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Environmental pollutant Cd^{2+} biphasically and differentially regulates myosin light chain kinase and phospholipid/Ca^{2+}-dependent protein kinase

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Cd^{2+} was found to mimic effectively, potentiate and antagonize the stimulatory action of Ca^{2+} on myosin light chain kinase (MLCK) and phospholipid-sensitive Ca^{2+}-dependent protein kinase (PL-Ca-PK, or protein kinase C). PL-Ca-PK, however, was slightly less sensitive to Cd^{2+} regulation than was MLCK. Cd^{2+} also biphasically regulates (i.e., stimulation followed by inhibition) phosphorylation, in the homogenates of the rat caudal artery, of myosin light chain and other endogenous proteins catalyzed by MLCK and PL-Ca-PK. The activation by Cd^{2+} of MLCK was inhibited by anticalmodulins (e.g., R-24571), whereas the inhibition by a higher Cd^{2+} concentration of MLCK and PL-Ca-PK was reversed by thiol agents (e.g., cysteine). The present findings may provide one mechanism underlying the vascular toxicity of Cd^{2+}, a major environmental pollutant.

Myosin light chain *Cd^{2+}* *Vascular toxicity* *Protein phosphorylation*

1. INTRODUCTION

A number of in vivo studies have linked exposure to environmental low levels of Cd^{2+} to pathophysiological changes in cardiovascular tissues without manifestations of generalized, overt systemic toxicity of the heavy metal [1,2]. The specific changes include hypertension and depressed cardiac excitability. Exposure to high levels of Cd^{2+}, however, reportedly produces hypotensive or normotensive responses in human or experimental animals [1–4]. The acute in vitro treatments with Cd^{2+} have been shown to decrease contractile response of cardiac muscle [5,6] and aorta [7]. Although several modes of action of Cd^{2+} have been suggested [5–10], the molecular mechanisms underlying the toxic effects of Cd^{2+} on blood pressure remain largely unknown. It has been shown that phosphorylation of smooth muscle myosin P light chain (MLC) by myosin light chain kinase (MLCK, a calmodulin-sensitive Ca^{2+}-dependent protein kinase) causes an increased actomyosin ATPase and muscle contraction [11]. More recent evidence indicates that phospholipid-sensitive Ca^{2+}-dependent protein kinase (PL-Ca-PK, protein kinase C) counteracts the stimulatory effects of MLCK by phosphorylating MLC at different sites [12]. In view of the reported cardiovascular toxicity of Cd^{2+}, investigations into its ability to substitute for Ca^{2+} in the regulation of these two Ca^{2+}-dependent protein kinases seem warranted. We report here that Cd^{2+} biphasically and differentially modulated MLCK and PL-Ca-PK and phosphorylation of MLC in the homogenate of rat caudal artery catalyzed by these two enzymes.

2. EXPERIMENTAL

2.1. *Materials*

Phosphatidylserine (bovine brain) and histone H1 (lysine-rich histone, type III-S) were purchased from Sigma, St. Louis; calmodulin was from Sciogen, Detroit.

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2.2. *Methods*

MLCK was purified to apparent homogeneity from bovine heart as in [13]. PL-Ca-PK was partially purified from bovine heart through the Sephacryl step [14]. MLCK (0.15 μg) was assayed essentially as in [13]. Briefly, the reaction mixture (0.2 ml) contained 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 0.05 mM EGTA, 1 mM 2-mercaptoethanol, 80 μg cardiac MLC, 100 μM [γ-^{32}P]-ATP (containing about 1×10^6 cpm), 0.5 μg calmodulin, and various concentrations of $CdSO_4$ and/or $CaCl_2$, as indicated. PL-Ca-PK (5.0 μg) was assayed as in [14]. Briefly, the reaction mixtures (0.2 ml) contained 50 mM Pipes (pH 6.5), 10 mM $MgCl_2$, 0.05 mM EGTA, 1 mM 2-mercaptoethanol, 20 μg histone H1, 5 μM [γ-^{32}P]ATP (containing about 1×10^6 cpm), and various concentrations of $CdSO_4$ and/or $CaCl_2$, as indicated. The reactions for the enzymes were carried out at 30°C for 5 min. Cd^{2+} was without effect on MLCK and PL-Ca-PK in the absence of calmodulin and phosphatidylserine, respectively. To study the ability of Cd^{2+} to substitute for Ca^{2+} in the activation of MLCK and PL-Ca-PK and phosphorylation of their endogenous substrates in vascular smooth muscle, rat caudal artery (160 mg) was cut into small pieces and homogenized with a glass homogenizer in 1 ml of 50 mM Tris–HCl (pH 7.5) containing 10% glycerol, 50 mM 2-mercaptoethanol and 2 mM EGTA. The homogenate (30 μg protein), serving as the source of endogenous protein kinases and their substrates, was incubated in 0.2 ml of 50 mM Tris–HCl (pH 7.5) containing 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.05 mM EGTA, 14 μM [γ-^{32}P]ATP (containing 2×10^7 cpm) and various additions as indicated in fig.2. The reaction was carried out at 30°C for 15 s. A short reaction time is essential to demonstrate the effects of the activators. Electrophoresis of the phosphoproteins in SDS-polyacrylamide gel and subsequent autoradiography were performed as in [15].

3. RESULTS AND DISCUSSION

Phosphorylation of MLC by MLCK has been shown to increase actomyosin ATPase activity, a mechanism responsible for Ca^{2+}-induced smooth muscle contraction [11]. We found that $CdSO_4$ could effectively substitute for $CaCl_2$ to stimulate nearly maximally MLCK and, moreover, could potentiate the stimulatory effect of a suboptimal concentration of $CaCl_2$ (fig.1A). Unlike $CaCl_2$, the plateau of the MLCK activity was maintained only over a narrow range of $CdSO_4$ concentration; the enzyme activity was markedly inhibited by high concentrations of $CdSO_4$ (fig.1A). The concentration-related biphasic effects of $CdSO_4$ shown

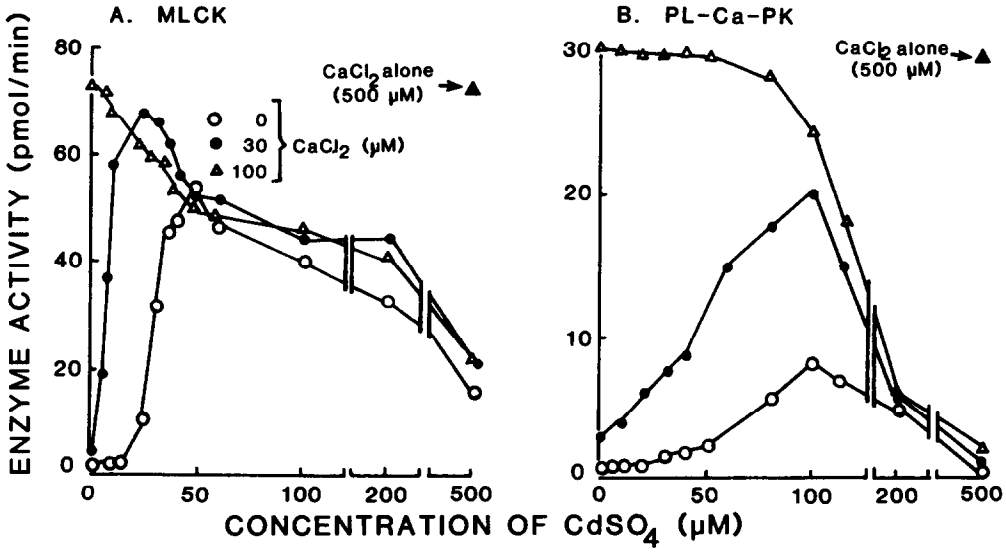

Fig.1. Stimulatory and inhibitory effects of Cd^{2+} on MLCK and PL-Ca-PK assayed in the presence or absence of Ca^{2+}. MLCK and PL-Ca-PK was assayed as described in section 2 with various concentrations of $CdSO_4$ and/or $CaCl_2$.

above seem to be in agreement with previous findings that exposure to Cd^{2+} at low levels produces hypertension whereas at high levels produces hypotensive (or normotensive) responses in rats [1–4]. Our data also could explain in part why high Cd^{2+} concentrations decrease contractile responses of the isolated vascular tissue. It should be noted here that the concentrations of $CaCl_2$ and $CdSO_4$ indicated in fig.1A and all other studies reported herein were those added to the reaction mixtures containing 50 μM EGTA; therefore, they were not the actual free Ca^{2+} and Cd^{2+} concentrations present in the reaction mixtures.

As shown in fig.1B, Cd^{2+} also regulated PL-Ca-PK in a manner quite similar to that seen above for MLCK; one notable difference was that PL-Ca-PK was less sensitive to Cd^{2+} regulation than was MLCK. It has been shown that PL-Ca-PK also phosphorylates smooth muscle MLC but at sites different from those by MLCK [12], resulting in attenuation (about 50%) of actomyosin ATPase activity which is activated when MLC is phosphorylated by MLCK [12]. It appears that Cd^{2+}, like Ca^{2+}, could differentially modulate, in a concentration-related manner, the vascular reactivity via phosphorylation by the two Ca^{2+}-dependent enzymes which have seemingly opposing effects.

The data shown in fig.1 strongly suggest that Cd^{2+} could mimic Ca^{2+} in activating MLCK, presumably by binding to calmodulin in a manner similar to Ca^{2+}. This contention was supported by our findings that calmodulin antagonists, such as trifluoperazine [15], W-7 [16] and R-24571 [17], indeed inhibited to similar extents MLCK activity stimulated either by Ca^{2+}, Cd^{2+}, or both (not shown).

To investigate further the biochemical basis of the vascular toxicity of Cd^{2+}, we examined and compared the effects of Cd^{2+} and Ca^{2+} on phosphorylation of endogenous proteins in the homogenate of rat caudal artery. Autoradiography of the phosphoproteins of such studies indicated that, in the presence of calmodulin, $CaCl_2$ stimulated phosphorylation of MLC (M_r = 20000) and two other proteins having higher M_r of 60000 and 55000 (fig.2). The identities and the functional roles of these higher M_r proteins are unknown; the presence of similar phosphoproteins in tracheal smooth muscle has been reported [18]. In the

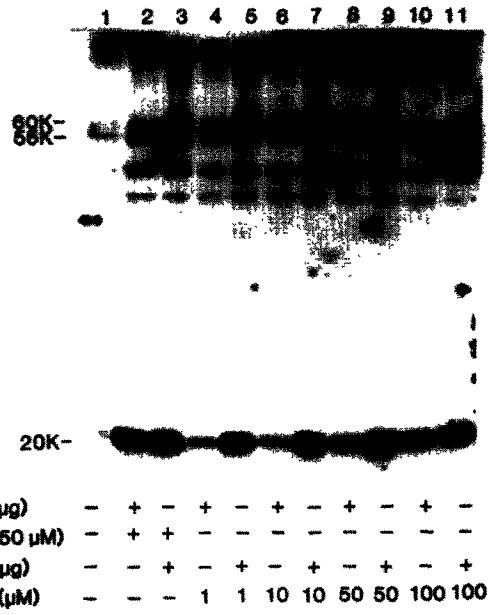

Fig.2. Autoradiograph showing effects of Cd^{2+} and Ca^{2+} on phosphorylation of MLC and other endogenous proteins in rat caudal artery. The homogenate, serving as the source of the endogenous protein kinases and their substrate, was assayed as described in section 2 with various additions of $CdSO_4$, $CaCl_2$, phosphatidylserine (PS) and calmodulin (CaM).

presence of calmodulin, $CdSO_4$ at a concentration as low as 1 μM could effectively replace $CaCl_2$ to stimulate phosphorylation of the 3 proteins. The phosphorylation, however, was inhibited by a high concentration (100 μM) of $CdSO_4$ (fig.2). Although phosphatidylserine appeared to be as effective as calmodulin in supporting the Ca^{2+}-dependent phosphorylation of MLC, it was less effective than calmodulin in stimulating the phosphorylation of the other proteins. Moreover, $CdSO_4$ was less effective than $CaCl_2$ in supporting the phospholipid-sensitive phosphorylation of all endogenous proteins. These findings, consistent with those made with the purified enzyme shown above in fig.1, clearly indicated that Cd^{2+} could regulate the phosphorylation of MLC and other arterial proteins catalyzed by MLCK and PL-Ca-PK.

The interactions of Cd^{2+} with sulfhydryl groups in cellular proteins have been suggested as a possible mechanism by which it produces more

Table 1

Reversal of Cd^{2+} inhibition of MLCK and PL-Ca-PK by cysteine

Addition	[$CdSO_4$] (μM)	MLCK (pmol/min)		PL-Ca-PK (pmol/min)	
		Basal	+ $CaCl_2$	Basal	+ $CaCl_2$
None (control)	0	1.6	55.0	1.5	44.1
	50	46.3	48.2	4.3	46.2
	500	13.0	2.1	0.9	0.7
Cysteine (10 mM)	0	1.8	62.7	1.1	46.4
	50	44.1	61.3	3.9	50.3
	500	52.8	47.0	4.8	30.9

Incubation conditions (in 0.2 ml) were the same as in fig.1, with or without cysteine, $CdSO_4$ and $CaCl_2$, as indicated. EGTA (50 μM) was present in all incubations

generalized cytotoxicity [5,7,19]. In support of this contention is the finding that the Cd^{2+} inhibition of the contractile response of cardiovascular tissues is reversed by cysteine [5,7]. Here, we found that the inhibition of MLCK and PL-Ca-PK by 500 μM $CdSO_4$ was also effectively reversed by 10 mM cysteine (table 1). Other thiol agents, such as 2-mercaptoethanol (10 mM), dithiothreitol (1–5 mM), and 2,3-dimercaptopropanol (0.5–1.0 mM), were also similarly effective (not shown).

Despite investigations over the years, the molecular mechanisms underlying the presser effects of Cd^{2+} are still unclear. The present evidence suggests one mechanism for such effects. We found recently that PL-Ca-PK phosphorylates troponin I and troponin T from cardiac [20,21] and skeletal muscle [21]. It remains to be seen whether regulation by Cd^{2+} of troponin phosphorylation by the enzyme is related to the cardiotoxicity of the metal.

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