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CORRELATION OF METAL TOXICITY WITH IN VITRO CALMODULIN INHIBITION

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A fundamental biochemical process that is directly and universally related to the toxicity of metals and metal compounds has yet to be identified. Results of studies reported here indicate that the toxicity of a series of divalent metal cations correlates well with the metals' ability to inhibit the Ca^{2+} receptor protein calmodulin ($r = 0.986$). Because calmodulin regulates a variety of cellular enzymes and processes including intracellular Ca^{2+} concentrations, calmodulin inhibition may have value for predicting metal toxicity and for revealing information about the mechanism by which metals induce toxic effects.

There are few, if any, satisfying hypotheses of a unifying mechanism of metal-induced cell damage and death. One recent example was a report relating the toxicity of a series of metal cations to their chemical softness (1). Although this approach indicated some predictive value, the biological significance of metal softness is questionable, especially with respect to alkaline earth metals (1). However, the ranked toxicity data (mouse LD_{50} values) reported by Williams et al. (1) prompted us to compare this order with a ranking of metals according to their ability to mimic Ca^{2+} in stimulating calmodulin (2). Calmodulin is an intracellular Ca^{2+} receptor protein which regulates a variety of cellular enzymes and processes including cyclic nucleotide phosphodiesterase, adenylate cyclase, phospholipase A_2, Ca^{2+}-ATPase, phosphorylase kinase, neurotransmitter release, phosphorylation of membranes, the disassembly of microtubules, and Ca^{2+} transport (3). Because alterations in cellular Ca^{2+} fluxes have been postulated to be involved in steps leading to irreversible cell damage (4,5), and because calmodulin participates in the regulation of these fluxes (6), the relationship between toxicity and alterations in calmodulin activity was explored. We have determined that the mouse LD_{50} of divalent metal cations (1) corre-

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lates well with the IC_{50} for metal-induced calmodulin inhibition in vitro (7).

MATERIALS AND METHODS

Bovine brain calmodulin was the generous gift of Dr. R. Wallace, University of Alabama, Birmingham, AL. Bovine brain cyclic 3',5'-nucleotide phosphodiesterase was prepared according to the method of Wallace et al. (3). Salts of lead [$(CH_3CO_2)_2Pb \cdot 3H_2O$], palladium ($K_2PdCl_4$), platinum ($K_2PtCl_4$), and strontium ($SrCl_2 \cdot 6H_2O$) were obtained from Aldrich Chem. Co., Milwaukee, WI. All other salts ($BaCl_2 \cdot 2H_2O$, $BeSO_4 \cdot 4H_2O$, $CaCl_2$, $CdCl_2$, $CoCl_2 \cdot 6H_2O$, $CuCl_2 \cdot 2H_2O$, $MnCl_2 \cdot 4H_2O$, $MgSO_4 \cdot 7H_2O$, $NiCl_2 \cdot 6H_2O$, $HgCl_2$, and $ZnCl_2$) and chemicals were obtained from Sigma Chem. Co. except where noted.

Aqueous solutions of metal salts of varying concentrations were incubated with calmodulin (without added Ca^{2+}) for 18 hr at 25°C. Calmodulin activity was subsequently assayed by measuring stimulation of phosphodiesterase activity (3). The reaction mixture contained Tris-HCl (40 mM, pH 8.0), $CaCl_2$ (50 µM), $MgSO_4$ (5 mM), phosphodiesterase (60 µg), and calmodulin (880 ng) in a final volume of 0.1 ml. Protein concentrations were determined by a modification of the Lowry method (8). The reaction was started by the addition of [^{3}H]cyclic AMP (2 mM, 0.01 µCi, New England Nuclear, Boston, MA). For each metal concentration, basal phosphodiesterase activity was determined in the absence of calmodulin. After a 10 min incubation at 30°C, the reaction was terminated by placing tubes in boiling water for 2 min. After an additional 10 min incubation with 5'-nucleotidase (50 µg) at 30°C, [^{3}H]adenosine was separated from unreacted [^{3}H]cyclic AMP using AG1-X2 resin (33% slurry, pH 5.0; Biorad, Richmond, CA) and quantified by liquid scintillation spectrometry with a Packard A300CD spectrometer. Quench correction was by external standard.

RESULTS

The initial observation of correlation between metal-induced toxicity and calmodulin activity is presented in Table 1. With seven metals common to both data sets, the correlation coefficient is 0.92 ($p < 0.005$).

Table 1. Comparison between published LD_{50} values and calmodulin stimulation for divalent metals

Metal	LD_{50}[1] (mmol/Kg)	Calmodulin Stimulation[2] (units x 10^3)
Cu	0.063	2.42
Zn	0.18	2.87
Ba	0.21	2.27
Ni	0.29	2.43
Co	0.48	3.60
Mn	0.73	3.83
Sr	4.7	5.75
$r = 0.921$ ($p < 0.005$)		

[1] 14-Day mouse LD_{50} from ref. 1. [2] From ref. 2.

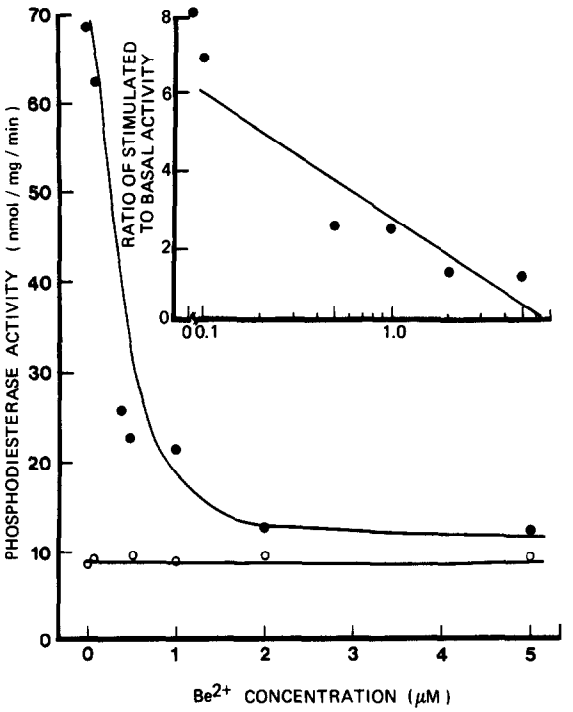

Fig. 1. Effect of Be^{2+} on bovine brain calmodulin in vitro reflected as reduced activation of calmodulin-dependent cyclic nucleotide phosphodiesterase. Closed circles (●) represent calmodulin-stimulated activity and open circles (○) represent basal phosphodiesterase activity. Inset: Log concentration vs ratio of stimulated to basal phosphodiesterase activity yields linear results (r = -0.92), and the IC_{50} is 0.47 mM.

For 13 divalent metals of biologic interest, the IC_{50}'s for calmodulin were determined by linear regression analysis (9) of the log concentration vs calmodulin activation plot. Calmodulin activation was defined as the ratio of calmodulin activity to basal phosphodiesterase activity for each metal concentration. The correlation coefficient for each of the metals examined was between -0.99 and -0.82 ($|r| > 0.82$). An illustrative example is presented for Be^{2+} in Fig 1.

The IC_{50} value for each metal was then compared to the corresponding mouse LD_{50} value (1) by linear regression analysis (9). Table 2 lists the IC_{50} values, LD_{50} values, and softness parameter for each of the 13 metals along with the correlation coefficients obtained from regression analyses of these values. Fig 2 is a graphical representation of the data comparing IC_{50} values and LD_{50} values, excluding Sr^{2+} because of the extremely large

Table 2. Effect of divalent metals on calmodulin activity

Metal	Calmodulin Inhibition IC_{50} (mM)	LD_{50}[1] (mmol/Kg)	σp[2]
Cd	0.47	0.020	0.081
Hg	0.12	0.024	0.064
Cu	0.19	0.063	0.104
Pt	0.43	0.16	0.051
Zn	0.48	0.18	0.115
Ba	1.20	0.21	0.184
Be	0.47	0.23	0.172
Ni	1.34	0.29	0.126
Pb	0.90	0.46	0.131
Pd	1.20	0.47	0.069
Co	1.23	0.48	0.130
Mn	3.81	0.73	0.124
Sr	1059	4.7	0.172

LD_{50} vs IC_{50}	$r = 0.986$	$(p < 0.001)$
LD_{50} vs σp	$r = 0.545$	$(p = 0.05)$
IC_{50} vs σp	$r = 0.386$	N.S.

[1]14-Day mouse LD_{50} from ref. 1. [2]Softness parameter from ref. 1.

difference in the magnitude of values obtained for that cation. However, without Sr^{2+} the correlation coefficient remained quite good ($r = 0.85$).

DISCUSSION

Very little information is available that would suggest an underlying, unifying mechanism of metal-induced cell damage and death, if indeed such a mechanism exists (10). The correlation obtained between divalent metal softness values and mouse LD_{50} values was statistically significant (1), but not as striking as the one obtained herein. The biological significance of the relationship between metal softness and toxicity is open to question. The softness parameter did not adequately predict the toxicity of four me-

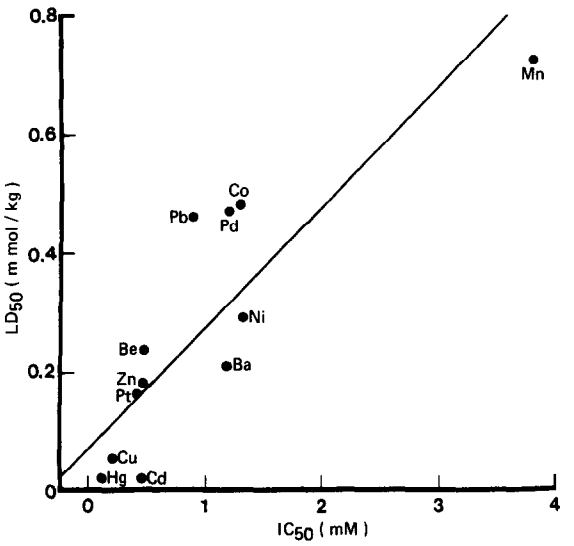

Fig. 2. Relationship between IC_{50} for calmodulin and 14-day mouse LD_{50}. Results for Sr are not included in this graph, however the correlation coefficient remains high (r = 0.846).

tals, Ba^{2+}, Be^{2+}, Pd^{2+}, and Pt^{2+} (1). Williams et al. also pointed out that the correlation between the softness and toxicity of the alkaline earth metals (Ba^{2+}, Be^{2+}, Mg^{2+}, and Sr^{2+}) was not significant and that some other characteristic must distinguish their toxicity (1). All of these metals, Pd^{2+}, Pt^{2+}, and the alkaline earths (with the exception of Mg^{2+}, which was not included in this study because it was used in the phosphodiesterase assay), fit the present regression between calmodulin inhibition and toxicity.

Increasing evidence suggests that alterations in Ca^{2+} fluxes may be an irreversible step in the process of cell death (4,5,11-13). A net flux of Ca^{2+} into the cytosol from either extracellular fluid or a cellular redistribution of Ca^{2+} (4,14) may result from a decrease in the rate of Ca^{2+} exit from the cytosol rather than an increase in Ca^{2+} influx (13,15). Ca^{2+}-ATPase is a calmodulin-dependent enzyme which pumps Ca^{2+} from the cytosol into organelles (mitochondria, sarcoplasmic reticulum) or out of the cell (6). Inhibition of calmodulin activity could result in decreased Ca^{2+}-ATPase activity, which would then decrease Ca^{2+} efflux and result in a net flux of Ca^{2+} into the cell, perhaps leading to cell death.

The results presented in this study indicate that inhibition of calmodulin activity correlates well with mouse LD_{50} values for a series of divalent metals. These results support the hypothesis that calmodulin may be a target for metal induced cell death, but correlation does not necessarily imply a cause and effect relationship (9). Calmodulin inhibition may be superior to chemical softness for predicting the toxicity of divalent metals, and it has the advantage of potential relevance to the mechanism of metal toxicity.

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REFERENCES

1. Williams, M. W., Hoeschele, J. D., Turner, J. E., Jacobson, K. B., Christie, N. T., Paton, C. L., Smith, L. H., Witschi, H. R., and Lee, E. H. (1982) Toxicol. Appl. Pharmacol. 63, 461-469.
2. Teo, T. S. and Wang, J. H. (1973) J. Biol. Chem. 248, 5950-5955.
3. Wallace, R. W., Tallant, E. A., and Cheung, W. Y. (1980) Calcium and Cell Function, Vol. 1, (W. Y. Cheung, ed.) pp. 13-40, Academic Press, New York.
4. Schanne, F. A. X., Kane, A. B., Young, E. E. and Farber, J. L. (1979) Science 206, 700-702.
5. Mergner, W. J., Shamsuddin, A. M. and Trump, B. F. (1981) Basic Mechanisms of Gastrointestinal Mucosal Cell Injury and Protection, (J. W. Harmon, ed.), pp. 3-30, Williams and Wilkins, Baltimore.
6. Vincinzi, F. F. and Hinds, T. R. (1980) Calcium and Cell Function, Vol. 1, (W. Y. Cheung, ed.), pp. 127-165, Academic Press, New York.
7. Cox, J. L. and Harrison, S. D. Jr. (1983) Fed. Proc. 42, 1087.
8. Schacterle, G. R. and Pollack, R. L. (1973) Anal. Biochem. 51, 654-655.
9. Snedecor, G. W. and Cochran, W. G. (1980) Statistical Methods, The Iowa State University Press, Ames, Iowa.
10. Hammond, P. B. and Beliles, R. P. (1980) Toxicology: The Basic Science of Poisons, (J. Doull, C. D. Klaassen, and M. O. Amdur, eds.), pp. 409-467, Macmillan, New York.
11. Gallagher, C. H., Gupta, D. N., Judah, J. D. and Rees, K. R. (1956) J. Pathol. Bacteriol. 72, 193-201.
12. Magee, P. N. (1964) Lab. Invest. 15, 111-131.
13. Judah, J. D. (1969) Brit. Med. Bull. 25, 274.
14. Smith, M. T., Thor, H. and Orrenius, S. (1981) Science 213, 1257-1259.
15. Jewell, S. A., Bellomo, G., Thor, H., Orrenius, S. and Smith, M. T. (1982) Science 217, 1257-1259.