

# Endogenous Heavy Metal Ions Perturb Fura-2 Measurements of Basal and Hormone-Evoked $\text{Ca}^{2+}$ Signals

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**ABSTRACT** Using the membrane-permeant chelator of heavy metal ions, *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylene diamine (TPEN), we demonstrate that in pancreatic acinar cells, hepatocytes, and a variety of mammalian cell lines, endogenous heavy metal ions bind to cytosolic fura-2 causing basal cytosolic free  $[\text{Ca}^{2+}]_i$  ( $[\text{Ca}^{2+}]_i$ ) to be overestimated. TPEN had most effect in cells lightly loaded with fura-2, suggesting the presence of a limited pool of heavy metal ions ( $\geq 12 \mu\text{M}$  in pancreatic acinar cells) that does not rapidly exchange across the plasma membrane. In fura-2-loaded hepatocytes, vasopressin failed to evoke a detectable change in fluorescence, but after preincubation of cells with TPEN, it caused fluorescence changes characteristic of an increase in  $[\text{Ca}^{2+}]_i$ . We conclude that in many mammalian cells, a slowly exchanging mixture of cytosolic heavy metal ions binds to fura-2 both to quench its fluorescence and to mimic the effects of  $\text{Ca}^{2+}$  binding, thereby causing basal  $[\text{Ca}^{2+}]_i$  to be overestimated. By chelating endogenous heavy metal ions, TPEN allows basal  $[\text{Ca}^{2+}]_i$  to be accurately measured and, by preventing competition between heavy metal ions and  $\text{Ca}^{2+}$  for binding to fura-2, unmasks the full effect of agonists in increasing  $[\text{Ca}^{2+}]_i$ .

## INTRODUCTION

Current understanding of both intracellular  $\text{Ca}^{2+}$  regulation and its modulation by extracellular stimuli relies heavily on the use of fluorescent  $\text{Ca}^{2+}$  indicators derived from EGTA and BAPTA. The first of these indicators to find widespread use was quin 2, but it has been largely superseded by fura-2, which has many advantages over its predecessor (Tsien, 1989). These powerful tools are not, however, without their pitfalls, many of which were highlighted in the original publications (Tsien et al., 1982; Grynkiewicz et al., 1985; reviewed in Poenie, 1992). The potential problems include inefficient intracellular hydrolysis of fura-2 acetoxymethyl ester (AM) to fura-2 (Roe et al., 1990), dye leakage, extrusion or compartmentalization (Di Virgilio et al., 1990; Roe et al., 1990), the difficulties of reliably calibrating fura-2 fluorescence to cytosolic free  $[\text{Ca}^{2+}]_i$  ( $[\text{Ca}^{2+}]_i$ ) (Poenie, 1992), and interactions of fura-2 with inositol trisphosphate receptors (Richardson and Taylor, 1993). Another potential problem, which has received less consideration, is the interaction of fura-2 with other intracellular cations. The

binding of these cations to fura-2 may either quench its fluorescence (e.g.,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$ ) or cause fluorescence changes similar to those evoked by  $\text{Ca}^{2+}$  binding (e.g.,  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$ ) (Grynkiewicz et al., 1985). Previous work with quin 2-loaded cells suggested that endogenous heavy metal ions could cause  $[\text{Ca}^{2+}]_i$  to be substantially underestimated by quenching quin 2 fluorescence, particularly in cells loaded with low concentrations of the indicator (Arslan et al., 1985). It has been generally assumed that this problem is restricted to certain cells, notably differentiated tumor cell lines (Arslan et al., 1985) loaded with quin 2 (Tsien and Pozzan, 1989).

Although fura-2 binds heavy metal ions with lower affinity (typically  $\sim 10$ -fold lower) than does quin 2 (Arslan et al., 1985), it nevertheless binds many such ions with substantially greater affinity than it binds  $\text{Ca}^{2+}$  (Grynkiewicz et al., 1985). The brighter fluorescence of fura-2 relative to quin 2 allows measurements from cells loaded with lower intracellular concentrations of indicator, but it may also increase the likelihood of endogenous heavy metal ions binding to a significant fraction of the intracellular indicator. There is, therefore, no reason to assume that binding of endogenous heavy metal ions to indicator might not also be a problem in fura-2-loaded cells.

## MATERIALS AND METHODS

### Isolation and primary culture of hepatocytes

Hepatocytes were isolated from the livers of male Wistar rats (150–200 g) by collagenase digestion (Taylor et al., 1989). For studies of freshly isolated cells, hepatocytes were stored for up to 1 h in cold Eagle's medium supplemented with  $\text{NaHCO}_3$  (26 mM) and bovine serum albumin (BSA, 20 mg/ml). They were then resuspended ( $2\text{--}5 \times 10^6$  cells/ml) at  $20^\circ\text{C}$  in Hepes-buffered medium (HBM:  $\text{NaCl}$ , 120 mM;  $\text{KCl}$ , 5.9 mM;  $\text{MgCl}_2$ , 1.2 mM;  $\text{CaCl}_2$ , 1.5 mM; glucose, 11.5 mM; Hepes, 27 mM, pH 7.4) supplemented with BSA (1 mg/ml) and fura-2 AM (1  $\mu\text{M}$ ). After 10 min, the cells were washed twice and kept in oxygenated HBM containing BSA (1

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**Abbreviations:** AM, acetoxymethyl ester; AVP, Arg<sup>8</sup>-vasopressin; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid;  $[\text{Ca}^{2+}]_i$ , cytosolic free  $\text{Ca}^{2+}$  concentration; CCK-8, cholecystokinin octapeptide; DTPA, diethylene triaminepentaacetic acid; EGTA, ethylene glycol-bis( $\beta$ -aminoethyl ether) *N,N,N',N'*-tetraacetic acid;  $F_{340/380}$ , fura-2 fluorescence ratio at excitation wavelengths of 340 and 380 nm; HBM, Hepes-buffered medium; Hepes, N-[2-hydroxyethyl]piperazine-*N'*-2-ethanesulfonic acid;  $\lambda_{ex}$ ,  $\lambda_{em}$ , excitation and emission wavelengths, respectively; Pipes, piperazine-*N,N'*-bis[2-ethanesulfonic acid]; TPEN, *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylene diamine.

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mg/ml) at 20°C for up to 1 h. Using this protocol to load hepatocytes in suspension, ~65% of the cellular fura-2 fluorescence was from cytosolic fura-2 (Fig. 2). Hepatocytes were used in suspension (Fig. 1, Table 1), after a 30-min incubation to allow attachment to coverslips (Fig. 2) or after primary culture (Table 1).

Primary cultures of hepatocytes were prepared by first separating hepatocytes from nonhepatocytes by centrifugation through medium (NaCl, 116 mM; KCl, 5.4 mM; MgSO<sub>4</sub>, 0.8 mM; NaH<sub>2</sub>PO<sub>4</sub>, 0.96 mM; NaHCO<sub>3</sub>, 25 mM; CaCl<sub>2</sub>, 1.8 mM; glucose, 15 mM; O<sub>2</sub>:CO<sub>2</sub>, 95:5; pH 7.4) containing Percoll (density = 1.08 g/ml). The cells were resuspended in Williams' medium E (1.7 × 10<sup>5</sup> cells/ml) supplemented with penicillin (87 units/ml), streptomycin (87 μg/ml), neomycin (87 μg/ml), and insulin (8.7 μg/ml), L-glutamine (3.5 mM) and fetal calf serum (8.7%). Cells (2 ml) were plated onto two glass coverslips (22 × 9 mm, Number 2) in a 35-mm plastic culture dish and allowed to attach for 60 min; the medium was then replaced with supplemented Williams' medium E containing cytosine arabinoside (10 μM). After 24 h at 37°C in 5% CO<sub>2</sub>, the medium was replaced with supplemented Williams' medium E lacking cytosine arabinoside, and after a further 1–2 days in culture, the cells were used for experiments. Cultured hepatocytes were preincubated (10 min) in cold loading medium (NaCl, 135 mM; KCl, 5.9 mM; MgCl<sub>2</sub>, 1.2 mM; CaCl<sub>2</sub>, 1.5 mM; Hepes, 11.6 mM; glucose, 11.5 mM, pH 7.3) (Roe et al., 1990) and then loaded with fura-2 by incubation at 20°C in the same medium supplemented with fura-2 AM (2 μM), BSA (1 mg/ml), and Pluronic F-127 (200 μg/ml) for 2 h. The fura-2-loaded hepatocytes were then washed and incubated for a further 1–3 h at 20°C to allow complete hydrolysis of the intracellular fura-2 AM. This method optimized conditions for culture of primary hepatocytes, while minimizing contamination with other cell types. It allowed the cells to be reliably loaded with fura-2 AM with 80 ± 1% (*n* = 8) of the indicator in the cytosol, and the cells responded to a range of agonists (Arg<sup>8</sup>-vasopressin, ATP, angiotensin II) and thapsigargin with an increase in [Ca<sup>2+</sup>]<sub>i</sub> that resulted from both mobilization of intracellular Ca<sup>2+</sup> stores and stimulated Ca<sup>2+</sup> entry (not shown).

### Isolation of pancreatic acinar cells

Acinar cells were isolated from pancreata removed from male outbred TO mice (40–45 g) (Osipchuk et al., 1990). Briefly, an isolated pancreas was perfused with 2 ml of HBM containing collagenase (200 units/ml) and soybean trypsin inhibitor (1 mg/ml), and then incubated in the same medium for 20–30 min at 37°C. The digestion was terminated by fivefold dilution of the cell suspension into HBM containing BSA (0.1 mg/ml) and soybean trypsin inhibitor (0.1 mg/ml). Cells were dispersed by gentle pipetting and then washed by centrifugation before loading with fura-2 by incubation with fura-2 AM (1 μM) in HBM for 10 min at 20°C. At the end of the loading period, the cells were washed, incubated at 20°C for 15 min

**TABLE 1 Effects of TPEN on fura-2 fluorescence in unstimulated mammalian cells**

	Initial fluorescence/ fluorescence after TPEN addition at λ <sub>ex</sub> (%)			Initial F <sub>340/380</sub> / F <sub>340/380</sub> after TPEN addition (%)
	340 nm	360 nm	380 nm	
Pancreatic acinar cells	82 ± 1	72 ± 1	47 ± 0.5	184 ± 7 (42)
Hepatocytes				
Fresh	86 ± 3	81 ± 1	70 ± 2	122 ± 3 (4)
Cultured	92 ± 4	81 ± 4	68 ± 4	136 ± 3 (3)
BC <sub>3</sub> H1 cells				
Undifferentiated	66 – 75	63 – 70	56 – 67	112 – 116 (2)
Differentiated	79 ± 2	69 ± 3	48 ± 10	197 ± 50 (4)
HEK293 cells	67 ± 2	69 ± 3	63 ± 2	106 ± 1 (3)
CPAE cells	79 ± 3	79 ± 5	78 ± 8	108 ± 15 (5)

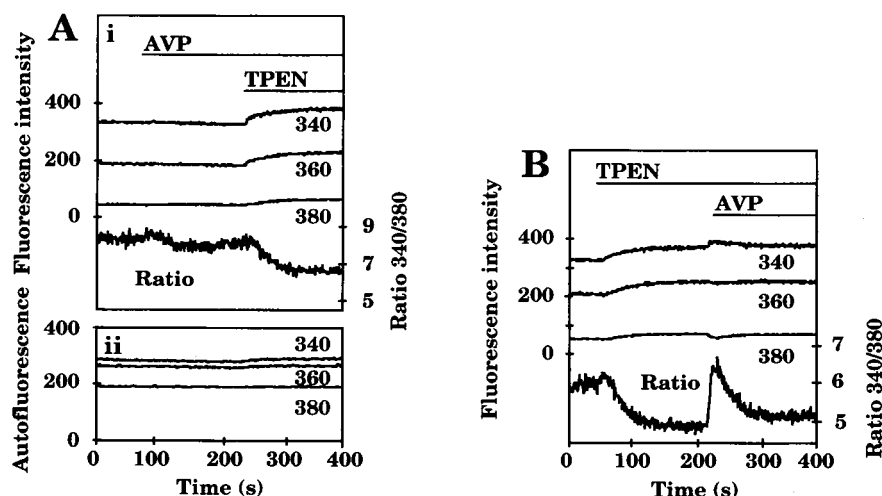
The table shows the effects of TPEN on the fluorescence of fura-2 in a variety of unstimulated cells. The fluorescence intensity of fura-2 in populations of each of the cell types shown was measured before (initial fluorescence) and 4 min after addition of TPEN (100 μM), except for cultured hepatocytes, which were incubated with TPEN (20 μM) for 20 min. All fluorescence intensities were corrected for autofluorescence. Fluorescence intensities and F<sub>340/380</sub> measurements before TPEN addition are expressed as percentages of their values after TPEN addition. Results are expressed as means ± SEM of (*n*) independent determinations.

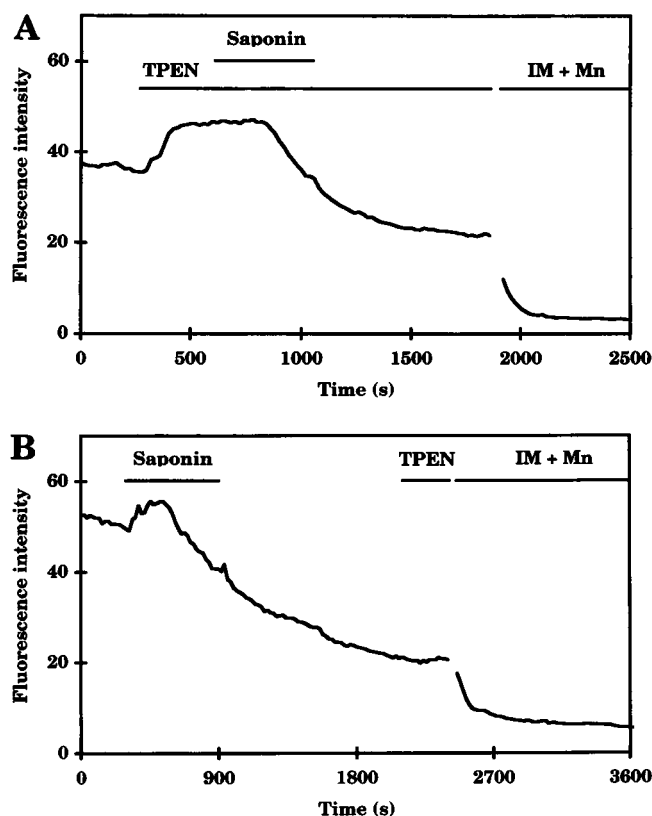
to allow de-esterification of the indicator and then stored for up to 3 h at 4°C; similar results were obtained from cells stored at 20°C. Cells were either used in suspension (Figs. 3, 4, 5 C, and 6) or after a 30-min incubation to allow attachment to coverslips (Fig. 5, A and B). Cell viability, assessed by trypan blue exclusion, was ≥95%, and ~90% of the intracellular fura-2 was cytosolic.

### Cell culture

BC<sub>3</sub>H1 cells (passages 13–16 after receipt) were grown (37°C, 5% CO<sub>2</sub>) on glass coverslips in Dulbecco's modified Eagle's medium supplemented with fetal calf serum (20%), penicillin (85 units/ml), streptomycin (85 μg/ml), L-glutamine (3.5 mM), and nonessential amino acids (Broad et al., 1996). The cells were used either while still subconfluent and in their undifferentiated state or after differentiation to smooth muscle-like cells (Spizz et al., 1986) by further incubation in medium containing 0.5% serum

**FIGURE 1** AVP-evoked increases in [Ca<sup>2+</sup>]<sub>i</sub> are detectable in freshly isolated fura-2-loaded hepatocytes only after pretreatment with TPEN. Suspensions of fura-2-loaded hepatocytes were treated with AVP (100 nM) before (A(i)) or after (B) preincubation with TPEN (100 μM). The results, typical of recordings from at least three independent cell preparations, show the fluorescence (in arbitrary units and corrected for autofluorescence) recorded at each of the three excitation wavelengths (λ<sub>ex</sub> = 340, 360, and 380 nm) and the fluorescence ratio (F<sub>340/380</sub>). After addition of TPEN, the ratio of autofluorescence/total fluorescence at 340, 360, and 380 nm was 26 ± 3%, 35 ± 3%, and 61 ± 5% (*n* = 5), respectively. The fluorescence signals recorded from a parallel incubation of cells that had not been loaded with fura-2 are shown in A(ii).





**FIGURE 2** TPEN increases the fluorescence of cytosolic fura-2 in freshly isolated hepatocytes. Fura-2-loaded hepatocytes were attached to glass coverslips and the fluorescence ( $\lambda_{ex} = 360$  nm) of single cells was recorded from intact cells or from cells after permeabilization with saponin in a cytosol-like medium. Saponin (100  $\mu$ g/ml), TPEN (100  $\mu$ M), and ionomycin (IM, 10  $\mu$ M) with  $MnCl_2$  (10 mM) were included for the periods shown. Whereas TPEN caused a substantial increase in fura-2 fluorescence when applied to intact cells (A), it had no effect on the fluorescence of fura-2 trapped within the intracellular stores (B). Each panel shows the average fluorescence intensity recorded from 10–20 cells in a single field. Similar results were obtained from two independent fields of hepatocytes and from two independent fields of pancreatic acinar cells, although in the latter a much smaller fraction of the fura-2 ( $\sim 10\%$ ) was sequestered within intracellular stores.

for 9–12 days (Broad et al., 1996). Calf pulmonary artery endothelial (CPAE) cells (passage 28) were grown (37°C, 5%  $CO_2$ ) on glass coverslips in minimal essential medium supplemented with fetal calf serum (20%), penicillin (100 units/ml), streptomycin (100  $\mu$ g/ml), and L-glutamine (2 mM). Human embryonic kidney cells were cultured as previously described (Hargreaves et al., 1994). The cell lines were loaded with fura-2 AM using the same methods used for freshly isolated cells.

### Fluorescence measurements

Measurements from populations of fura-2-loaded cells were obtained either from cells in suspension (pancreatic acinar cells,  $10^6$ – $10^7$  cells/ml; freshly isolated hepatocytes,  $2$ – $5 \times 10^6$  cells/ml) or from cells attached to glass coverslips (hepatocytes and cell lines) mounted vertically in a cuvette as previously described (Byron and Taylor, 1993). Additions to cell suspensions were made directly via a syringe needle, whereas continuous perfusion of the mounted coverslips (10–15 ml/min) allowed repeated changes of media with a half-time for exchange of  $\sim 10$ –15 s. Cuvettes were mounted in the light path of an Hitachi F-4500 fluorescence spectrometer,

which allowed rapid switching (1 cycle/s) between excitation wavelengths while recording emitted fluorescence ( $\lambda_{em} = 510$  nm). All traces show the results of recordings corrected for autofluorescence, which was measured after addition of ionomycin (10  $\mu$ M) and  $MnCl_2$  (5–20 mM).

Fluorescence measurements of single cells were performed on the stage of a Diaphot inverted epifluorescence microscope (Nikon, Tokyo, Japan). Excitation light of different wavelengths was provided by a rapidly rotating filter wheel (0.2–1 cycle/s) mounted in front of a xenon arc lamp (100 W). Emitted fluorescence passed via the objective through a dichroic mirror (400 nm) and high-pass barrier filter (480 nm) before capture by an intensified charge-coupled device (CCD) camera (Photonic Science, Tunbridge Wells, UK). Images were digitized and stored for subsequent analysis using IonVision software (Improvision, Coventry, UK).

### Materials

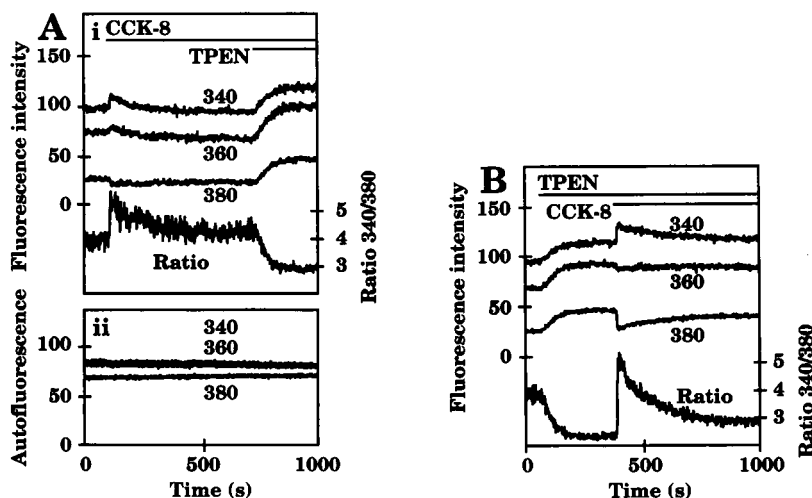
Fura-2 AM and indo 1 AM were from Molecular Probes Europe BV (Leiden, Netherlands). Collagenase was from Lorne Laboratories (Twyford, Reading, UK). Cell culture media, with the exception of fetal calf serum (Advanced Protein Products, W. Midlands, UK), were from Gibco BRL (Paisley, Scotland). Cell lines were from the European Collection of Animal Cell Cultures (Salisbury, UK) or (CPAE cells) the American Type Culture Collection (Rockville, MD). ATP, creatine phosphate and creatine kinase were from Boehringer (Lewes, UK). Percoll was from Pharmacia (Uppsala, Sweden). All other reagents were from Sigma (Posle, UK).

### RESULTS

#### Effects of TPEN on the fura-2 fluorescence of hepatocytes

In suspensions of freshly isolated hepatocytes loaded with fura-2,  $Arg^8$ -vasopressin (AVP, 100 nM) failed to evoke a detectable change in fura-2 fluorescence, although subsequent addition of the membrane-permeant chelator of heavy metals, TPEN (100  $\mu$ M), substantially increased fluorescence at each excitation wavelength ( $\lambda_{ex} = 340$ , 360, and 380 nm) (Fig. 1 A(i)), without affecting autofluorescence (Fig. 1 A(ii)). In unstimulated hepatocytes, the fluorescence ratio ( $F_{340/380}$ ) normally used to assess  $[Ca^{2+}]_i$  decreased from  $7.29 \pm 0.90$  to  $5.94 \pm 0.61$  ( $n = 4$ ;  $p < 0.05$ , paired  $t$ -test) after addition of TPEN (Table 1). When cells were first incubated with TPEN (100  $\mu$ M, 160 s) to relieve the quenching of fura-2, subsequent addition of AVP (100 nM) caused the changes in fura-2 fluorescence characteristic of an increase in  $[Ca^{2+}]_i$  (Fig. 1 B). Similar results were obtained from hepatocytes loaded with indo 1 (not shown). To determine whether the fraction of fura-2 ( $\sim 35\%$ ) trapped within the intracellular stores contributed disproportionately to the ability of TPEN to relieve the quenching of fura-2 fluorescence, the effect of TPEN on intact and permeabilized cells was compared. Freshly isolated fura-2-loaded hepatocytes were attached to coverslips and the effect of TPEN (100  $\mu$ M) on fura-2 fluorescence ( $\lambda_{ex} = 360$  nm) was examined in intact single cells and in cells permeabilized by incubation with saponin (100  $\mu$ g/ml) in a cytosol-like medium (Marshall and Taylor, 1994) containing ATP (1.5 mM), creatine phosphate (5 mM), creatine kinase (5 units/ml), EGTA (1 mM), and  $CaCl_2$  (free  $[Ca^{2+}] \sim 200$  nM) to preserve the  $Ca^{2+}$  content of the intracellular stores.

**FIGURE 3** TPEN increases the amplitude of CCK-8-evoked changes in fura-2 fluorescence in pancreatic acinar cells. Suspensions of fura-2-loaded pancreatic acinar cells were stimulated with CCK-8 (10 nM) before (A*i*) or after (B) preincubation with TPEN (100  $\mu$ M). The results show the fluorescence (in arbitrary units and corrected for autofluorescence) recorded at each of the three excitation wavelengths ( $\lambda_{ex}$  = 340, 360, and 380 nm) and the fluorescence ratio ( $F_{340/380}$ ). The results are typical of recordings from at least three independent cell preparations in showing larger CCK-8-evoked changes in fluorescence ( $\lambda_{ex}$  = 340 and 380 nm) after addition of TPEN. After addition of TPEN, the ratio of autofluorescence/total fluorescence at 340, 360, and 380 nm was  $35 \pm 2\%$ ,  $41 \pm 2\%$ , and  $59 \pm 1\%$  ( $n = 4$ ), respectively. The fluorescence signals recorded from a parallel incubation of cells that had not been loaded with fura-2 are shown in A(*ii*).



The results demonstrate that TPEN increased the fluorescence of cytosolic fura-2 (Fig. 2 A), but it had no effect on the fluorescence of the fura-2 trapped within intracellular stores (Fig. 2 B).

In primary cultures of fura-2-loaded hepatocytes, TPEN (20  $\mu$ M, 20 min) increased fura-2 fluorescence at each excitation wavelength and again the effect was most pronounced at the longer wavelengths thereby causing a decrease in the  $F_{340/380}$  ratio (Table 1). Prolonged incubation (2 h at 20°C) of cultured hepatocytes with TPEN (20  $\mu$ M) did not affect cell viability whether assessed by trypan blue exclusion, retention of lactate dehydrogenase, morphology, or the ability of cells to reversibly respond to AVP with an increase in  $[Ca^{2+}]_i$  (not shown).

#### Effects of TPEN on the fura-2 fluorescence of other mammalian cells

Addition of TPEN (100  $\mu$ M) to fura-2-loaded BC<sub>3</sub>H1 smooth muscle cells, human embryonic kidney 293 cells, CPAE cells (Table 1), or freshly isolated mouse pancreatic acinar cells (Fig. 3 A*i*) caused increases in fura-2 fluorescence at each excitation wavelength ( $\lambda_{ex}$  = 340, 360, and 380 nm). In most cell types, the increases in fluorescence that followed addition of TPEN were proportionally greater at  $\lambda_{ex}$  = 380 nm than at  $\lambda_{ex}$  = 340 nm (Fig. 3 B and Table 1). TPEN therefore caused a decrease in the fura-2 fluorescence ratio ( $F_{340/380}$ ), although the magnitude of the decrease varied considerably between cell types (Table 1). The decrease in  $F_{340/380}$  was not a consequence of inappropriate correction for autofluorescence, because when we exaggerated any possible error by assuming that the autofluorescence at 340 nm was double its measured value and that the autofluorescence at 380 nm was zero, TPEN still caused a significant decrease in the  $F_{340/380}$  ratio. The effects of TPEN were mimicked by another membrane-permeant chelator of heavy metal ions, *o*-phenanthroline (10–100  $\mu$ M) (not shown), but not by DTPA (100  $\mu$ M), which is membrane

impermeant (Fig. 4 B). DTPA did, however, relieve the quenching of fura-2 fluorescence in pancreatic acinar cells that had been permeabilized with saponin (100  $\mu$ g/ml) in nominally  $Ca^{2+}$ -free cytosol-like medium (free  $[Ca^{2+}] = 15$ –20  $\mu$ M), and subsequent addition of TPEN (100  $\mu$ M) had no further effect (not shown). In pancreatic acinar cells, as in hepatocytes (Fig. 2), TPEN affected only the fluorescence of cytosolic fura-2 (not shown). TPEN, under the conditions used for these and all subsequent experiments, did not affect the ability of pancreatic acinar cells to either recover from increases in  $[Ca^{2+}]_i$  or to exclude trypan blue; cell viability always exceeded 90%.

#### Characterization of the effects of TPEN in pancreatic acinar cells

The effect of TPEN on the basal fura-2 fluorescence of populations of pancreatic acinar cells was both time- and concentration-dependent (Fig. 4). Low concentrations of TPEN ( $\leq 5$   $\mu$ M for  $\leq 15$  min) failed to significantly affect fluorescence, whereas higher concentrations ( $\geq 10$   $\mu$ M) relieved the fluorescence quench at rates that increased with TPEN concentration (Fig. 4 A). When cells were treated with TPEN for 4 min, its half-maximal effect ( $EC_{50}$ ) on fura-2 fluorescence occurred with a TPEN concentration of 18  $\mu$ M (Fig. 4B). DTPA (100  $\mu$ M) neither mimicked TPEN nor appreciably affected its concentration-effect relationship ( $EC_{50} = 12$   $\mu$ M) (Fig. 4 B). These results demonstrate that the heavy metal ions that bind to fura-2, bind with high affinity to TPEN and that they cannot readily cross the plasma membrane.

Previous studies with quin 2-loaded cells suggested that perturbation of  $[Ca^{2+}]_i$  measurements by heavy metal ions was most acute in cells loaded with low concentrations of the indicator (Arslan et al., 1985), presumably reflecting stoichiometric binding of a limited pool of exchangeable heavy metal ions. Measurements of single pancreatic acinar cells demonstrate that even when loaded under identical

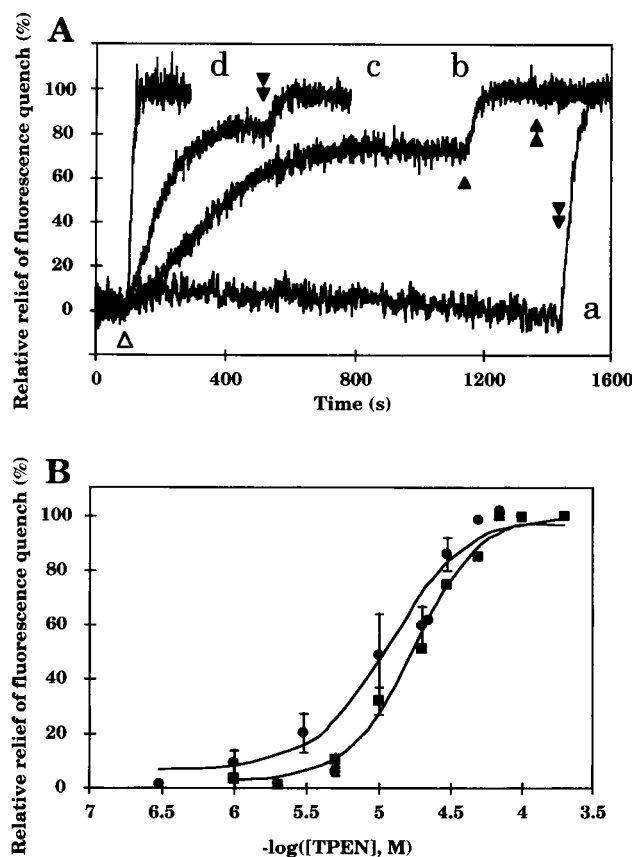
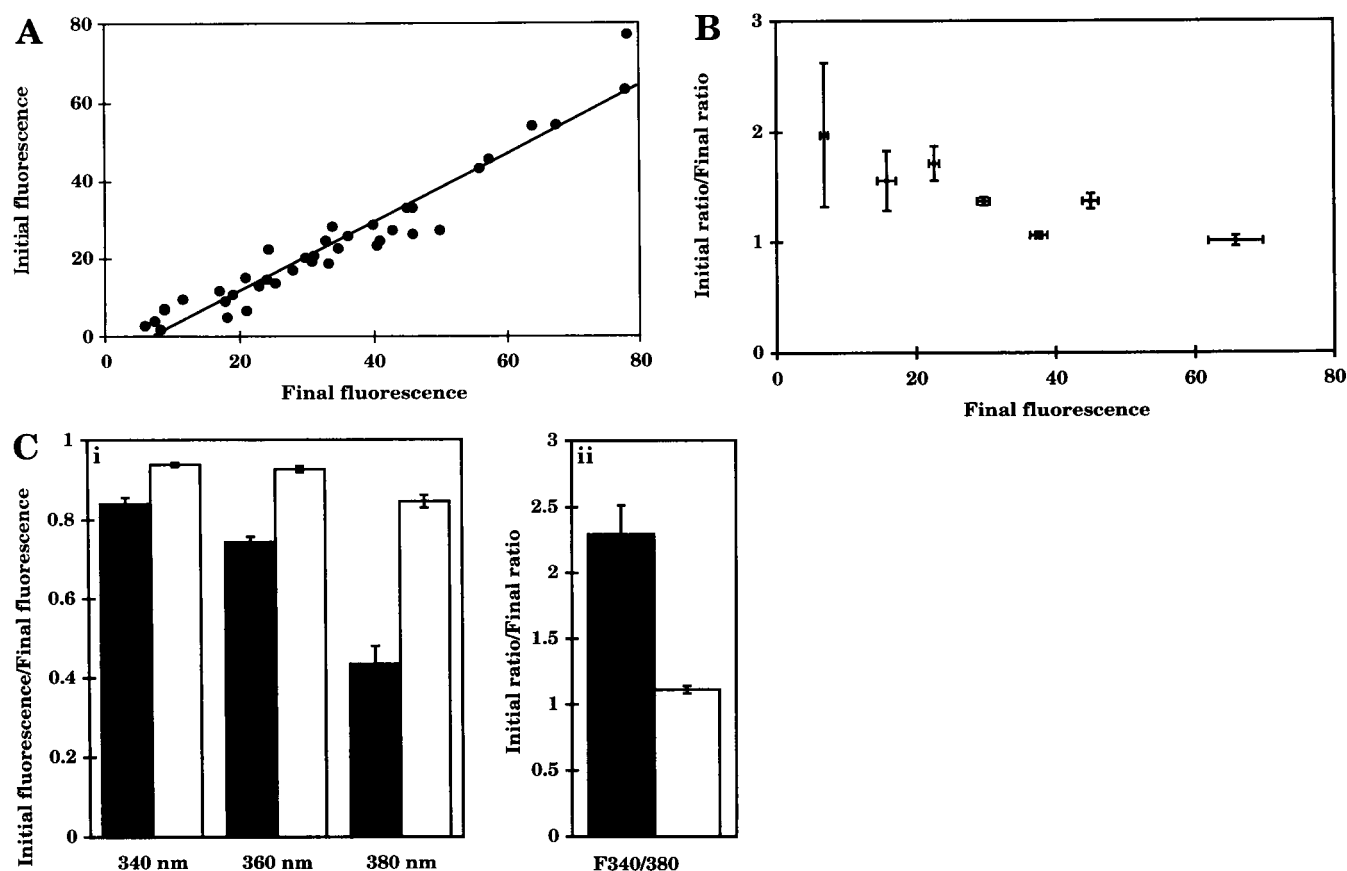


FIGURE 4 Concentration-dependent effect of TPEN on quenching of fura-2 in pancreatic acinar cells by endogenous heavy metal ions. Fluorescence ( $\lambda_{\text{ex}} = 360$  nm) was recorded from populations of fura-2-loaded pancreatic acinar cells after addition of TPEN. A. Addition of increasing concentrations of TPEN (open arrow: a, 5  $\mu\text{M}$ ; b, 15  $\mu\text{M}$ ; c, 30  $\mu\text{M}$ ; d, 1 mM) caused an increase in the rate at which the quench of fura-2 fluorescence was relieved. The TPEN concentration was increased to 100  $\mu\text{M}$  or 200  $\mu\text{M}$  (single and double solid arrows, respectively) at the times shown. The results show the increase in fluorescence intensity as a percentage of that observed after addition of a maximal concentration of TPEN. B. Cells were incubated with the indicated concentrations of TPEN either alone (■) or in the simultaneous presence of DTPA (100  $\mu\text{M}$ , ●), and the increase in fura-2 fluorescence was recorded after 4 min. Points, showing the increase in fluorescence intensity as a percentage of that observed after addition of a maximal concentration of TPEN, represent the results of one or three (means  $\pm$  SEM) independent experiments and the lines were fitted to a logistic equation.

conditions, cells differ by up to 10-fold in their final intracellular fura-2 concentration (Fig. 5 A). By measuring fura-2 fluorescence ( $\lambda_{\text{ex}} = 360$  nm) before and after TPEN (100  $\mu\text{M}$ ) addition, it is apparent that in all but the most lightly loaded of cells, TPEN evoked a similar absolute increase in fura-2 fluorescence over a wide range of intracellular fura-2 concentrations (Fig. 5 A). Lightly loaded cells therefore showed the greatest proportional increase in fura-2 fluorescence after TPEN addition, suggesting that a limited pool of intracellular heavy metal ions is available to bind to fura-2. Estimates of basal  $[\text{Ca}^{2+}]_i$  derived from  $F_{340/380}$  are, therefore, most likely to substantially overestimate  $[\text{Ca}^{2+}]_i$  in cells that are lightly loaded with fura-2 (Fig.

5 B). The effects of fura-2 loading on the fluorescence ratio were further examined by deliberately overloading (by  $8.9 \pm 1.0$ -fold) populations of pancreatic acinar cells with fura-2 by extending the period of incubation with fura-2 AM to 60 min and increasing the fura-2 AM concentration to 10  $\mu\text{M}$ . We were concerned that the more prolonged incubations with fura-2 AM might lead either to less complete hydrolysis of fura-2 AM or to more substantial sequestration of fura-2 into intracellular stores. Neither potential problem appears to be significant in our experiments. The fluorescence intensities ( $\lambda_{\text{ex}} = 360$  nm) from which we estimated intracellular fura-2 concentrations were all corrected for autofluorescence by addition of ionomycin and  $\text{MnCl}_2$ . Because the fluorescence of fura-2 AM is not quenched by  $\text{Mn}^{2+}$ , our measurement is directly related to the intracellular concentration of fura-2-free acid. Furthermore, the autofluorescence ( $\lambda_{\text{ex}} = 360$  nm) recorded after addition of ionomycin and  $\text{MnCl}_2$  to lightly ( $72 \pm 3$  units,  $n = 4$ ) and heavily ( $99 \pm 10$  units,  $n = 4$ ) loaded cells differed by  $<20\%$ , suggesting that each contained similar amounts of incompletely hydrolyzed fura-2 AM. Two lines of evidence suggest that the extended loading period did not lead to substantially greater sequestration of fura-2 into intracellular stores. Firstly, the  $F_{340/380}$  ratios recorded after addition of TPEN to lightly ( $3.8 \pm 0.7$ ,  $n = 4$ ) and heavily ( $4.8 \pm 0.8$ ,  $n = 4$ ) loaded cells were not significantly different, suggesting that in each the fura-2 is exposed to a similar free  $[\text{Ca}^{2+}]$ . Secondly, using saponin (100  $\mu\text{g/ml}$ ) to selectively permeabilize the plasma membrane, the fraction of the fura-2 fluorescence derived from cytosolic fura-2 was found to be similar in lightly (7 and 9%,  $n = 2$ ) and heavily (7 and 8%,  $n = 2$ ) loaded cells. Our methods therefore allow both overloading of the cytosol with fura-2 and reliable estimation of the relative loadings of lightly and heavily loaded cells. The results of these experiments with deliberately overloaded cells confirmed those from single cells: addition of TPEN (100  $\mu\text{M}$ ) caused the basal  $F_{340/380}$  ratio to decrease from  $7.9 \pm 2.0$  to  $3.8 \pm 0.7$  ( $n = 4$ ) in lightly loaded cells and from  $5.3 \pm 0.8$  to  $4.8 \pm 0.8$  ( $n = 4$ ) in cells overloaded with fura-2 (Fig. 5 C). The results from experiments in which the fura-2 loading of cells was either serendipitously or deliberately manipulated therefore suggest that estimates of basal  $[\text{Ca}^{2+}]_i$  are most likely to be overestimated in cells lightly loaded with fura-2.

By permeabilizing populations of fura-2-loaded pancreatic acinar cells with saponin and then calibrating the intensity of the fluorescence to fura-2 concentration by addition of known concentrations of fura-2, we estimated the fura-2 content of the lightly loaded cells to be  $\sim 10^{-16}$  mol/cell (not shown). From the estimated volume of a mouse pancreatic acinar cell ( $1.8 \times 10^{-12}$  l) and the fraction of guinea pig pancreatic acinar cells occupied by cytosol (54%) (Bollender, 1974), we estimated the average cytosolic fura-2 concentration to be  $\sim 56$   $\mu\text{M}$  in lightly loaded cells (1  $\mu\text{M}$  fura-2 AM, 10 min) and  $\sim 500$   $\mu\text{M}$  in heavily loaded cells (10  $\mu\text{M}$  fura-2 AM, 60 min).

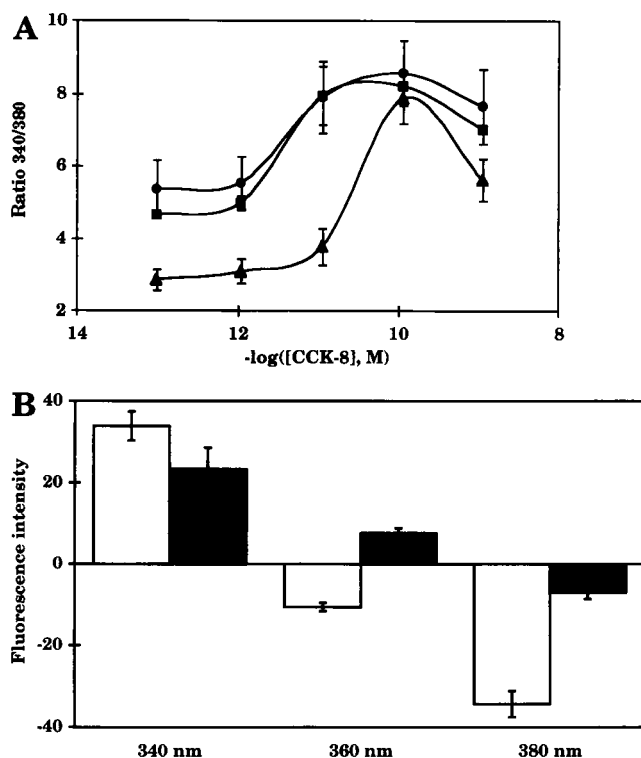


**FIGURE 5** Quenching of fura-2 fluorescence by heavy metal ions is worst in cells lightly loaded with fura-2. **A**, Pancreatic acinar cells were loaded with fura-2 by incubation for 10 min with fura-2 AM (1  $\mu$ M) at 20°C, they were attached to glass coverslips, and the fluorescence of single cells ( $\lambda_{ex} = 360$  nm) was recorded before and 4 min after TPEN (100–500  $\mu$ M) addition. Results from 39 cells taken from six independent experiments show the fluorescence intensity (arbitrary units) of each fura-2-loaded cell before addition of TPEN (initial fluorescence) plotted against the fluorescence intensity of the same cell after addition of TPEN (final fluorescence). The final fluorescence provides an index of the cytosolic fura-2 concentration (see Results). All recordings were corrected for autofluorescence. **B**,  $F_{340/380}$  ratios were determined for 36 of the 39 cells shown in panel A before and after TPEN addition (fluorescence from the remaining three cells was only recorded at  $\lambda_{ex} = 360$  nm). The ratio of those two measurements (initial  $F_{340/380}$ /final  $F_{340/380}$ ) is plotted against the fluorescence intensity (arbitrary units) of each cell after addition of TPEN ( $\lambda_{ex} = 360$  nm). The results show the means  $\pm$  SEM of five cells (six cells for the final group) grouped according to their final fluorescence intensity. In the five most lightly loaded and six most heavily loaded cells, the ratio of autofluorescence/total fluorescence at 340 nm was 59% and 2%, respectively, and 10% and 1% at 380 nm. **C**, Populations of pancreatic acinar cells were lightly loaded with fura-2 by incubation with 1  $\mu$ M fura-2 AM for 10 min (solid bars) or heavily loaded by incubation with 10  $\mu$ M fura-2 AM for 60 min (open bars). The initial fluorescence was recorded before addition of TPEN and the final fluorescence was recorded 4 min after addition of TPEN (100  $\mu$ M). The results (means  $\pm$  SEM of 4 independent experiments) show the ratios of the initial fluorescence/final fluorescence intensities at each excitation wavelength (i) and the ratios of the initial  $F_{340/380}$ /final  $F_{340/380}$  (ii). In the lightly and heavily loaded cells, the ratios of autofluorescence/total fluorescence after TPEN addition were  $38 \pm 1\%$  and  $7 \pm 1\%$  at 340 nm,  $45 \pm 1\%$  and  $11 \pm 1\%$  at 360 nm, and  $66 \pm 4\%$  and  $24 \pm 2\%$  at 380 nm ( $n = 4$ ).

### Effects of TPEN on hormone-evoked fluorescence signals

We next examined the influence of heavy metal ions on the  $F_{340/380}$  fluorescence ratio in pancreatic acinar cells stimulated with cholecystokinin octapeptide (CCK)-8. Preincubation (3 min) with the membrane impermeant chelator, DTPA (100  $\mu$ M; Fig. 4 B), affected neither the basal fluorescence ratio nor the response to CCK-8 (Fig. 6 A). TPEN (100  $\mu$ M), however, significantly decreased the basal  $F_{340/380}$  ratio (Fig. 3), increased the changes in fluorescence at  $\lambda_{ex} = 340$  and 380 nm evoked by a maximal concentration of CCK-8 (Figs. 3 and 6 B), and reduced the sensitivity of the cells to CCK-8 (Fig. 6 A). Furthermore, whereas the fluo-

rescence recorded at  $\lambda_{ex} = 360$  nm increased after addition of agonists to control cells, it decreased after stimulation of cells in the presence of TPEN (Figs. 3 B and 6 B). The agonist-evoked increase in fluorescence ( $\lambda_{ex} = 360$  nm) in control cells may reflect displacement of quenching heavy metal ions from fura-2 by  $Ca^{2+}$  following the increase in  $[Ca^{2+}]_i$ . The decrease in fluorescence in TPEN-treated cells is more difficult to explain. Both in hepatocytes, where interleukin-6 evokes an increase in  $[Zn^{2+}]_i$  (Coyle et al., 1994), and in cortical slices, where an increase in  $[Ca^{2+}]_i$  is accompanied by an increase in  $[Zn^{2+}]_i$  (Badar-Goffer et al., 1994), there is evidence to suggest mobilization of  $Zn^{2+}$ . If similar changes occur in acinar cells, the  $Zn^{2+}$  might dis-



**FIGURE 6** TPEN, but not DTPA, affects the fluorescence signals recorded from fura-2-loaded pancreatic acinar cells. **A.** Populations of fura-2-loaded pancreatic acinar cells were stimulated with the indicated concentrations of CCK-8 under control conditions (●) or 5 min after addition of DTPA (100  $\mu$ M, ■) or TPEN (100  $\mu$ M, ▲). The results, showing the integrated change in fluorescence ratio recorded during the 150 s after each addition of CCK-8, are the means  $\pm$  SEM of three independent experiments. **B.** Pancreatic acinar cells were stimulated with CCK-8 (10 nM) in the absence (solid bars) or presence of 100  $\mu$ M TPEN (open bars). The combined results (means  $\pm$  SEM) of four to five experiments are shown to illustrate the changes in the peak amplitudes of the fluorescence signals recorded at each excitation wavelength.

place less tightly bound ions from TPEN (e.g.,  $\text{Fe}^{2+}$ ,  $K_D^{\text{Fe}} = 2.5 \times 10^{-15}$  M) because  $\text{Zn}^{2+}$  binds with such high affinity to TPEN ( $K_D^{\text{Zn}} = 2.6 \times 10^{-16}$  M) (Arslan et al., 1985) allowing the displaced ions to quench fura-2. Whatever the mechanisms underlying agonist-evoked changes in fura-2 fluorescence at  $\lambda_{\text{ex}} = 360$  nm, endogenous heavy metal ions perturb measurements made at this wavelength as well as measurements of  $F_{340/380}$ .

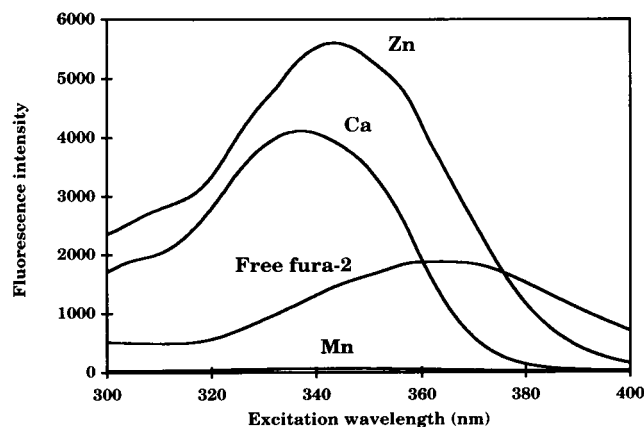
### Mechanisms underlying the effect of TPEN on fura-2 fluorescence

The ability of TPEN and *o*-phenanthroline to relieve quenching of fura-2 fluorescence in a variety of cells and to exert more profound effects in lightly loaded cells is consistent with previous studies of quin 2-loaded cells (Arslan et al., 1985; Tsien and Pozzan, 1989). However, quenching of fura-2 fluorescence by endogenous heavy metal ions cannot alone provide an explanation for the greater effect of TPEN on  $\lambda_{\text{ex}} = 380$  nm relative to  $\lambda_{\text{ex}} = 340$  nm and the

commensurate decrease in  $F_{340/380}$  after addition of TPEN (Table 1). Those heavy metal ions (e.g.,  $\text{Mn}^{2+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Fe}^{2+}$ ) that quench fura-2 fluorescence do so by almost completely quenching the fluorescence at each excitation wavelength (Fig. 7); binding of such metals to intracellular fura-2 would not, therefore, significantly affect  $F_{340/380}$ . The most likely explanation for the decrease in  $F_{340/380}$  observed in all fura-2-loaded cells after addition of TPEN is that the cells also contain an endogenous heavy metal ion (e.g.,  $\text{Zn}^{2+}$ ) that binds to fura-2 and increases its fluorescence at the short excitation wavelengths. The problem is likely to be most acute with  $\text{Zn}^{2+}$  because its binding to fura-2 causes more intense fura-2 fluorescence, especially at the longer excitation wavelengths, than does binding of  $\text{Ca}^{2+}$  (Fig. 7) (Grynkiewicz et al., 1985).

### DISCUSSION

Although a previous report highlighted the need to consider interactions between heavy metal ions and quin 2 when attempting to calibrate fluorescence signals to  $[\text{Ca}^{2+}]_i$ , it also suggested that the problems were unlikely to be significant in most cell types (Arslan et al., 1985). In the present study, we have demonstrated that in a variety of mammalian cells, heavy metal ions also substantially perturb measurements of  $[\text{Ca}^{2+}]_i$  with fura-2. The heavy metal ions responsible are endogenous and do not readily exchange across the plasma membrane because the membrane impermeant chelator, DTPA, is unable to mimic the effects of the permeant chelators, TPEN (Figs. 4 B and 6 A) (Arslan et al., 1985) and *o*-phenanthroline, nor does DTPA appreciably affect the sensitivity of cells to TPEN (Fig. 4 B). There appears to be a small limited pool of endogenous heavy metal ions because their effects on fura-2 fluorescence saturate as the intracellular fura-2 concentration is



**FIGURE 7** Effects of  $\text{Zn}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Fe}^{2+}$ , and  $\text{Mn}^{2+}$  on the fluorescence excitation spectra of fura-2. Fluorescence excitation spectra of fura-2 (1  $\mu$ M) in  $\text{Mg}^{2+}$ -free cytosol-like medium were recorded ( $\lambda_{\text{em}} = 510$  nm) after addition of 1 mM EGTA (= Free fura-2),  $\text{CaCl}_2$ ,  $\text{MnCl}_2$ ,  $\text{ZnCl}_2$ , or  $\text{FeCl}_2$ . In the presence of the latter, there was no detectable fluorescence from fura-2.

increased (Fig. 5). From our estimates of the average cytosolic fura-2 concentration ( $\sim 56 \mu\text{M}$  in lightly loaded cells, see Results) and, from extrapolation of Fig. 5 A, the amount of fura-2 quenched by heavy metal ions, we estimate the cytosolic concentration of the exchangeable heavy metal ion that quenches fura-2 to be at least  $12 \mu\text{M}$  in pancreatic acinar cells.

Endogenous heavy metal ions cause quin 2 to substantially underestimate basal  $[\text{Ca}^{2+}]_i$  (Arslan et al., 1985; Tsien and Pozzan, 1989), whereas our results from a variety of mammalian cell types at different stages of differentiation, each maintained in different media and examined under different experimental conditions, indicate that endogenous heavy metal ions cause fura-2 to overestimate basal  $[\text{Ca}^{2+}]_i$  (Figs. 1–6; Table 1) and underestimate the amplitudes of stimulus-evoked increases in  $[\text{Ca}^{2+}]_i$  (Figs. 1, 3, and 6). To explain the more pronounced enhancement of fura-2 fluorescence at the longer excitation wavelengths after TPEN addition (Table 1), we propose that cells contain a mixture of endogenous heavy metal ions some of which quench and some of which increase fura-2 fluorescence at the short excitation wavelengths (Fig. 7). Because cells are likely to differ in their complement of such ions, the extent to which TPEN decreases the basal  $F_{340/380}$  measurement will also vary between cells (Table 1).  $\text{Zn}^{2+}$ , one of the most abundant intracellular heavy metal ions, is a prime candidate for the ion that causes an increase in fura-2 fluorescence. The total  $\text{Zn}^{2+}$  concentration in liver is comparable to that of  $\text{Ca}^{2+}$  (Hughes, 1987), and labile pools of  $\text{Zn}^{2+}$  in rat hepatocytes are estimated to be  $\sim 3 \text{ nmol}/10^6$  cells ( $\sim 600 \mu\text{M}$ ) (Coyle et al., 1994).  $\text{Zn}^{2+}$  quenches the fluorescence of quin 2 (Grynkiewicz et al., 1985), but it binds to fura-2 with high affinity ( $K_D = 1.5 \text{ nM}$ ) (Hechtenberg and Beyersmann, 1993) causing similar shifts in excitation spectrum to those evoked by  $\text{Ca}^{2+}$ , although the fluorescence intensity is greater when  $\text{Zn}^{2+}$  is bound (Hechtenberg and Beyersmann, 1993) and the isosbestic wavelength is increased to  $376 \text{ nm}$  (Fig. 7). One factor that may contribute to the different effects of endogenous heavy metal ions on estimates of  $[\text{Ca}^{2+}]_i$  derived from quin 2 and fura-2 measurements is therefore the different effects of  $\text{Zn}^{2+}$  on the two indicators.

Competition between heavy metal ions and  $\text{Ca}^{2+}$  for binding to fura-2 will decrease the apparent affinity of fura-2 for  $\text{Ca}^{2+}$ . Furthermore, chelation of those heavy metal ions (e.g.,  $\text{Zn}^{2+}$ ) that mimic  $\text{Ca}^{2+}$  binding to fura-2 will, by decreasing the basal  $F_{340/380}$  ratio, increase the amplitude of the changes in fluorescence evoked by an increase in  $[\text{Ca}^{2+}]_i$ . Both effects are likely to have contributed to the ability of TPEN both to unmask the increase in  $F_{340/380}$  evoked by AVP in freshly isolated hepatocytes (Fig. 1) and to substantially increase the fluorescence changes evoked by CCK in pancreatic acinar cells (Figs. 3 and 6).

By overloading cells with fura-2, the problems associated with endogenous heavy metal ions are much reduced (Fig. 5), but only at the expense of significantly increasing the  $\text{Ca}^{2+}$ -buffering capacity of the cytosol and thereby damping

the kinetics of  $\text{Ca}^{2+}$  signals. In a variety of cells, the concentration of intracellular  $\text{Ca}^{2+}$  buffers with  $\leq 1 \mu\text{M}$  affinity for  $\text{Ca}^{2+}$  has been estimated to be  $\leq 1 \text{ mM}$  (Poenie, 1992), substantially more than the fura-2 concentration of lightly loaded cells ( $\sim 50 \mu\text{M}$ ), but comparable to the fura-2 concentration ( $\sim 500 \mu\text{M}$ ) of the heavily loaded cells in which the problems of heavy metal ions were minimized (Fig. 5). In addition to interfering with  $\text{Ca}^{2+}$  regulation, excessive loading of cells with fura-2 may also antagonize the binding of  $\text{InsP}_3$  to its receptor (Richardson and Taylor, 1993) and the toxic effects of formaldehyde released by intracellular hydrolysis of fura-2 AM may become problematic (Poenie, 1992). Indeed, the decreased sensitivity of pancreatic acinar cells to CCK-8 after TPEN addition (Fig. 6 A) may, in part, result from the increased concentration of free fura-2, the affinity of which for  $\text{InsP}_3$  receptors ( $K_D \sim 120 \mu\text{M}$ ) (Richardson and Taylor, 1993) may be sufficient to allow appreciable antagonism of  $\text{InsP}_3$  binding. TPEN provides a better remedy than overloading with fura-2 to the problems resulting from endogenous heavy metal ions. TPEN, with its very low affinity for  $\text{Ca}^{2+}$  ( $K_D = 40 \mu\text{M}$ ) (Arslan et al., 1985), is unlikely to directly perturb  $[\text{Ca}^{2+}]_i$ , but its use to chelate cytosolic heavy metal ions, and thereby prevent their binding to fura-2, is not without potential problems. Many heavy metal ions, including  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Fe}^{2+}$ , are important cofactors of enzymes, and whereas TPEN is unlikely to remove such ions from their very high-affinity sites, it may chelate more loosely bound heavy metal ions. Previous studies have shown that TPEN does not affect epidermal growth factor-stimulated phosphoinositidase C activity (Wahl and Carpenter, 1988), and our results suggest that acute exposure ( $\leq 2 \text{ h}$ ) to concentrations of TPEN ( $20\text{--}100 \mu\text{M}$ ) sufficient to prevent binding of heavy metal ions to fura-2 are not toxic; all cells retained the ability to exclude trypan blue, to respond to  $\text{Ca}^{2+}$ -mobilizing stimuli, and to recover from increases in  $[\text{Ca}^{2+}]_i$ . Furthermore, because even intracellular concentrations of fura-2 below those normally used to make  $[\text{Ca}^{2+}]_i$  measurements are capable of chelating the entire intracellular pool of free heavy metal ions (Fig. 5), perturbation of free heavy metal ion homeostasis may be no worse with TPEN than with fura-2. TPEN did, however, significantly decrease the sensitivity of pancreatic acinar cells to CCK-8; this may result from an increase in the free intracellular fura-2 concentration and, consequently, greater antagonism of  $\text{InsP}_3$  receptors (Richardson and Taylor, 1993); or it may reflect a role for heavy metal ions in signal transduction.

In conclusion, we suggest that many mammalian cells contain substantial pools of endogenous heavy metal ions some of which increase and some of which quench fura-2 fluorescence. These ions cause substantial errors in measurements of both basal and hormone-evoked  $[\text{Ca}^{2+}]_i$  that are most severe in cells lightly loaded with fura-2.

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