Induction of c-fos Proto-oncogene in Mesangial Cells by Cadmium*

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Cadmium is mitogenic under some circumstances and has been shown to cause accumulation of transcripts for several proto-oncogenes in a variety of cells, but the mechanism(s) remain to be delineated. Here we show that CdCl₂ causes an increase in c-fos mRNA within 30 min of exposure of mesangial cells. At 10 μM Cd²⁺, this increase persists for at least 8 h in both rat and human cells. The half-life of c-fos mRNA is the same whether it accumulates following 4 h of treatment with Cd²⁺ or is induced transiently by phorbol ester. Cycloheximide, which stabilizes the transcript, causes a synergistic increase when administered with CdCl₂. Nuclear run-on analysis confirms that Cd²⁺ causes transcriptional activation of the c-fos gene. Calmodulin and Ca²⁺/calmodulin-dependent kinase, and classical protein kinase C (PKC) isoforms represent two Ca²⁺-dependent signaling pathways that can lead to induction of c-fos, and Cd²⁺ has been shown to activate both calmodulin and PKC in vitro, possibly by virtue of the similar ionic radii of Cd²⁺ and Ca²⁺. Therefore, we investigated the effect of Cd²⁺ on these pathways in vivo. 10 μM CdCl₂ did not increase total PKC activity or Ca²⁺/calmodulin-dependent kinase II activity and inhibited the latter at higher concentrations, ruling out either pathway in the Cd²⁺-dependent induction of c-fos. However, Cd²⁺ did lead to a sustained activation of the Erk family mitogen-activated protein kinases (MAPK) that correlated with induction of c-fos. A specific inhibitor of the MAPK kinases, PD98059, partially inhibited the induction of c-fos by Cd²⁺. We conclude that Cd²⁺ induces c-fos at least in part by causing a sustained activation of MAPK independent of its ability to activate PKC and calmodulin in vitro.

Cadmium is a carcinogen in animals (1, 2) and has been classified as a category 1 carcinogen in humans by the International Agency for Research on Cancer (3). Oral CdCl₂ induces malignant prostatic tumors in rats (2), and myoblasts treated with Cd²⁺ become transformed and give rise to malignant sarcomas when injected into nude mice (4). At least in part these effects would seem to be a result of Cd²⁺-induced DNA damage. Single strand DNA breaks accumulate in cultured testicular cells treated with CdCl₂ (5). An enhanced rate of mutation in response to a number of chemical mutagens and UV light has been found in the presence of cadmium, supporting the view that Cd²⁺ adversely affects DNA repair (6). Cadmium has been shown to inhibit DNA polymerase β, decrease transcription of O⁶-methylguanine-DNA methyl transferase, increase the UV mutation rate in competent but not repair-deficient xeroderma pigmentosum cells, and decrease the removal of pyrimidine dimers (for review, see Ref. 6).

When quiescent cells enter the cell cycle, expression of immediate early response genes is necessary for progression through G₁ and subsequent proliferation (7). Many of these are proto-oncogenes that encode nuclear transcription factors and determine subsequent expression of other genes. For example, Fos and Jun protein heterodimers constitute the AP-1 transcription factor that leads to transcriptional activation of many genes (8). Cadmium causes accumulation of transcripts of c-jun and c-myc in myoblasts (9); c-fos and egr-1 in fibroblasts (10); c-fos, c-jun, and c-myc in normal rat kidney fibroblasts (11); and c-fos, c-jun, c-myc, and egr-1 in LLC-PK1 proximal tubular cells (12). Thus, Cd²⁺ has the potential to act as a mitogenic stimulus in some cells. Whereas DNA damage is associated with tumor initiation, effects on oncogene expression and mitogenesis are more likely to be associated with promotion and tumor progression (13).

On the other hand, Cd²⁺ can induce apoptosis in isolated T lymphocytes (14) and cultured LLC-PK1 cells (12) and lead to apoptotic cell death in canine proximal tubules (15) and rat testicular tissue (16). Genetic damage triggers apoptosis by a p53-mediated pathway, and a current concept of the action of p53 is that it arrests the cell cycle in late G₁ in response to damage, while DNA repair occurs (17, 18). If repair is unsuccessful within a certain period of time, apoptosis will be initiated. Thus, Cd²⁺ may initiate apoptosis by causing DNA damage and inhibiting DNA repair. Alternatively, mitogenic stimulation by Cd²⁺ against a background of genetic damage may circumvent apoptosis and lead to escape and proliferation of cells destined for transformation. Therefore, Cd²⁺ may be an interesting agent to explore the factors determining the balance between mitogenesis and apoptosis.

Cadmium-related effects on oncogene expression may play a critical role in deciding cell transformation or removal. For example, c-fos expression is part of a mitogenic response that is required for cell proliferation (8). On the other hand, numerous authors have described an association between c-fos expression and apoptosis (19), and overexpression of c-fos in transfected fibroblasts increased the apoptotic response to serum deprivation 10-fold (20). The outcome may in part relate to the time and amplitude of expression and sustained levels of c-fos mRNA appear to favor apoptosis over proliferation. The present study was undertaken to examine the response of c-fos to Cd²⁺ in smooth muscle-like mesangial cells, and the role of two major Cd²⁺-dependent signal transduction pathways involved in c-fos induction.

EXPERIMENTAL PROCEDURES

Materials—Fetal bovine serum (FBS) was obtained from CanSera (Rexdale, Ont.); other cell culture reagents were from Life Technologies.

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1 The abbreviations used are: FBS, fetal bovine serum; CaMK, Ca²⁺-calmodulin-dependent protein kinase; HMC, human mesangial cell; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; RMC, rat mesangial cell; SRE, serum-response element; TPA, 12-O-tetradecanoylphorbol-13-acetate.
Leupeptin, aprotinin, actinomycin D, cycloheximide, staurosporine, thrombin, and RNase-free DNase 1 were products of Pharmacia. Phosphocellulose paper circles (P81, 2 cm) were from Whatman. 

Cell Culture—Rat mesangial cells (RMC) were cultured from glomeruli obtained from kidneys of male Wistar rats, and characterized as described previously (21, 22). They were maintained in 10-cm Petri dishes in a 5% CO2 environment at 37 °C, in RPMI 1640 medium with penicillin, streptomycin, and 10% FBS, passed by trypsinization, and used between the 5th and 15th passages for all experiments reported here. Quiescence was induced by exchanging minimal medium on cells at 60–80% confluence (approximately 5 × 104 cells per well, and after 16 h the FBS content of the medium was decreased to 0.4% FBS followed by 48 h of incubation). Cells were sonicated for 5 s and centrifuged at 100,000 × g for 15 min. Aliquots of cytosol containing 500 μg of protein were preclarified by adding 1 μl of normal rabbit IgG with 20 μl of protein A-Sepharose and centrifuging in a microcentrifuge for 5 min. The supernatant was incubated with 2 μg of anti-Erk-2 antibody (3 h, 4 °C) and immunoprecipitates were recovered by incubation (2 h) with 10% (v/v) 10 mM CaCl2 at 37 °C for 15 min. The sample was subsequently treated with 10% (v/v) 10 mM CaCl2, 1 mM dithiothreitol, 0.5 mg/ml myelin basic protein, 2 μg AMP-dependent protein kinase inhibitor, 50 μM ATP, and 5 μCi of [γ-32P]ATP. After incubation at 30 °C for 30 min, reaction mixtures were added to an equal volume of 2 × sample buffer and subjected to 16% SDS-polyacrylamide gel electrophoresis, according to Laemmli (27). Incorporation of 32P into myelin basic protein was determined by autoradiography and densitometry.

CaMK II Assay—Cells in 10-cm Petri dishes were treated with CdCl2 for various times before being scraped into 800 μl of lysis buffer (50 mM Tris-HCl, pH 7.4, 1% (v/v) Nonidet P-40, 0.25% (w/v) sodium deoxycholate, 150 mM NaCl, 5 mM EGTA, protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin), and phosphatase inhibitors (1 mM Na3VO4, 1 mM NaF). Cells were sonicated for 5 s and centrifuged at 100,000 × g for 15 min. Aliquots of cytosol containing 500 μg of protein were preclarified by adding 1 μl of normal rabbit IgG with 20 μl of protein A-Sepharose and centrifuging in a microcentrifuge for 5 min. The supernatant was incubated with 2 μg of anti-Erk-2 antibody (3 h, 4 °C) and immunoprecipitates were recovered by incubation (2 h) with 10% (v/v) 10 mM CaCl2 at 37 °C for 15 min. The sample was subsequently treated with 10% (v/v) 10 mM CaCl2, 1 mM dithiothreitol, 0.5 mg/ml myelin basic protein, 2 μg AMP-dependent protein kinase inhibitor, 50 μM ATP, and 5 μCi of [γ-32P]ATP. After incubation at 30 °C for 30 min, reaction mixtures were added to an equal volume of 2 × sample buffer and subjected to 16% SDS-polyacrylamide gel electrophoresis, according to Laemmli (27). Incorporation of 32P into myelin basic protein was determined by autoradiography and densitometry.

RESULTS

When RMC are serum-starved (0.4% FBS) for 48 h they become quiescent, reverting to G0, and then when stimulated to re-enter the cell cycle with serum or phorbol ester they begin to express c-fos mRNA within 15 to 30 min (29). To test if Cd2+ could mimic this mitogenic response, quiescent cells were treated with CdCl2 at 1 or 10 μM. Both concentrations caused an appearance of c-fos mRNA in 30 min (Fig. 1). The higher concentration caused a sustained increase that plateaued at 2 h and remained elevated for at least 8 h. HMC showed similar behavior to RMC with respect to accumulation of c-fos mRNA in response to 10 μM Cd2+. The higher concentration of Cd2+ may initiate additional mechanisms affecting c-fos mRNA, or a larger initial response to the higher concentration could trigger a self-sustaining response. Alternatively, 10 μM Cd2+ may cause inhibition of mechanisms (e.g. phosphorylation activity) normally limiting activation of c-fos induction. In keeping with the latter view, TPA (50 nM), an activator of PKC and potent inducer of c-fos mRNA in these cells, caused a transient appearance of c-fos mRNA that peaked at 30–60 min and rapidly declined thereafter (Fig. 1).

Because c-fos mRNA is rapidly degraded, changes in its
hybridization to 18 S cDNA. A typical slot blot is shown in Fig. 4. treatment alone in both RMC and HMC (Fig. 2). This suggested that relative amount of c-fos mRNA by different mechanisms, the rate of decay of the message was measured in actinomycin D-treated cells (Fig. 3). Subconfluent RMC were first treated with CdCl₂ for 4 h or TPA for 30 min, and then RNA synthesis was stopped with actinomycin D (5 μg/ml). Whether c-fos mRNA was induced by TPA or allowed to accumulate in the presence of CdCl₂, it was degraded at a comparable rate. However, cycloheximide prevented any detectable degradation over 90 min (Fig. 3). Because c-fos mRNA turnover is fully blocked by cycloheximide, the large synergistic effect of CdCl₂ plus cycloheximide on the mRNA level cannot be due to further inhibition of degradation by CdCl₂ but must result from induction by CdCl₂ together with prevention of turnover by cycloheximide. To demonstrate transcriptional activation of c-fos by CdCl₂, a nuclear run-on assay was performed. In four separate experiments, labeled transcripts hybridizing to c-fos cDNA in nuclear extracts of cells treated with 10 μM CdCl₂ for 4 h exceeded hybridization from extracts of control cells by 1.8–4.9-fold (mean 3.4-fold) when corrected for loading. Alternatively, cells were treated with phorbol ester (TPA, 50 ng/ml). a, Autoradiograms of the c-fos slots; b, histograms of the intensity ratios of c-fos to 18 S signals determined by laser densitometry for RMC treated with 1 (□) or 10 μM CdCl₂; c, histograms of c-fos mRNA:18 S rRNA ratios for RMC treated with TPA (□) and HMC treated with 10 μM CdCl₂ (■). Each blot is from a single experiment. The results with CdCl₂ are implicitly repeated under several different protocols in subsequent figures and Table 1.

The experiment was performed once with human cells (HMC) and three times with rat cells (RMC). The histogram shows the signal intensities for RMC (mean ± S.D.), corrected for loading by probing for 18 S rRNA, and expressed relative to the value for CdCl₂ treatment alone taken as 100%.

Several kinase inhibitors were used to examine the possible involvement of kinase cascades in the response to CdCl₂ at 4 h. None of the inhibitors alone caused a significant increase in c-fos mRNA above basal levels (data not shown). Genistein, an inhibitor of tyrosine kinases, caused a significant decrease (Table I). Genistein had no effect on the uptake of CdCl₂ by the
Fig. 3. Turnover of c-fos mRNA. RMC were pretreated with either 10 μM CdCl₂ for 4 h (○) or 50 ng/ml TPA for 30 min (●) to allow accumulation of c-fos mRNA. At time 0, 5 μg/ml actinomycin D was added to stop RNA synthesis, and total RNA was isolated at intervals as indicated on the abscissa. Levels of c-fos mRNA were determined by laser densitometry of autoradiograms of Northern blots, corrected for 18 S rRNA taking the value at time 0 as 100%. Values are mean ± S.D. of three separate experiments with TPA and six separate experiments with Cd²⁺. Linear regression lines are shown. A single experiment was done to determine the effect of cycloheximide (4 h, 50 μg/ml) on mRNA stability (□).

Fig. 4. Representative blot from nuclear run-on assays. Quiescent RMC were transferred to serum-free medium (Control) or medium containing 10 μM CdCl₂ for 4 h, and then nuclei were isolated for transcription run-on as described under “Experimental Procedures.” Labeled transcripts were hybridized to immobilized cDNAs for c-fos or 18 S rRNA as indicated and subjected to autoradiography. Bars show the mean ± S.E. of the ratio of intensity of the c-fos signal to the 18 S rRNA signal from four separate experiments.

cells (data not shown). At concentrations that inhibited ionomycin-dependent CaMK-mediated induction of c-fos but did not affect induction in response to serum stimulation,² neither the general CaMK inhibitor, KT 5926, nor the CaMK II-specific inhibitor, KN-93, caused any significant diminution in the response of c-fos mRNA to Cd²⁺. The PKC inhibitor, calphostin C, was also without effect. However, staurosporine, a relatively nonspecific inhibitor of several kinases including PKC (32) and CaMK (33), decreased c-fos induction by about 70%. Therefore, to confirm that neither PKC nor CaMK were involved in the response to Cd²⁺, we measured the activity of PKC and CaMK directly.

When calmodulin is activated, one consequence is autophosphorylation of CaMK II, with the development of autonomous (i.e. calmodulin-independent) CaMK II activity. Transfer of synchronized RMC to serum-free medium with or without 10 μM CdCl₂ resulted in an apparent early increase in autonomous CaMK II activity within about 15 min that nevertheless failed to reach significance (data not shown). Thereafter, autonomous activity declined with time, irrespective of the presence of Cd²⁺, suggesting an effect of serum deprivation rather than Cd²⁺ on basal CaMK II activity. Furthermore, 10 μM Cd²⁺ added to the assay reaction mixture in vitro had no effect on either autonomous or total CaMK II activity (data not shown). This decrease was paralleled by a decrease in total CaMK II activity, measured by activation with the excess Ca²⁺ and calmodulin in vitro, suggesting decreased synthesis or increased degradation of CaMK II in serum-free conditions and indicating that activation of CaMK II by Cd²⁺ is not the mechanism responsible for induction of c-fos by Cd²⁺. To further confirm this, the concentration dependence of autonomous CaMK II activity after 1 h treatment with Cd²⁺ was measured (Fig. 5). After 1 h, when serum deprivation was beginning to cause a decrease in both autonomous and total CaMK II activity, Cd²⁺ exposure caused a dose-dependent decrease in autonomous activity that became significant at 5 μM. Under these conditions, Cd²⁺ had no significant effect on total CaMK II activity, up to the highest concentration tested (20 μM).

When RMC growing in 10% FBS were transferred to serum-free medium, there was an initial decrease in total PKC activity, followed by a rebound to a variable and somewhat higher than control value over several hours. This pattern was unaffected by the presence of Cd²⁺ (data not shown). The increase was insufficient to induce c-fos and does not account for the induction seen with Cd²⁺, for example in Fig. 1, because no increase in c-fos mRNA was detected on Northern blots from serum-free controls. To further rule out activation of PKC by Cd²⁺, cells were treated for 4 h with between 5 and 30 μM CdCl₂ in serum-free conditions. PKC activity was not increased above the serum-free control. Furthermore, a similar treatment with Cd²⁺ failed to augment the increase in PKC activity caused by treatment with TPA (50 ng/ml) during the last 30 min of the 4 h treatment period or to overcome inhibition of PKC by staurosporine (data not shown). In keeping with the failure of calphostin C to inhibit the induction of c-fos by Cd²⁺, PKC appears not to be involved in the process.

Although Cd²⁺ did not activate PKC, it might activate

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Table I

| Treatment | c-fos mRNA/18 S rRNA mean ± S.D. | Significance |
|-----------|-------------------------------|--------------|
| Control   | 100                           | N.S.         |
| Cd²⁺      | 425 ± 38                      | p = 0.02     |
| Cd²⁺ + genistein | 318 ± 34             | N.S.         |
| Cd²⁺ + KN-93 | 528 ± 139            | N.S.         |
| Cd²⁺ + KT 5926 | 407 ± 250            | N.S.         |
| Cd²⁺ + calphostin C | 381 ± 94        | N.S.         |
| Cd²⁺ + staurosporine | 133 ± 35      | p = 0.0003   |

* N.S., not significant.

² Miralem, T., and Templeton, D. M. (1997) Biochem. J., in press.
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FIG. 5. Cadmium concentration and CaMK II activity. RMC were transferred to serum-free medium for 1 h with the indicated concentrations of CdCl₂, from 0 to 20 μM, and autonomous (▲) and total (□) activities were determined as in Fig. 5. Values are mean ± S.D., n = 3, * significantly different from Cd²⁺-free control, p < 0.05 by Student’s t test.

MAPK via other pathways. Therefore, we measured MAPK activity in RMC at different times after treating quiescent cells with 10 μM Cd²⁺. Cadmium caused an increase in immunoprecipitable MAPK activity that peaked at 15 min and then declined but remained above control levels for several hours (Fig. 6). To determine whether a sustained increase in MAPK was responsible for the sustained increase in c-fos mRNA, we used the MEK-1/2-specific inhibitor PD98059. This flavone prevents the activation of MAPK by inhibiting the MAPK kinases, MEK-1 and MEK-2 (34). At 25 μM, PD98059 caused a marked decrease in the level of c-fos mRNA induced by a 4 h treatment with CdCl₂ (Fig. 7). However, it did not prevent the induction completely, nor did higher concentrations show any greater effect.

DISCUSSION

The c-fos gene is well characterized, and its transcription is regulated by two DNA regulatory elements, the cAMP-response element and the serum-response element (SRE) (35, 36). MAPK can be activated by PKC through Raf-1 and MEK (37), and it then phosphorylates the Ets-like transcription factor, Elk, that forms part of the transcription factor complex that binds to SRE (36, 38–40). CaMK II phosphorylates another factor in this complex, the serum response factor (41, 42), so activation of either PKC or CaMK II can induce c-fos through SRE. CaMK II can also phosphorylate the cAMP-response element-binding protein, CREB, and phosphorylation of CREB is itself sufficient to induce c-fos (43). It has recently been shown that c-fos transcription through the SRE can be activated by an increase in cytoplasmic Ca²⁺, whereas CREB is dependent upon intranuclear Ca²⁺ (44). In view of earlier reports that Cd²⁺ can activate both PKC and calmodulin (see below), we considered both as possible candidates for mediating induction of c-fos by Cd²⁺.

The ionic radii of Cd²⁺ and Ca²⁺ are very similar (0.97 and 0.99 Å, respectively (45)), and Cd²⁺ can substitute for Ca²⁺ in a number of Ca²⁺-binding proteins including calmodulin and Ca²⁺-dependent PKC isoforms. Calmodulin is a highly conserved and widely distributed peptide containing four Ca²⁺-binding loops. Occupancy of these sites activates the Ca²⁺-calmodulin complex to interact with a number of other proteins including CaMK II and myosin light chain kinase and in turn affect their activity (46). In vitro Cd²⁺ can induce conformational changes in calmodulin and activate calmodulin-dependent phosphodiesterase (47–49), a property it shares with other metal ions with similar ionic radii (47). Activation occurs even in the presence of physiological concentrations of glutathione (50) but requires a minimum concentration of Cd²⁺ of approximately 10 μM to become significant (47, 48). It has been postulated that increased activity of myosin light chain kinase contributes to the vascular toxicity of cadmium (51), although even higher Cd²⁺ concentrations are required to activate myosin light chain kinase, another Ca²⁺-calmodulin partner, in vitro (51). Intracellular Cd²⁺ concentrations in cadmium-exposed cells are in the range of 1 pM (52, 53) with higher amounts of Cd²⁺ inducing and becoming sequestered by metallothioneins. Therefore, it is unlikely that Cd²⁺ would directly activate calmodulin in vivo, although prevention of Cd²⁺-induced testicular necrosis in mice by a series of calmodulin inhibitors (54) suggests a role of altered calmodulin activity in Cd²⁺ toxicity perhaps secondary to changes in Ca²⁺ metabolism (55). One consequence of calmodulin activation is auto-phosphorylation of CaMK II with the development of autonomous CaMK II activity (56). The failure of Cd²⁺ treatment to activate autonomous CaMK II activity in this study is consistent with insufficient intracellular Cd²⁺ concentrations being attainable to activate calmodulin in cultured RMC.

FIG. 6. Effect of Cd²⁺ on MAPK activity. Quiescent RMC were treated with serum-free medium with or without 10 μM CdCl₂ for the times indicated prior to measurement of MAPK activity. Activity was measured as the ability of an anti-Erk-2 immunoprecipitate to incorporate label from [γ-³²P]ATP into myelin basic protein as described under “Experimental Procedures,” and the autoradiogram is of labeled myelin basic protein after electrophoresis. The histogram shows the band intensity determined by laser densitometry, relative to the value at 15 min of Cd²⁺ treatment taken as 100%. The autoradiogram is from one representative experiment, and the bars show the mean ± S.D. from five separate experiments. Activity in Cd²⁺-treated cells is significantly greater than control cells (p < 0.05 by Student’s t test) at all time points.

FIG. 7. Effect of MAPK kinase inhibition on c-fos induction by Cd²⁺. Quiescent RMC cultures were treated with PD98059 at the indicated concentrations or with 0.1% (v/v) Me₂SO as vehicle control for 1 h prior to addition of 10 μM CdCl₂ where indicated. 4 h after addition of Cd²⁺, total RNA was collected and the c-fos mRNA level was determined by Northern blotting. A representative blot is shown, and the histogram shows the intensity of the signal corrected for 18 S rRNA relative to cultures treated with 10 μM CdCl₂, without inhibitor taken as 100%. Bars are mean ± S.D. from six separate experiments. The decrease in c-fos mRNA is significant (p < 0.03 by Student’s t test) at each concentration of inhibitor relative to Cd²⁺ treatment alone.
The “classical” isoforms (α, β, βII, γ) of PKC are Ca2+-dependent (57). In *in vitro* assays with unfractionated PKC, Cd2+ stimulated PKC activity at concentrations up to 100 μM in low Ca2+ medium and was inhibitory only at higher concentrations (51). Cd2+ also enhanced the interaction of PKC with isolated rat liver nuclei (58) and increased histone S-III phosphorylation significantly at about 30 pm when added to an *in vitro* assay mixture (59). Therefore, in the intracellular environment, any effect of Cd2+ on PKC should be stimulatory. Induction of c-fos in Cd2+-treated proximal tubule cells was partially inhibited by the PKC inhibitor, H-7, and those authors (12) attributed this induction to activation of PKC secondary to a Cd2+-mediated increase in [Ca2+]i, rather than to a direct effect of Cd2+. Although they did not measure [Ca2+]i, Cd2+ binding to an orphan receptor has been reported to cause mobilization of intracellular Ca2+ stores in cultured fibroblasts (60). On the other hand, Epner and Herschman (10) showed that PKC down-regulation with phorbol ester pretreatment did not attenuate induction of several phorbol ester-inducible oncoproteins, which include c-fos, and they concluded that PKC signaling was not involved in the mechanism. In RMC under our experimental conditions, 8 h of exposure to 10 μM CdCl2 caused an increase in [Ca2+]i from 137 ± 25 nM to only 259 ± 59 nM (53), which is insufficient to induce c-fos. Nor did the present study show any effect of Cd2+ on total PKC activity above that caused by a change of medium alone. Together with the failure of the PKC inhibitor, calphostin C, to inhibit c-fos induction, we consider these data to rule out either primary or secondary activation of PKC as underlying c-fos induction by Cd2+ in RMC.

There is no general consensus of opinion on the mechanism(s) involved in the induction of proto-oncoproteins by Cd2+. It has been suggested that metal regulatory elements like those that bind the Zn2+-dependent metal transcription factor, MTF-1, required for metallothionein induction, may be involved in the induction of early response phorbol ester-inducible oncogenes (10), but none have been identified in these genes to date. In fact, only metallothionein (61) and metalresponsive heme oxygenase (62) genes have been found to contain consensus metal regulatory element sequences, and to our knowledge those of heme oxygenase have not been shown to be functional. Zinc can cause accumulation of c-fos mRNA in fibroblasts by increasing mRNA stability secondary to a non-specific effect on protein synthesis (30). Cadmium, under the conditions of our experiments, causes a depression in the rate of protein synthesis but rather suggests an independent mechanism mediated through a signal transduction pathway. Whereas sustained elevation of MAPK activity appears to be a major contributor to induction of c-fos by Cd2+, the incomplete inhibition of induction by PD98059 and the partial inhibition by genistein and staurosporine indicate that additional pathways (mechanisms) also operate.

A notable result of this study is the sustained time of elevation of MAPK activity and c-fos expression in Cd2+-exposed cells. MAPK activation by serum is accompanied by measurable c-fos mRNA levels at 15 min that peak at 0.5–1 h and decline to nearly undetectable levels by 2 h (22). In keeping with a sustained increase in MAPK activity, 10 μM Cd2+ causes a prolonged increase in c-fos mRNA. At lower concentrations of Cd2+ (1 μM) (Fig. 1), the increase in c-fos mRNA appears more transient. Whereas MAPK activity is maximal at 1–2 min after stimulation of quiescent RMC with serum or TPA and declines thereafter (29), the increase in response to Cd2+ reaches a maximum level sometime after 5 min but remains at 60% of the value at 15 min for at least a further 8 h (Fig. 6). This apparent delay in activation relative to serum or TPA likely reflects the rate of entry of Cd2+ into the cells. We have previously found that exposure of RMC to 10 μM CdCl2 produces cytosolic concentrations of ionic Cd2+ of about 1 pm (53), consistent with values reported in other cells (52). Although intracellular cadmium continues to accumulate (31), induction of metallothionein and its sequestration of Cd(II) prevents further increases in cytosolic [Cd2+]. The early activation of MAPK and c-fos transcription suggests that this ionic pool is possible. Persistence of these processes is consistent with kinetically inert Cd2+ binding to an as yet unidentified effector target.

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