Transmembrane Signals and Protooncogene Induction Evoked by Carcinogenic Metals and Prevented by Zinc

Jeffrey Bingham Smith, Lucinda Smith, Vivian Pijuan, Yingxin Zhuang, and Yueh-Chu Chen

Department of Pharmacology, Schools of Medicine and Dentistry, University of Alabama at Birmingham, Birmingham, Alabama

Cd^{2+} provokes an immediate production of inositol trisphosphate and the release of Ca^{2+} from internal stores in human fibroblasts and some other mammalian cells. Ni^{2+}, Co^{2+}, Fe^{2+}, and Mn^{2+} evoke the release of stored Ca^{2+}, but are less potent than Cd^{2+} (apparent $K_{0.5}$ = 40 nM). Zn^{2+} and Cu^{2+} competitively inhibit Ca^{2+} release evoked by Cd^{2+} without affecting Ca^{2+} release by hormones such as bradykinin. Zn^{2+} has the same apparent K_i value (80–90 nM) towards the five agonist metals, which suggests that the metals interact with the same site. Many other divalent cations neither released stored Ca^{2+} nor affected Cd^{2+}-evoked Ca^{2+} release. The agonist metals appear to activate phospholipase C via a G protein rather than a tyrosine kinase. The production of reactive oxygen species is probably not involved in Ca^{2+} release by the metals. Cd^{2+} and other stimuli that raise cytosolic-free Ca^{2+} induce cyclic (AMP) production, apparently by activating a calmodulin-dependent adenylyl cyclase. We suggest that an orphan receptor mediates the hormonelike responses to Cd^{2+} and the other agonist metals. The receptor is referred to as an orphan because its physiological stimulus is unknown. Growth of the fibroblasts in high Zn^{2+} desensitizes them to the five agonist metals without affecting Ca^{2+} release by bradykinin or histamine. A several hour incubation in culture medium with normal Zn^{2+} fully restores responsiveness to the five active metals. Growth in high Zn^{2+} appears to repress the synthesis of the putative orphan receptor because inhibitors of RNA or protein synthesis, or asparagine-linked glycosylation, prevented the restoration of metal responsiveness. Experiments with lectins and neuraminidase support the view that a cell surface sialoprotein mediates Cd^{2+} responsiveness. Cd^{2+} evokes rapid changes in [^{32}P] incorporation by certain proteins, as would be expected for the activation of a phospholipase C-coupled receptor. Cd^{2+} and the other metals that trigger hormonelike messenger production, also induce protooncogenes. These observations have revealed a new target for certain metals which is extraordinary with respect to metal potency and specificity. Additionally, the work reviewed here supports the view that certain metals can promote cell growth, which results in part from the fortuitous induction of hormonelike signals. — Environ Health Perspect 102(Suppl 3):181–189 (1994).

Key words: xenobiotic receptor, inositol, zinc, nickel, iron, manganese, oncogene, cadmium, cyclic AMP, c-*fos*, c-*jun*, c-*myc*, *egr*-1, calcium, protein kinase C, cobalt

Introduction

Cadmium and nickel are modern environmental contaminants that are toxic and carcinogenic (*1–3*). Industrial exposure, food, and cigarette smoking are the major sources of body cadmium and nickel (*2*). Cd^{2+} in whole blood is 5 to 15 times higher in smokers than in nonsmokers in nonoccupationally exposed adults (*4*). Cadmium avidly binds to polythiol groups in proteins such as metallothionein as well as zinc sites in metalloenzymes and transcription factors (*5–8*). Although the substitution of Cd^{2+} for Zn^{2+} in metalloenzymes and DNA-binding proteins may produce a functional enzyme, Cd^{2+} has no known biological role and is regarded as a xenobiotic (*1,5,6*). The functions of nickel are largely confined to enzyme systems of primordial organisms and their close relatives (*9*). Ni^{2+} is a cofactor of three bacterial enzymes—hydrogenases, CO dehydrogenase, and methyl-CoM reductase—as well as bacterial and plant urease (*9*).

Carcinogenicity of Cadmium and Nickel

Cadmium and nickel are carcinogenic in laboratory animals (*1–3*). Occupational exposure to nickel predisposes workers to lung and nasal cancer (*1*). Exposure of rats to an aerosol containing 25 μg/m^3 $CdCl_2$ produced a 50% incidence of lung tumors (*10*). A single subcutaneous injection of 40 μmole/kg $CdCl_2$ in rats produced a high incidence of Leydig cell adenomas in the testes, prostatic neoplasia, and sarcomas at site of injection (*11*). Oral administration of $CdCl_2$ to rats also potently induced tumors in the prostate, testes, and the hematopoietic system (*12*). Cadmium and nickel compounds are inactive or weakly active in gene mutation assays (*2,13,14*). Therefore, epigenetic mechanisms probably play a significant role in the carcinogenicity of Cd^{2+} and Ni^{2+}, although the mechanisms are not well understood.

In vitro treatment of fibroblasts or prostatic epithelial cells with $CdCl_2$ produced transformed cell lines that are tumorigenic (*15,16*). Cultured skin fibroblasts from Indian muntjac are highly sensitive to the toxic effects of Cd^{2+} (*17*). Long-term exposure to low levels of Cd^{2+} produced transformed muntjac cells with normal karyotypes that were 58-fold more resistant to Cd^{2+} than the parental cells (*17*). The development of resistance to Cd^{2+} apparently occurs concurrently with transformation.

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Address correspondence to Dr. Jeffrey B. Smith, Department of Pharmacology, Schools of Medicine and Dentistry, University of Alabama at Birmingham, Birmingham, Alabama 35294. Telephone (205) 934-7434. Fax (205) 934-8240.

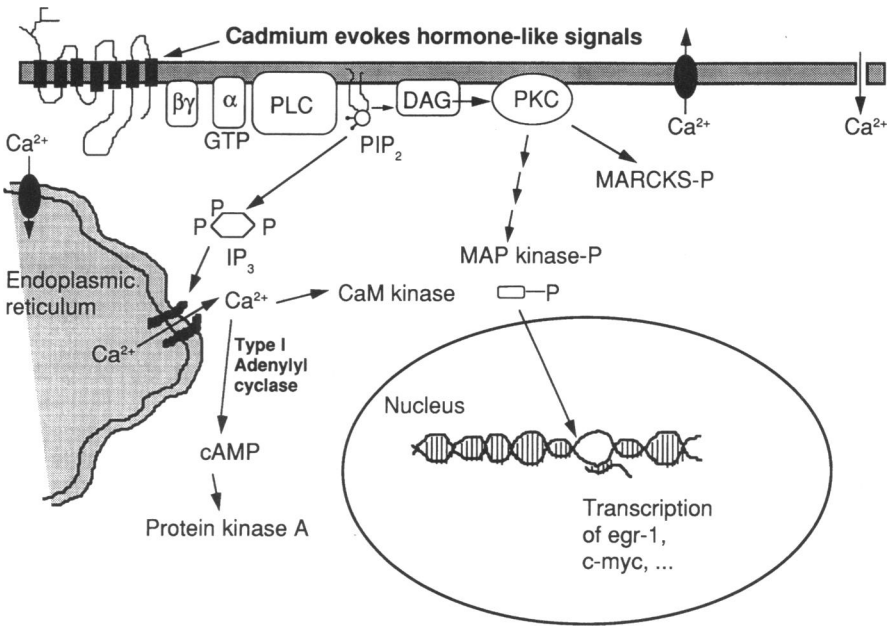

Figure 1. Diagram of transmembrane-signaling events and protooncogene induction via the orphan receptor triggered by cadmium. The key features of the hypothesis are: a) a seven transmembrane domain receptor (upper left) is coupled to an enzyme, phospholipase C (PLC), via an heterotrimeric GTP-binding protein composed of subunits α, β, and γ; b) two second messengers, inositol trisphosphate (IP_3) and diacylglycerol (DAG) are produced simultaneously by the hydrolysis of phosphatidylinositol bisphosphate (PIP_2) when Cd^{2+} binds to a Zn^{2+} site in the external domain of the receptor; c) IP_3 opens an intracellular Ca channel which releases Ca from the endoplasmic reticulum; and d) DAG activates protein kinase C (PKC) which phosphorylates an actin crosslinking protein called myristoylated alanine-rich C-kinase substrate (MARCKS) and translocates from the cytoplasm to the plasma membrane. The diagram depicts the activation of Ca/calmodulin (CaM) and mitogen-activated (MAP) protein kinases, which occur in human fibroblasts stimulated with bradykinin or epidermal growth factor (*99,100*). It is not yet known whether orphan receptor stimuli affect these two kinases. Cd^{2+} induces "immediate/early" protooncogenes (*egr*-1 and c-*myc*) (*33*; Smith, unpublished). The diagram shows the activation of Ca/CaM-activated adenylyl cyclase (Type I adenylyl cyclase), which apparently causes the cAMP increases produced by Cd^{2+}, calcium ionophores, and bradykinin in human dermal fibroblasts (*53*).

Transmembrane Signaling and Cell Transformation

A variety of mitogenic stimuli (e.g., neuropeptides and peptide growth factors) trigger receptors that activate phospholipase C (*18–22*). Phospholipase C activation concomitantly produces inositol trisphosphate (IP_3), which releases stored Ca^{2+}, and diacylglycerol (DAG), which activates protein kinase C (PKC) as illustrated in Figure 1. Heterotrimeric G proteins belonging to the G_q class regulate the β isoform of phospholipase C (*23*). Malignant transformation by several different oncogenes causes alterations in the phosphoinositide pathway (*24,25*). One mechanism responsible for the transformed phenotype may be persistently elevated levels of diacylglycerol (*25,26*). Expression of a continuously activated mutant form of the α subunit of G_q transforms NIH3T3 cells (*27*).

We have proposed that Cd^{2+} may promote tumor development by fortuitously triggering an orphan receptor (*28*). Figure 1 summarizes the key features of the orphan receptor hypothesis. An orphan receptor is one for which the physiological stimulus is unknown. The putative orphan receptor was provisionally called a "Cd^{2+} receptor" because Cd^{2+} was the most potent stimulus known (*28*), although it was realized that Cd^{2+} was a xenobiotic and therefore not the physiological stimulus. Whether or not the site of action of the metals is a cell surface orphan receptor, the hormone-like responses to the metals appear to be unprecedented and remarkable with respect to metal potency and specificity. Here we review the evidence that Cd^{2+} fortuitously activates an orphan receptor which raises cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) and activates certain protein kinases, including PKC, the target of tumor promoting phorbol esters (*22*). Additionally, we discuss recent observations on the role of the orphan receptor in protooncogene induction by cadmium.

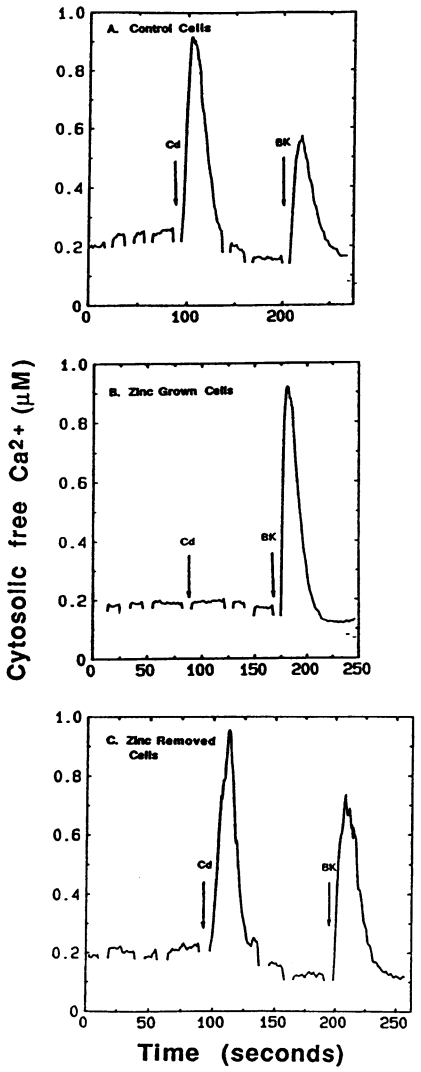

Figure 2. Growth in high Zn^{2+} reversibly abolishes the $[Ca^{2+}]_i$ response to Cd^{2+} without affecting the response to bradykinin. Modified from Smith et al. (*55*). Cd^{2+} (1 μM) or 0.1 μM bradykinin (BK) was added as indicated by the arrows. The other interruptions of the tracings were caused by the time required to remove and replace the physiological salts solution with fresh solution. From Smith et al. (*55*); reproduced with permission of the publisher.

Cadmium Triggers Hormone-like Responses in Certain Mammalian Cells

While investigating Ca^{2+} release from intracellular stores that was evoked by the replacement of extracellular Na^+ (*29*), Dwyer tested Co^{2+} and Ni^{2+} as potential inhibitors. Co^{2+} or Ni^{2+} alone produced an immediate and marked release of stored Ca^{2+} (*29*). Subsequently, a variety of monovalent and divalent cations were surveyed to see if they provoked Ca^{2+} release from internal stores. Cd^{2+} was observed to be the most potent (apparent $K_{0.5}$ of 40 nM) among the 5 metals (Cd^{2+}, Co^{2+},

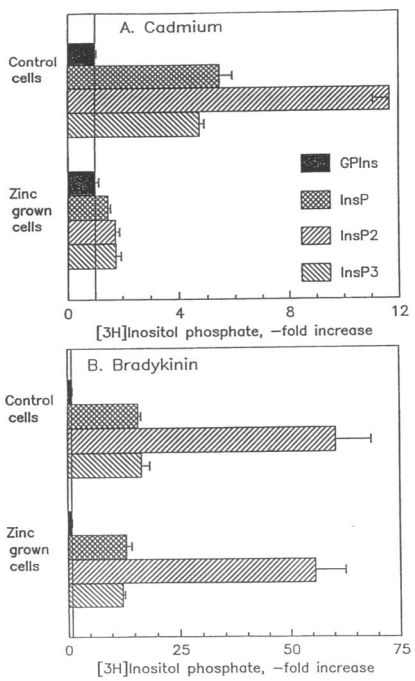

Figure 3. Growth in high Zn^{2+} almost abolishes [^{3}H]inositol phosphate production by Cd^{2+} without affecting the response to bradykinin. Human fibroblasts were grown in culture medium without (control cells) or with (zinc grown cells) 100 μM Zn^{2+} and labeled with [^{3}H]inositol as described (*55*). The indicated cultures were incubated for 1 min with 10 μM Cd^{2+} or 40 nM bradykinin. Growth in high Zn^{2+} had no effect on the basal levels of any of the [^{3}H]inositol phosphates. GPIns, glycerolphosphorylinositol; InsP, inositol monophosphate; InsP2, inositol bisphosphate; InsP3, inositol trisphosphate. From Smith et al. (*55*); reproduced with permission of the publisher.

Ni^{2+}, Fe^{2+}, Mn^{2+}) that mobilized stored Ca^{2+}, which was determined by assaying $^{45}Ca^{2+}$ efflux at 10-sec intervals (*28–30*). Ni^{2+}, Co^{2+}, Fe^{2+}, and Mn^{2+} are 6, 7, 17, and 380 times less potent than Cd^{2+} (*28,30*; Smith, unpublished). Zn^{2+} and Cu^{2+} competitively inhibit Ca^{2+} release evoked by Cd^{2+} with apparent K_i values of 80 and 100 nM, respectively. Zn^{2+} has the same apparent K_i value (80 to 90 nM) towards each of the five "agonist" metals, (Smith, unpublished data). Therefore, the metals appear to bind to the same site. Many other divalent metals including Ca^{2+}, Mg^{2+}, Ba^{2+}, Sr^{2+}, Be^{2+}, and Pd^{2+} neither release stored Ca^{2+} nor inhibit Ca^{2+} release evoked by Cd^{2+} (*28–30*). Additionally several monovalent cations had no effect on Ca^{2+} release.

The potency order of the "agonist" and "antagonist" metals is similar to the Irving-Williams stability order ($Cu^{2+} > Cd^{2+} > Zn^{2+} > Ni^{2+} > Co^{2+} > Fe^{2+} > Mn^{2+}$) for the coordination of divalent metals by compounds containing both nitrogen and oxygen donors (*9*). Notably, the relative potencies of the metals span a 400-fold range from Cd^{2+} to Mn^{2+} as indicated above, which is similar to the range of the stability constants of the metals for model compounds containing both amino and carboxyl groups (*9*).

Cd^{2+} and the other four active metals also evoke Ca^{2+} release in human neuroblastoma cells and dog coronary endothelial cells (*30,31*). The potency order of the metals in the neuroblastoma and endothelial cells is the same as in human dermal fibroblasts (*28–31*). Additionally, Cd^{2+} evokes the release of stored Ca^{2+} in human lung fibroblasts and human aortic and intestinal smooth muscle cells (*31*). Cd^{2+} fails to release stored Ca^{2+} in rat aortic myocytes, rat skin fibroblasts, and human A431 cells (*31*). The target of Cd^{2+}, a putative orphan receptor, may have widespread significance in mammals because it occurs in different cell types and species.

$[Ca^{2+}]_i$ and IP_3 Increases Produced by Cd^{2+} and Other Metals

Cd^{2+} evoked similar several-fold increases in $[Ca^{2+}]_i$ in human fibroblasts and coronary endothelial cells (*28,30*). Moreover, Cd^{2+} produces a $[Ca^{2+}]_i$ spike similarly to bradykinin rather than a hyperbolic rise in $[Ca^{2+}]_i$ (Figures 2, 5). The $[Ca^{2+}]_i$ spike is largely caused by the release of stored Ca^{2+} because Cd^{2+} evoked similar spikes in the presence and absence of extracellular Ca^{2+} (*28,30*). A prior incubation with bradykinin, which depletes the IP_3-sensitive Ca^{2+} store, abolished the effect of Cd^{2+} on $[Ca^{2+}]_i$ (*28*). The initial spike produced by Cd^{2+} is followed by a sustained $[Ca^{2+}]_i$ increase, which is dependent on external Ca^{2+} and probably is caused by Ca^{2+} influx (*28,30*).

$[Ca^{2+}]_i$ was determined on monolayers of fura-2-loaded cells (*28,30*). Fura-2 has a K_d for Cd^{2+} which is greater than 10^{-12} M (*32*), and ~10^5-fold greater than the K_d of fura-2 for Ca^{2+}. Although Cd^{2+} shifts the excitation spectrum of fura-2 similarly to Ca^{2+} (*28,32*), Cd^{2+} would not be expected to dissociate from fura-2 during the time of the Ca^{2+} measurements. Accordingly, Cd^{2+} accumulation by the cells would produce a sustained shift in the excitation spectrum of fura-2. Such a sustained spectral change is produced by incubating the cells with millimolar Cd^{2+} (*28*). In contrast to Cd^{2+}, Fe^{2+} quenches the fluorescence of fura-2. Fe^{2+}, however, produces a $[Ca^{2+}]_i$ spike similarly to Cd^{2+} (*28*).

The addition of 5 or 10 μM Zn^{2+} just prior to 1 μM Cd^{2+} prevented Cd^{2+} from increasing $[Ca^{2+}]_i$ without affecting the $[Ca^{2+}]_i$ response to hormones such as bradykinin (*28,33*). Rinsing the cells with a physiologic salt solution fully restored the $[Ca^{2+}]_i$ response to a subsequent addition of Cd^{2+} (*28,33*). The rapid reversibility inhibition by Zn^{2+} is consistent with the competitive mechanism of Zn^{2+} inhibition discussed above.

Cd^{2+} and the other active metals evoke net Ca^{2+} efflux similarly to bradykinin or angiotensin (*34,35*). The net Ca^{2+} efflux is probably caused by the plasma membrane

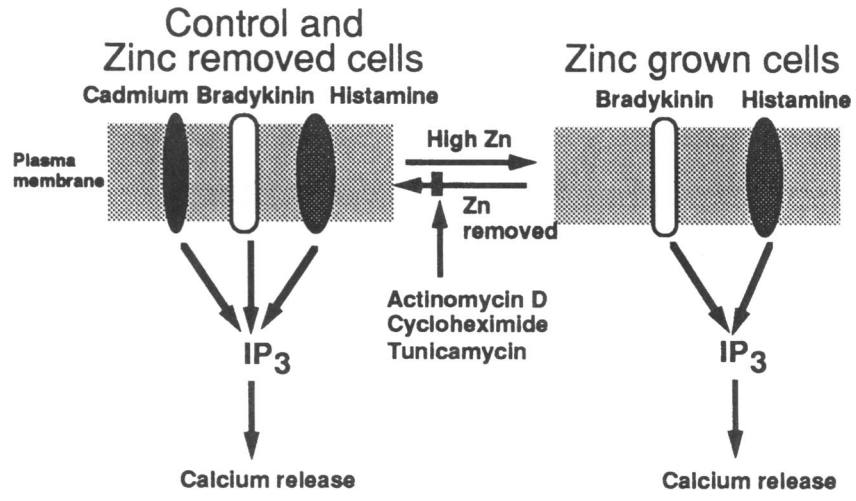

Figure 4. Growth in high zinc selectively and reversibly desensitizes fibroblasts to cadmium. Depiction of a mechanism by which growth in high zinc may selectively and reversibly desensitize human fibroblasts to cadmium and other stimuli of the putative orphan receptor.

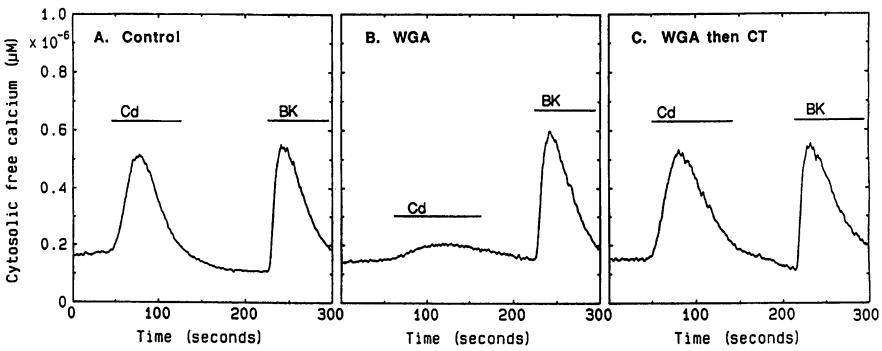

Figure 5. Wheat germ agglutinin (WGA) inhibits the $[Ca^{2+}]_i$ response to Cd^{2+}, and chitotriose reverses the inhibition. The cover glasses of fibroblasts were loaded with fura-2 and incubated for 30 min in the absence panel (A) or presence panels (B and C) of 50 μg/ml WGA. Then the cover glasses were incubated for 4 min without panels (A and B) or with 200 μg/ml chitotriose. The horizontal bars show the duration of the incubation of the cells with 0.1 μM Cd^{2+} or 40 nM bradykinin (BK). (*39*; reproduced with permission of the publisher).

Ca^{2+} ATPase of human fibroblasts (*28, 34*). The endoplasmic reticulum probably does not rapidly reaccumulate the released Ca^{2+} because of the prolonged active state of the IP_3-gated Ca^{2+} channel. Verbost and coworkers have shown that Cd^{2+} inhibits the Ca^{2+} ATPase of inside out red cells by binding to a site in its cytoplasmic domain (*36*). The fact that Cd^{2+} produces a net decrease in the total Ca^{2+} content of fibroblasts indicates that Cd^{2+} neither inhibits the plasma membrane Ca^{2+} ATPase nor markedly increases Ca^{2+} diffusion down its several thousand fold electrochemical gradient.

IP_3 probably causes Ca^{2+} release evoked by Cd^{2+} and the other metals that trigger the release of stored Ca^{2+}. Cd^{2+} (5 μM) increased $[^3H]IP_3$ 3- to 4-fold in 15 sec (Figure 3) (*28*). A 1-min incubation with 20 μM Fe^{2+} or Co^{2+} increased $[^3H]IP_3$ 3- and 5 fold, respectively (*28*). Zn^{2+} abolished the increases in $[^3H]IP_3$ produced by Cd^{2+} or Fe^{2+}. The $[^3H]IP_3$ data agree well with the Ca^{2+} mobilization data with respect to agonist and antagonist metal specificity.

Evidence that Reactive Oxygen Species Are Not Involved in the Ca^{2+}-Mobilizing Response to Cd^{2+}

Oxidative stress is known to increase $[Ca^{2+}]_i$ in some mammalian cells (*37*). Initially we considered the production of reactive oxygen species to be an attractive mechanism of Ca^{2+} mobilization evoked by Cd^{2+} and the other active metals. The following observations, however, indicated that the production of reactive oxygen is not involved in the release of stored Ca^{2+} by the metals. First, production of reactive oxygen species by xanthine oxidase or addition of H_2O_2 (0.11 mM) failed to release stored Ca^{2+} in human fibroblasts (*28*). Second, agents that quench reactive oxygen (superoxide dismutase, mannitol) or antioxidants (butylated hydroxyanisole or butylated hydroxytoluene) had no effect on Ca^{2+} release evoked by Cd^{2+} or Fe^{2+} (*28*). Trump and coworkers (*38*) have reported that $[Ca^{2+}]_i$ increases in renal epithelial cells play a role protooncogene induction by oxidative stress. The role of reactive oxygen species in protooncogene induction by Cd^{2+} has not yet been addressed in human fibroblasts, although, as indicated below, protooncogene induction correlates with the Ca^{2+}-mobilizing response to the metals.

Cd^{2+} Acts at an External Site

The following observations suggest that the "agonist" and "antagonist" metals trigger the release of stored Ca^{2+} by binding to an external site on the cell surface. First, there was no detectable lag between the addition of 0.1 μM $CdCl_2$ and the $[Ca^{2+}]_i$ increase as might be expected for an external site of action (*28,30,39*). Second, loading the cells with a heavy metal chelator (*N,N,N',N'*-tetrakis-(2-pyridylmethyl)-ethylenediamine) did not delay the onset or decrease the extent of Cd^{2+}-evoked $^{45}Ca^{2+}$ efflux (*28,30*). Third, no intracellular Cd^{2+} or Zn^{2+} was detected with fura-2 (*28,30*), whose fluorescence is exquisitely sensitive to these metals (*28,32,40*). Fourth, as described below, a cell surface sialoprotein appears to mediate metal responsiveness (*40,41*). It is unlikely that Cd^{2+} and the other metals that evoke the release of stored Ca^{2+} directly activate phospholipase C. Cd^{2+} potently inhibits one isoform of phospholipase C and has no effect on another isoform (*42*). Furthermore, phospholipase C is an intracellular enzyme which does not span the plasma membrane (*43*).

Cadmium Activates Protein Kinase C

We have observed that a 2 min incubation of human dermal fibroblasts with 1 μM $CdCl_2$ increased the incorporation of $[^{32}P]$ into myristoylated alanine-rich C-kinase

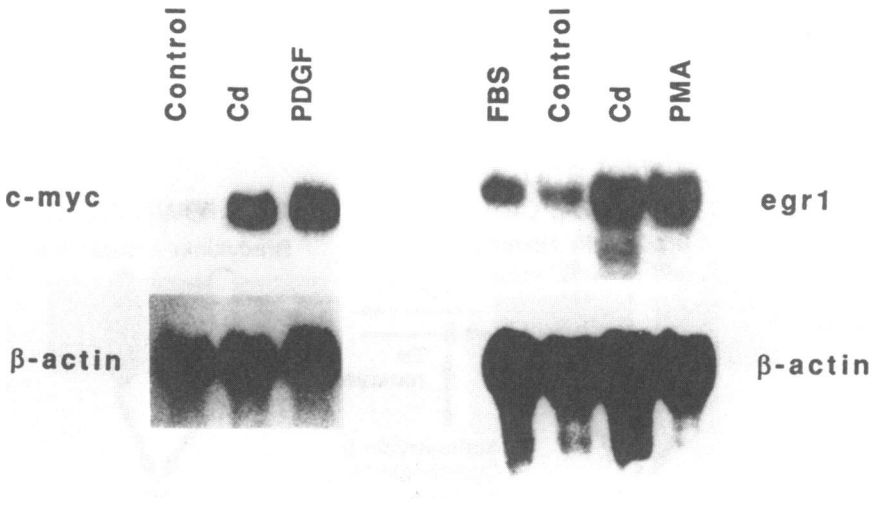

Figure 6. Induction of c-*myc* and *egr*-1 by Cd^{2+}, platelet-derived growth factor (PDGF), fetal bovine serum (FBS), or phorbol myristate acetate (PMA). Human fibroblasts were incubated in a physiologic salt solution containing glucose (*28*) for 1 hr before adding 2 μM $CdCl_2$, 10 ng/ml PDGF, 10% (v/v) FBS, or 0.1 μM PMA. Two hr later, total RNA was extracted and size fractionated on an agarose-formaldehyde gel. The RNA was transferred to a nylon membrane and hybridized to a c-*myc* cDNA probe that was $[^{32}P]$ labeled by the Klenow large fragment of DNA polymerase I primed with random hexamers. The membrane was stripped and reprobed for β-actin as a control for RNA quantity and quality.

Agonists: $Cd^{2+} > Co^{2+} > Ni^{2+} > Fe^{2+} > Mn^{2+}$
$K_{0.5}$ Cd^{2+} = 40 nM
Antagonists: $Zn^{2+} = Cu^{2+}$; apparent K_i = 80 - 100 nM
Second messengers: IP_3 and DAG
Primary effector: phospholipase C
Cell types that express the receptor:
human skin and lung fibroblasts
human aortic and intestinal smooth muscle cells
human neuroblastoma cells
canine coronary endothelial cells and others

Figure 7. Key features of a putative orphan receptor that is activated by cadmium and inhibited by zinc.

substrate (MARCKS) as determined by immunoprecipitation and two-dimensional gel electrophoresis (Chen and Smith, unpublished data). The phosphorylation of MARCKS, an actin cross-linking protein (*44*), is a prominent and widespread response of mammalian cells to mitogenic stimuli (*18,45*). The increase in MARCKS phosphorylation evoked by Cd^{2+} was similar to that produced by phorbol myristate acetate (PMA) or bradykinin. In contrast to Cd^{2+}, $ZnCl_2$ did not affect MARCKS phosphorylation. These findings indicate that Cd^{2+} activates PKC *in vivo* because MARCKS is known to be a specific substrate of PKC in human fibroblasts (*46,47*). Presumably, PKC is activated by DAG produced by bradykinin or stimulation of the putative orphan receptor by Cd^{2+}.

PKC has a Zn^{2+} binding domain (*48*), and Zn^{2+} apparently modulates the interaction of the kinase with the plasma membrane (*49,50*). A 1-hr incubation of mouse fibroblasts with 50 μM Cd^{2+} had no effect on PKC activity, but it potentiated the association of PMA-activated PKC with the nucleus (*51*). Cd^{2+} does not evoke Ca^{2+} release in mouse fibroblasts (Swiss 3T3 cells) (Smith, unpublished data). The influence of Cd^{2+} on the association of PMA-activated PKC with the nucleus of mouse fibroblasts appears to be mechanistically unrelated to the activation of PKC by Cd in human fibroblasts.

Cadmium Increases Cyclic AMP Production

Bradykinin evokes cyclic AMP (cAMP) production in human fibroblasts (*52*). In these cells, $CdCl_2$ (2 μM) increases cAMP production similarly to bradykinin (*53*). Ni^{2+} and Fe^{2+} also increased cAMP, whereas Zn^{2+} did not. Zn^{2+} blocked the effect of Cd^{2+}, but not that of bradykinin, on cAMP production (*53*). Additionally, growth of the cells in high Zn^{2+} reversibly abolished cAMP production by Cd^{2+} without affecting the bradykinin response (*53*). Growth in high Zn^{2+} appears to selectively and reversibly repress the synthesis of the orphan receptor (see the next section). The metal specificity of cAMP production is the same as the metal specificity of Ca^{2+} mobilization. Calcium ionophores (A23187 or ionomycin) also markedly increase cAMP production. Raising $[Ca^{2+}]_i$ may increase cAMP in human fibroblasts via the Ca^{2+}-calmodulin activated (Type I) adenylyl cyclase as depicted in Figure 1 (*54*), although the expression of the Type I cyclase has not yet been directly demonstrated in these cells. By contrast to human fibroblasts, Cd^{2+} has no effect on cAMP in dog coronary endothelial cells, although Cd^{2+} raises $[Ca^{2+}]_i$ in these cells (*53*). Other stimuli that raise $[Ca^{2+}]_i$ in the endothelial cells also had no affect on cAMP, suggesting that they lack the Type I adenylyl cyclase.

Selective Desensitization of Fibroblasts to Cd^{2+} and Other Stimuli of the Putative Orphan Receptor

Growth of human fibroblasts in culture medium containing 100 μM Zn^{2+} selectively and reversibly desensitizes them to Cd^{2+} (*55*). Note that the desensitization produced by growth in high Zn^{2+} is mechanistically distinct from competitive inhibition by Zn^{2+}. Removing the Zn^{2+} immediately reverses competitive inhibition; however, a 10-hr incubation in culture medium is required to restore Cd^{2+} responsiveness to cells that have been grown in high Zn^{2+} (*55*). Figure 4 depicts a plausible mechanism that may account for the desensitization–repression of the synthesis of the putative orphan receptor. Growth in high Zn^{2+} reversibly abolished the $[Ca^{2+}]_i$ response to Cd^{2+} without affecting the $[Ca^{2+}]_i$ response to bradykinin (Figure 4) (*55*). $^{45}Ca^{2+}$ efflux and [3H]inositol phosphate determinations also showed that growth in high Zn^{2+} reversibly and selectively desensitized the cells to Cd^{2+} (*55*). Growth in high Zn^{2+} almost abolished Cd^{2+}-evoked production of [3H]inositol mono-, bis-, and trisphosphate and had no effect on bradykinin-evoked [3H]inositol phosphate production (Figure 3) (*55*). Growth in high Zn^{2+} nearly prevented the stimulation of $^{45}Ca^{2+}$ efflux by Cd^{2+} and had no effect on the stimulation of efflux by bradykinin or histamine (*55*). The half-time for the disappearance of Cd^{2+} responsiveness after adding 100 μM Zn^{2+} was 17 hr (*55*).

Inhibition of RNA or protein synthesis with actinomycin D or cycloheximide, or asparagine-linked glycosylation with tunicamycin (*56*) prevented the restoration of Cd^{2+} responsiveness. Notably, tunicamycin B_1 blocked the restoration of Cd^{2+} responsiveness at 0.1 μg/ml, which only slightly affected leucine incorporation into protein (Chen and Smith, unpublished data). Brefeldin A, which reversibly and selectively disrupts Golgi stacks and prevents post-translational processing of nascent peptides (*57,58*), blocked the restoration of Cd^{2+} responsiveness (*41*). Half-maximal inhibition of the restoration of Cd^{2+} responsiveness occurred at ~10 ng/ml brefeldin A. The subsequent removal of brefeldin A and incubation in culture medium for 8 hr or more fully restored Cd^{2+} responsiveness. Adding Zn^{2+} back to the culture medium at the time of brefeldin A removal prevented the restoration of Cd^{2+} responsiveness (*41*). These findings suggest that asparagine-linked glycosylation is required for the restoration of Cd^{2+} responsiveness to cells that have been grown in high Zn^{2+}.

Zn^{2+} transiently induces metallothionein and heat shock proteins in mammalian cells (*59–61*). It seems unlikely that these proteins are responsible for desensitizing the cells to Cd^{2+} and the other stimuli of the putative orphan receptor (*55*), although this possibility has not been excluded.

A Zn^{2+} Site May Mediate the Hormonelike Responses

We hypothesize that Cd^{2+} activates a putative orphan receptor by binding to a site that is normally occupied by Zn^{2+}. Total Zn^{2+} in plasma ranges from 10 to 20 μM Zn^{2+} in adults (*62*). Most of the Zn^{2+} is loosely bound to plasma proteins, therefore, free Zn^{2+} may be 0.2 to 1 μM. Because the apparent affinity of the metal site for Zn^{2+} is ~0.1 μM, based on its K_i for metal-evoked Ca^{2+} release, the site would be occupied by Zn^{2+} at the levels present in plasma. Recall that Zn^{2+} does not elicit hormonelike responses, but rather competitively inhibits those evoked by Cd^{2+} and the other metals. Therefore, we speculate that Zn^{2+} plays a role in the binding of the physiologic (unknown) stimulus or in receptor internalization or cycling.

There does not appear to be any precedent for the occurrence of a Zn^{2+} site in the external domain of a cell surface receptor, although some cytoplasmic receptors (e.g., estrogen and glucocorticoid receptors) have Zn^{2+} finger motifs (*63*). Human growth hormone contains three ligands that coordinate Zn^{2+}, which forms a dimer

that is stabilized by the metal (*64*). Additionally, Zn^{2+} in the 10 to 50 μM range stabilizes the binding of human growth hormone to the human prolactin receptor (*65*), which contributes one of the four ligands that coordinate the metal. Although the physiological significance of the interaction between growth hormone and the prolactin receptor is unclear, the hormone-receptor "zinc sandwich" is a model system in which Zn^{2+} modulates the binding of a polypeptide hormone to a nonphysiologic receptor. The receptors for prolactin and growth hormone do not have a high affinity site for Zn^{2+} or other metals (*64,65*). Therefore, these receptors do not mediate the hormonelike responses to Cd^{2+}.

Two lines of evidence suggest that the metals interact with histidyl residues. First, decreasing extracellular, not intracellular, pH induces $[^3H]IP_3$ production and Ca^{2+} release in the same cell types that respond to Cd^{2+} (*31*). Moreover, Zn^{2+} desensitizes fibroblasts and endothelial cells to a decrease in external pH as well as to Cd^{2+} without affecting responsiveness to Ca^{2+}-mobilizing hormones (*30,55*). The imidazole group of histidine (pK_a *6–7*) is the principle functional group with a pK_a near the external pH (6.4) which half-maximally induced Ca^{2+} release from internal stores (*30,31*). Histidine is the most common amino acid in Zn^{2+} sites (*5*). Second, dye-sensitized production of singlet oxygen almost abolished Ca^{2+} mobilization evoked by a decrease in external pH as well as Cd^{2+} and the other agonist metals without affecting Ca^{2+} release evoked by bradykinin or thrombin (*66*). Histidine is the most sensitive amino acid to photooxidation.

The Putative Orphan Receptor Is a Plasma Membrane Sialoprotein

Cell-surface receptors for hormones usually contain oligosaccharides, which are attached to asparagine residues in the external domain of the receptor (*67–69*). Cell-agglutinating, sugar-specific lectins bind the *N*-linked oligosaccharides of hormone receptors (*67,70–73*). Chen (*39*) screened a variety of lectins for an effect on Cd^{2+}-evoked Ca^{2+} mobilization. She found that wheat germ agglutinin (WGA) markedly inhibited the $[Ca^{2+}]_i$ and $^{45}Ca^{2+}$ efflux responses to Cd^{2+} (Figure 5) (*41*). One-tenth micromolar WGA half-maximally inhibited Cd^{2+}-stimulated $^{45}Ca^{2+}$ efflux. Extensive rinsing with a physiologic salt solution failed to reverse the inhibition of Cd^{2+}-evoked Ca^{2+} release. A brief incubation with *N,N',N''*-triacetylchitotriose, however, completely reversed the inhibition by WGA (Figure 5) (*39*). Chitotriose has a high affinity for WGA and displaced >90% of fluorescein-WGA that was bound to the cells. WGA neither bound $^{109}Cd^{2+}$ nor affected $^{109}Cd^{2+}$ uptake by the cells (*39*). WGA binds both *N*-acetylglucosamine and sialic acid. Succinylated WGA, which binds only *N*-acetylglucosamine, had no effect on Cd^{2+}-evoked Ca^{2+} release (*39*). These findings indicate that WGA reversibly inhibits Cd^{2+}-evoked Ca^{2+} release by binding to the sialic acid in the external domain of cell-surface protein.

Experiments with neuraminidase have provided further evidence that the Cd^{2+} receptor is a cell-surface sialoprotein. Incubating the cells with neuraminidase (0.075 /ml) decreased the binding of fluorescein-WGA to the cells by ~60% (Chen and Smith, unpublished data). Notably the treatment had no effect on the stimulation of $^{45}Ca^{2+}$ efflux by 0.2 μM Cd^{2+}, but it markedly decreased the inhibition of Cd^{2+}-stimulated efflux by WGA (*41*).

Genistein Blocks the $[Ca^{2+}]_i$ Response to Platelet-derived Growth Factor (PDGF) but Not to Cd^{2+} or Bradykinin

The isoflavone genistein selectively inhibits tyrosine kinases, such as those of the receptors for epidermal growth factor and PDGF (*74,75*). The PDGF receptor kinase activates phospholipase C-γ1 by phosphorylating certain tyrosine residues (*76,77*). Hill et al. (*75*) showed that genistein abolished the $[Ca^{2+}]_i$ response to PDGF without affecting the $[Ca^{2+}]_i$ responses to thrombin, phenylephrine, or ATP. In constrast to PDGF, the receptors for the latter compounds and bradykinin are coupled to phospholipase C via G proteins (*78*). We observed that 40 μM genistein almost abolished the $[Ca^{2+}]_i$ response to PDGF, but only slightly affected the $[Ca^{2+}]_i$ responses to Cd^{2+} or bradykinin (*79,80*). The relative insensitivity of the Cd^{2+} and bradykinin responses to genistein suggests that a G protein coupled receptor, rather than one belonging to the tyrosine kinase family, mediates Ca^{2+} release by Cd^{2+}. Recent studies with herbimycin A, a tyrosine kinase inhibitor (*81*), and staurosporine, which potently inhibits various classes of protein kinases (*82*), indicate that Ca^{2+} release by Cd^{2+} is not dependent on protein kinase activity.

Protooncogene Induction by Cadmium

Protooncogenes such as c-*myc*, c-*jun*, and c-*fos*, are rapidly induced by proliferative stimuli in a variety of diverse biological systems including regenerating liver and human diploid fibroblasts (*18,20,83–86*). Agonists of Ca^{2+}-mobilizing receptors also rapidly induce protooncogenes (*18,21,87*), many of which are regarded as "immediate-early genes" because induction occurs within minutes and is independent of protein synthesis (*88*). Cd^{2+} has recently been shown to increase c-*jun* and c-*myc* transcripts in L6 myoblasts (*89*), TIS genes in Swiss 3T3 cells (*60*), c-*myc* in NRK cells (*90*), and c-*myc*, c-*fos*, and *egr*-1 in human fibroblasts (Figure 6) (*33*).

c-*Fos* and c-*jun* are components of the AP-1 transcription factor, which mediates nuclear events elicited by extracellular stimuli (*91*). Phorbol esters, growth factors, and cytokines activate PKC and induce AP-1 responsive gene expression (*91*). c-*fos* and c-*jun* form a stable heterodimer via a coiled-coil interaction known as a leucine zipper. Phosphorylation regulates c-*jun* both positively and negatively (*92,93*). Thus, Cd^{2+} may induce protooncogenes by activating certain protein kinases.

Role of the Orphan Receptor in Protooncogene Induction by Cadmium

Two paradigms have been used to evaluate whether orphan receptor stimulation contributes to protooncogene induction by cadmium. First, the pharmacologic specificity of agonist and antagonist metals was used to determine whether receptor activation correlated with protooncogene induction. Second, human fibroblasts were grown in high Zn^{2+} to selectively and reversibly desensitize them to orphan receptor stimuli. Both approaches have produced correlative data that support the view that Cd^{2+} induces protooncogene expression, at least in part, by activating the calcium-mobilizing orphan receptor.

Briefly, the following observations implicate the orphan receptor in protooncogene induction by Cd^{2+}. Incubation of human fibroblasts with 0.2 to 2 μM $CdCl_2$ markedly and transiently increased c-*myc* and *egr*-1 expression as determined by northern analysis (Figure 6) (*33*). Cd^{2+}-evoked c-*myc* expression was maximal at 2 hr and then gradually decreased to the level of control cells (*33*). *egr*-1 expression evoked by Cd^{2+} was also transient, but preceded the increase in c-*myc* by about 30 min. Other metals that stimulate the

putative orphan receptor, such as Co^{2+}, Ni^{2+}, and Fe^{2+}, also increased c-*myc* and *egr*-1 transcripts. Zn^{2+} by itself had no effect on c-*myc* expression, but prevented c-*myc* induction by Cd^{2+} or Ni^{2+}. Zn^{2+} had no effect on c-*myc* expression evoked by fetal bovine serum (Pijuan and Smith, unpublished data). It is noteworthy that ferrous iron stimulates the growth of human fibroblasts (Smith, unpublished data) as well as HeLa and mouse melanoma cells by a transferrin receptor-independent mechanism (*94,95*). Further work is needed to determine whether or not the hormonelike responses evoked by Fe^{2+} contribute to the mitogenic response.

Growth of the cells in high Zn^{2+} almost abolished the effect of Cd^{2+} (0.2 to 2 μM) on *egr*-1 and c-*myc* expression (Pijuan and Smith, unpublished data). Incubating the cells for 24 hr in the usual culture medium (Dulbecco's modified Eagle's medium containing 1% fetal bovine serum) completely restored the induction of *egr*-1 and c-*myc* by Cd^{2+}. Growth of the cells in high Zn^{2+} had little or no effect on the induction of c-*myc* by platelet-derived growth factor, forskolin, or PMA. Thus, protooncogene induction correlates with the metal specificity of the orphan receptor as well as reversible manipulation of orphan receptor responsiveness by varying the Zn^{2+} level of the culture medium. Notably Zn^{2+} markedly decreases tumor induction by Cd^{2+} (*96–98*). Further work is needed to evaluate the roles of protein phosphorylation and the putative orphan receptor in protooncogene induction and mitogenic stimulation by divalent metals.

Conclusions

Figure 7 summarizes the principal features of Ca^{2+} mobilization evoked by Cd^{2+} and the other active metals. The following are the criteria on which we base the hypothesis that Cd^{2+} triggers Ca^{2+} mobilization via an orphan receptor: a) the target which mediates Ca^{2+} release exhibits remarkable affinity and specificity for divalent metals; b) the active metals evoke an immediate and marked production of IP_3 and other second messengers similarly to Ca^{2+} mobilizing hormones; c) the second messenger responses to the metals are cell-type specific; and d) the metals appear to act at an external site via a sialoprotein (*28,30,31,39,41,55*). Conclusive validation of the orphan receptor hypothesis awaits the cloning and expression of the putative receptor. The findings reviewed here indicate that two carcinogenic metals, Cd^{2+} and Ni^{2+}, evoke hormonelike responses in certain mammalian cells, apparently by binding to a site on the cell surface which exhibits extraordinary metal affinity and specificity.

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