dimethylamiloride of the enhanced ricin cytotoxicity in K^+-free medium. CHO *pro* cells were first subjected to a 5-min hypotonic shock, and then incubated for 60 min in isotonic α-MEM with or without potassium (plus or minus different concentrations of amiloride or dimethylamiloride). The cells were then exposed to different concentrations of ricin for 60 min. After 20 h further incubation, the inhibition of protein synthesis was measured as described in the legend to Fig. 1.

TABLE I. *Effect of Substitution on the 5-Amino Group and in the Guanidino Group of Amiloride on Its Potency for Inhibiting the Enhancement of Ricin Cytotoxicity in K^+-free Medium*

R_1*	R_2	R_3	Concentration for 50% inhibition of ricin cytotoxicity in K^+-free medium	Concentration for 50% inhibition of Na^+ influx through Na^+/H^+ exchange system
			μM	μM
NH_2—	H—	H—	150	4 (reference 36)
NH_2—	CH_3—	CH_3—	15	0.17 (reference 36)
NH_2—	$(CH_3)_2CHCH_2$—	CH_3—	5	ND
NH_2—	$(CH_3)_3C$—	H—	4	0.12 (reference 36)
NH_2—	$CH_3—C(=CH_2)—CH_2$—	CH_3—	3.4	ND
NH_2—		C_2H_5—	2.0	0.05 (reference 36)
$N(CH_3)_2$—	$(CH_3)_2CH$—	H—	≫200	150 (reference 29)
	$CH(CH_3)_2$—			

CHO *pro* cells were first subjected to a 5-min hypotonic shock, and then incubated for 60 min in isotonic α-MEM with or without potassium (plus or minus different concentrations of amiloride or amiloride analogues). The cells were then exposed to different concentrations of ricin for 60 min. After 20 h of incubation, the inhibition of protein synthesis was measured as described in the legend to Fig. 1. ND, not determined.
* See Scheme 1.



SCHEME 1 Structure of amiloride and its analogue. See Table I.

icity by K^+ depletion is prevented by amiloride and its analogues, which are known to be potent inhibitors of the Na^+/H^+ exchange system. Amiloride at 150 μM inhibits the enhancement of ricin cytotoxicity in K^+ free medium by 50%. Similarly, 15 μM dimethylamiloride is required to inhibit the enhancement of ricin cytotoxicity by 50% (Fig. 6). Table I and Scheme 1 show the correlation between the potency of amiloride analogues as inhibitors of the Na^+/H^+ exchange system and their efficacies in preventing the enhancement of ricin cytotoxicity in K^+ free medium. The potency of these compounds as inhibitors of the enhancement of ricin cytotoxicity in K^+-free medium increases with the presence of branched alkyl group (ethyl isopropyl amiloride) or alkenyl group (methyl isobutenyl amiloride); most potent among the analogues tested is the one bearing ethyl and isopropyl substituents (IC_{50} [concentration for 50% inhibition of ricin cytotoxicity in K^+-free medium] = 2 μM). These results are in agreement with the earlier report that substitution of the hydrogen atom on the 5-amino group of amiloride by alkyl or alkenyl groups produces compounds that are more potent than the parent compound in inhibiting the Na^+/H^+ exchange system (29, 35, 36). Earlier reports showed that substitution on the terminal nitrogen atom of the guanidino moiety of amiloride by alkyl or aryl groups reduces strikingly the potency of this compound as an inhibitor of the Na^+/H^+ exchange system, whereas it increases the potency of this compound as an inhibitor of the Na^+ channel of tight epithelia (45). Our result (Table I) showed that substitution on the terminal nitrogen atom of the guanidino moiety of amiloride by two methyl group produces a compound that is very poorly active in inhibiting the enhancement of ricin cytotoxicity in K^+-free medium. It is therefore clear that the Na^+ channel found in tight epithelia (45) does not contribute to the observed enhancement of ricin cytotoxicity, or to the increase in intracellular Na^+ concentration in K^+ free medium.

The inhibition of enhancement of ricin cytotoxicity in K^+-free medium by amiloride and amiloride analogues could be prevented by treating cells with 20 mM NH_4Cl, which increases the intravesicular pH (Fig. 7). This result clearly shows that amiloride or amiloride analogues prevent the extrusion of protons from the cells by blocking the Na^+/H^+ exchange

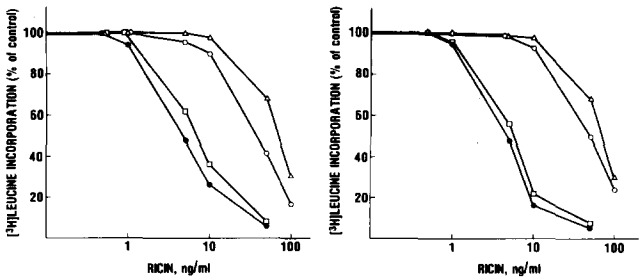


FIGURE 7 *Left:* amiloride. *Right:* dimethylamiloride. Reversal of the inhibitory effect of amiloride and dimethylamiloride on the enhancement of ricin cytotoxicity in K^+-free medium by NH_4Cl. CHO *pro* cells were subjected first to a 5-min hypotonic shock, then to a 60-min incubation in isotonic α-MEM with or without potassium (plus or minus 200 μM amiloride or 40 μM dimethylamiloride) and 20 mM NH_4Cl. The cells were then exposed to different concentrations of ricin for 60 min. After 20 h further incubation, the inhibition of protein synthesis was measured as described in the legend to Fig. 1. △, normal medium; ●, K^+-free medium; ○, K^+-free medium + 200 μM amiloride or 40 μM dimethylamiloride, □, K^+-free medium + 200 μM amiloride or 40 μM dimethylamiloride + 20 mM NH_4Cl.

TABLE II. *Binding and Internalization of ^{125}I-Ricin in CHO pro Cells in K^+-depleted Medium*

	Cell-associated ^{125}I-ricin (*cpm*)		
	Before galactose	After galactose	% of Internalization
K^+-free medium			
0°C	10,037	462	4.6
37°C	3,612	1,034	28.6
Normal medium			
0°C	11,805	402	3.4
37°C	4,771	1,323	27.7

CHO *pro* cells in suspension were subjected to a 5-min hypotonic shock, then incubated for 30 min in isotonic α-MEM with or without K^+ at 37°C. After 30 min, one set of tubes was incubated at 0°C. The cells were then incubated with ^{125}I-ricin (70,000 cpm/1 × 10^6 cells per tube) for 60 min, and washed with PBS or 0.1 M galactose in PBS three times, and the cell-associated radioactivity was measured.

system, thereby preventing the enhancement of ricin cytotoxicity in K^+-free medium. In normal medium, the addition of 200 μM amiloride slightly enhanced ricin cytoxicity (data not shown), whereas 40 μM dimethylamiloride slightly protects cells from ricin cytotoxicity (Fig. 5). This result may be attributed to an effect of amiloride as a weak base, similar to the effect of NH_4Cl on ricin cytotoxicity.

Effects of K^+ Depletion on the Binding, Internalization, and Degradation of ^{125}I-Ricin in CHO Cells

It was reported recently that the degradation of asialoglycoprotein by hepatocytes was enhanced by replacement of K^+ with Na^+ in the incubation medium (46). This was attributed to an increase in the delivery of ligands to lysosomes when Na^+ is present in lieu of K^+. To determine whether the enhanced cytotoxicity of ricin by K^+ depletion of CHO cells can be attributed to an increase in binding or internalization of ricin, or to a decrease in ricin degradation, we compared the binding, internalization, and degradation of ^{125}I-ricin in CHO cells in normal and K^+-depleted media. The data in Table II clearly indicate that binding and internalization of ^{125}I-ricin occur to nearly identical extents in CHO cells in K^+-free and normal medium. In addition, K^+ depletion does not result in an increase in ^{125}I-ricin degradation, as measured by trichloroacetic acid–soluble radioactivity in the culture supernatant (Fig. 8). In the cell pellets of CHO cells from normal or K^+-depleted medium, the trichloroacetic acid–soluble ^{125}I-ricin radioactivities were negligible (data not shown). These results indicate that the enhancement in ricin cytotoxicity by K^+ depletion cannot be attributed to an increase in binding or internalization of ricin, or a decrease in ricin degradation.

Kinetics of Ricin Cytotoxicity in K^+-depleted Medium

The lag period before the onset of inhibition of protein synthesis by ricin after the binding of ricin is generally assumed to correspond to the intracellular events that lead to the release of ricin into the cytosol. It was shown recently that the length of the lag period in intoxication by ricin can be affected by the doses of toxin and by pH; the lag period can be shortened by increasing ricin dosage or elevating the pH (47, 48). The effects of K^+ depletion, NH_4Cl, or nigericin on the kinetics of inhibition of protein synthesis by ricin were studied. At 1 μg/ml of ricin concentration, a lag period of 60 min was observed in control CHO cells with a T_{50} (time required to achieve a 50% inhibition of protein synthesis) of 180 min (Fig. 9). In K^+-depleted medium, the lag period was reduced to 30 min and T_{50} was reduced to 96 min. NH_4Cl, which is known to raise the intracellular pH, also shortened both the lag period and T_{50} to 30 and 80 min, respectively. Nigericin (10 nM) was even more effective in reducing the lag period and T_{50} for ricin intoxication to 30 and 60 min, respectively (Fig. 9). These reductions in both the lag periods before the onset of inhibition of protein synthesis by ricin and the T_{50}'s suggest that either K^+ depletion or the presence of NH_4Cl or nigericin during the intoxication process allows a more rapid and efficient release of ricin from endocytic vesicles to the cytosol.

DISCUSSION

The pertinent findings of the present study can be summarized as follows: K^+ depletion results in (*a*) an enhancement of cytotoxicities of ricin and *Pseudomonas* toxin in CHO cells and a reduction of the cytotoxicity of diphtheria toxin, and (*b*) an enhancement of Na^+ uptake through amiloride-sensi-

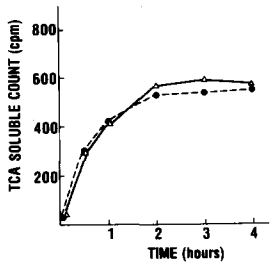


FIGURE 8 Effect of K^+-free medium on the degradation of ^{125}I-ricin in CHO *pro* cells. CHO *pro* cells in suspension culture were subjected to a 5-min hypotonic shock, then a 30-min incubation in isotonic α-MEM with or without potassium at 37°C. The cells were then incubated with ^{125}I-ricin (2 × 10^5 cpm/1 × 10^6 cells per ml) for 60 min, and the surface-bound ^{125}I-ricin was removed by washing with 0.1 M galactose. The cells were then suspended in α-MEM with or without potassium and incubated at 37°C in plastic tubes precoated with bovine serum albumin (1 × 10^6 cells/tube per 0.5 ml). After different intervals, cells were centrifuged and the 5% trichloroacetic acid-soluble count in the supernatant was measured. △, normal medium; ●, K^+-free medium.

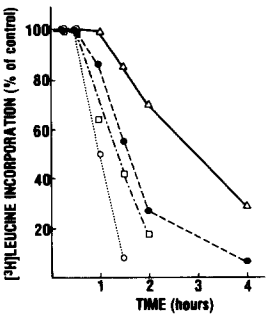


FIGURE 9 Kinetics of inhibition of protein synthesis in CHO *pro* cells in K^+-free medium and normal medium containing NH_4Cl and nigericin. CHO *pro* cells were subjected to a 5-min hypotonic shock, then to a 60-min incubation in isotonic α-MEM with or without potassium. The cells were then exposed to 1 μg/ml ricin for 10 min. After 10 min, the surface bound ricin was removed by treatment with 0.1 M galactose. After different intervals, the cells were labeled with [^{3}H]leucine for 30 min. When the kinetic studies were carried out in the presence of NH_4Cl and nigericin, the cells were preincubated with either of these chemicals for 60 min. △, normal medium; ●, K^+-free medium; □, normal medium + 10 mM NH_4Cl; ○, normal medium + 10^{-8} M nigericin.

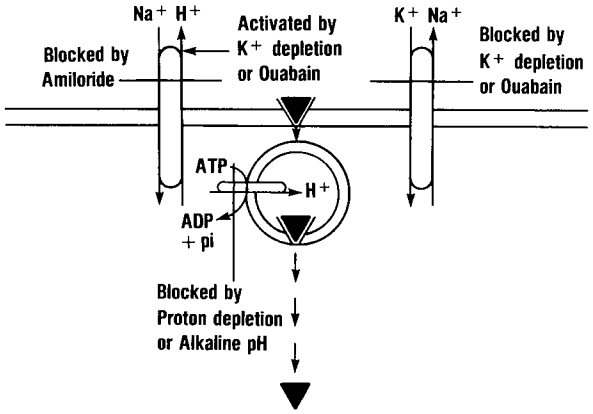


FIGURE 10 Schematic diagram of receptor-mediated endocytosis of ricin and a proposed mechanism for the enhancement of ricin cytotoxicity by K^+ depletion in CHO cells. ▼, ricin; V, ricin receptor.

tive Na^+/H^+ exchange system. The enhancement of ricin cytotoxicity of K^+ depletion requires a functional Na^+/H^+ antiport system, since amiloride and amiloride analogues, which are known to be potent inhibitors of the Na^+/H^+ exchange system, prevent the enhancement of ricin cytotoxicity and the increase in intracellular Na^+ concentration in K^+-depleted cells. The enhancement of ricin cytotoxicity by K^+ depletion can be reversed by the addition of K^+, Rb^+, or Cs^+, to the K^+-free medium but not by the addition of Li, and the reversal of these cations requires a functional Na^+/K^+ ATPase. Finally, K^+ depletion and NH_4Cl appear to alter the cytotoxicities of ricin, diphtheria toxin, and *Pseudomonas* toxin by a similar, if not identical, mechanism, inasmuch as their effects are qualitatively the same for *Pseudomonas* toxin and diphtheria toxin and are neither additive nor synergistic for ricin.

K^+ depletion may enhance the cytotoxicity of ricin in CHO cells by affecting one of the steps in the delivery of ricin from the cell surface receptors to the cytosol, an overall process usually referred to as receptor-mediated endocytosis. Neither binding nor internalization of ^{125}I-ricin into CHO cells appears to be affected by K^+ depletion. Nor can the enhanced cytotoxicity of ricin be attributed to a reduced degradation of ricin by CHO cells in K^+-depleted medium. On the other hand, both the lag period before the onset of inhibition of protein synthesis and the time required to achieve 50% inhibition by ricin were reduced by K^+ depletion. These results suggest a more rapid and efficient release of ricin from endocytic vesicles into the cytosol in CHO cells incubated in K^+-depleted medium than those in normal medium.

Fig. 10 depicts a working model that may account for these observations. K^+ depletion results in an influx of Na^+ through the amiloride-sensitive Na^+/H^+ exchange system, which extrudes protons from the cytosol. Soon after the formation of endosomes, these vesicles, which may contain various ligands are acidified, presumably by an ATP-dependent proton pump (2, 3). The depletion of protons in the cytosol would inhibit the acidification of endosomes by the ATP-dependent proton pump. Consequently, endosomes containing ricin would encounter an alkaline pH, which is optimal for the release of ricin into the cytosol. Amiloride and amiloride analogues, which are known to be potent inhibitors of the Na^+/H^+ exchange system, inhibit the extrusion of protons from the cytosol, thereby preventing the effect of K^+ depletion. This is in agreement with the report that the recovery of cells after acid load is inhibited by amiloride (23, 29, 32). Our model stipulates that the effect of K^+ depletion is mediated by a depletion of protons in the cytosol. Three predictions follow: (*a*) the effects of K^+ depletion and NH_4Cl on ricin cytotoxicity should be similar; (*b*) the effects of K^+ depletion on ricin cytotoxicity and on diphtheria toxin cytotoxicity should be opposite, since it has been shown previously that the cytotoxicity of diphtheria toxin requires acidic pH, whereas the optimal pH for ricin cytotoxicity is alkaline (7); and (*c*) the prevention of enhanced ricin cytotoxicity in K^+ free medium by amiloride or amiloride analogues could be reversed by NH_4Cl. The results described in this paper verify these predictions. Our results appear to contradict the earlier report by Larkin et al. (34) that depletion of intracellular K^+ results in an arrest of coated pit formation. Since it has been reported that *Pseudomonas* toxin is internalized through coated pits (49), it is possible that there may be more than one kind of coated pits; alternatively, the mechanism of formation of coated pits may be different in different cell lines.

Our results suggest that the site of action of nigericin may be distinct from those of K^+ depletion and NH_4Cl. Unpublished data indicate that the effects of nigericin and K^+ depletion on ricin cytotoxicity are additive, and the same is true for the effects of nigericin and NH_4Cl on ricin cytotoxicity. It appears that ricin-containing endosomes can be affected by nigericin and by NH_4Cl or K^+ depletion in two distinct ways, resulting in a more rapid and efficient release of ricin into the cytosol. Studies aimed at the elucidation of the sites of action of nigrericin and NH_4Cl and their temporal relationships are in progress.

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