Extracellular heavy-metal ions stimulate Ca^{2+} mobilization in hepatocytes

Tracy J. McNULTY and Colin W. TAYLOR¹

Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ, U.K.

Populations of hepatocytes in primary culture were loaded with fura 2 and the effects of extracellular heavy-metal ions were examined under conditions that allowed changes in fura 2 fluorescence ($R_{340/360}$, the ratio of fluorescence recorded at 340 and 360 nm) to be directly attributed to changes in cytosolic free $[\text{Ca}^{2+}]_i$ ($[\text{Ca}^{2+}]_i$). In Ca^{2+} -free media, Ni^{2+} $[\text{EC}_{50}$ (concentration causing 50% stimulation) $\approx 24 \pm 9 \mu\text{M}$] caused reversible increases in $[\text{Ca}^{2+}]_i$ that resulted from mobilization of the same intracellular Ca^{2+} stores as were released by $[\text{Arg}^8]\text{vasopressin}$. The effects of Ni^{2+} were not mimicked by increasing the extracellular $[\text{Mg}^{2+}]$, by addition of MnCl_2 , CoCl_2 or CdCl_2 or by decreasing the extracellular pH from 7.3 to 6.0; nor were they observed in cultures of smooth muscle, endothelial cells or pituitary cells. CuCl_2 (80 μM), ZnCl_2 (80 μM) and LaCl_3 (5 mM) mimicked the ability of Ni^{2+} to evoke Ca^{2+} mobilization. The response to La^{3+} was sustained even in the absence of extracellular

Ca^{2+} , probably because La^{3+} also inhibited Ca^{2+} extrusion. Although Ni^{2+} entered hepatocytes, from the extent to which it quenched fura 2 fluorescence the free cytosolic $[\text{Ni}^{2+}]_i$ ($[\text{Ni}^{2+}]_i$) was estimated to be $< 5 \text{ nM}$ at the peak of the maximal Ni^{2+} -evoked Ca^{2+} signals and there was no correlation between $[\text{Ni}^{2+}]_i$ and the amplitude of the evoked increases in $[\text{Ca}^{2+}]_i$. We conclude that extracellular Ni^{2+} , Zn^{2+} , Cu^{2+} and La^{3+} , but not all heavy-metal ions, evoke an increase in $[\text{Ca}^{2+}]_i$ in hepatocytes by stimulating release of the hormone-sensitive intracellular Ca^{2+} stores and that they may do so by interacting with a specific cell-surface ion receptor. This putative ion receptor may be important in allowing hepatocytes to contribute to regulation of plasma heavy-metal ions and may mediate responses to Zn^{2+} released into the portal circulation with insulin.

Key words: liver, nickel, receptor.

INTRODUCTION

The ability of hormones or neurotransmitters to regulate cellular activities by stimulating increases in cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is an ubiquitous feature of eukaryotic cells [1]. Such stimuli almost invariably cause an increase in $[\text{Ca}^{2+}]_i$ by opening Ca^{2+} channels in either the plasma membrane or the membranes of intracellular Ca^{2+} stores [1,2]. The same signalling machinery that allows cells to respond to hormones or neurotransmitters is also important in allowing some cells to respond to changes in extracellular $[\text{Ca}^{2+}]$ [3]. Ca^{2+} -sensing receptors were first characterized in parathyroid cells, where they mediate, via their ability to stimulate InsP_3 formation, the effects of changes in plasma $[\text{Ca}^{2+}]$ on secretion of parathyroid hormone [4,5]. The Ca^{2+} -sensing receptor is an unusual member of the G-protein-linked family of receptors in that it has components with structural similarity to both bacterial proteins involved in ion and nutrient transport and to more typical G-protein-linked receptors [3,4,6]. The receptor, which can also be stimulated by other cations, is now known to be more widely expressed in both tissues that contribute to plasma Ca^{2+} homeostasis (e.g. kidney and thyroid parafollicular cells) [3,7] and elsewhere (e.g. brain) [8]. The role of an extracellular Ca^{2+} -sensing system in these additional tissues is uncertain, although it has been speculated that it may allow local changes in extracellular $[\text{Ca}^{2+}]$ resulting from the activities of some cells to be communicated to their neighbours [3,8].

The immunological identification of another putative Ca^{2+} sensor [9] and the different pharmacological profiles of ion

sensors in different tissues [3,10] suggest that additional cation receptors may be expressed in other tissues. In endothelial cells, fibroblasts, neuroblastoma cells and smooth muscle, for example, micromolar concentrations of metal ions ($\text{Cd}^{2+} > \text{Co}^{2+} > \text{Ni}^{2+} > \text{Fe}^{2+} > \text{Mn}^{2+}$) stimulate polyphosphoinositide hydrolysis or Ca^{2+} mobilization [11,12] with a potency order that differs from that observed in osteoclasts ($\text{Cd}^{2+} > \text{La}^{3+} \geq \text{Ni}^{2+} > \text{Ca}^{2+}$) [13–15] or parathyroid cells ($\text{Gd}^{3+} > \text{Ca}^{2+} = \text{Sr}^{2+} = \text{Ba}^{2+} > \text{Mg}^{2+}$) [3]. In *Xenopus* oocytes, too, metal ions ($\text{Cd}^{2+} \geq \text{Ni}^{2+} \geq \text{Zn}^{2+} \geq \text{Co}^{2+} \geq \text{Mn}^{2+} > \text{Cr}^{2+} \geq \text{Sr}^{2+} = \text{Ca}^{2+} = \text{Ba}^{2+}$) stimulate release of intracellular Ca^{2+} stores [16]. However, with the exception of the parathyroid cation receptor, the means whereby these metal ions evoke Ca^{2+} signals are unknown and may reflect their actions at either cell-surface or intracellular targets. These observations, suggesting that receptors capable of transducing changes in extracellular ion concentration into intracellular signals may be widespread, has fostered speculation concerning their distribution, the ions they detect and their roles in both regulating and responding to the local and systemic environment [10].

In mammals, heavy-metal ions absorbed by the small intestine enter the portal circulation, where they are transported to the liver bound to amino acids, peptides or proteins [17]. The liver is therefore the first major organ to encounter both toxic and essential heavy-metal ions absorbed from the diet, and it plays a key role in regulating their plasma concentrations [17]. Furthermore, several heavy-metal ions are routinely used as either antagonists (e.g. Ni^{2+} , La^{3+}) or surrogates (e.g. Mn^{2+}) in experimental analyses of Ca^{2+} signalling. The existence of functional receptors for heavy-metal ions in hepatocytes would therefore

Abbreviations used: AVP, $[\text{Arg}^8]\text{vasopressin}$; $[\text{Ca}^{2+}]_i$ and $[\text{Ni}^{2+}]_i$, cytosolic free Ca^{2+} and Ni^{2+} concentrations respectively; CLM, cytosol-like medium; DTPA, diethylenetriaminepenta-acetic acid; EC_{50} and IC_{50} , concentrations causing 50% stimulation and inhibition respectively; EM, extracellular medium; λ_{em} and λ_{ex} , emission and excitation wavelengths respectively; $R_{340/380}$, ratio of fluorescence recorded at $\lambda_{\text{ex}} = 340$ and 380 nm (similar notation for other ratios); TPEN, *N,N,N',N'*-tetrakis-(2-pyridylmethyl)ethylenediamine; fura 2/AM, fura 2 acetoxymethyl ester.

¹ To whom correspondence should be addressed (e-mail cwt1000@cam.ac.uk).

have significant implications for the physiology and pathology of hepatic handling of these ions and for the use of heavy-metal ions in the analysis of Ca^{2+} signalling pathways.

MATERIALS AND METHODS

Primary culture and fura 2 loading of hepatocytes

Hepatocytes were isolated from the livers of male Wistar rats (body weight 150–200 g) by collagenase digestion [18], washed in cold (4 °C) medium and then separated from other liver cells by centrifugation (50 g, 10 min) through medium (NaCl, 116 mM; KCl, 5.4 mM; MgSO_4 , 0.8 mM; NaH_2PO_4 , 0.96 mM; NaHCO_3 , 25 mM; CaCl_2 , 1.8 mM; glucose, 15 mM; O_2/CO_2 , 19:1; pH 7.4) containing Percoll [density (ρ) = 1.08 g/ml]. After a further wash, they were resuspended in William's medium E (1.7×10^5 cells/ml) containing penicillin (87 units/ml), streptomycin (87 $\mu\text{g}/\text{ml}$), neomycin (87 $\mu\text{g}/\text{ml}$) and insulin (8.7 $\mu\text{g}/\text{ml}$), L-glutamine (3.5 mM) and foetal calf serum (8.7%). Cells (2 ml) were plated on to 2 glass coverslips (22 mm $\times$ 9 mm, No. 2), allowed to attach for 60 min at 37 °C, and the medium then replaced with supplemented William's medium E containing cytosine arabinoside (10 μM). After 24 h at 37 °C in 5% CO_2 , the medium was replaced with supplemented William's medium E lacking cytosine arabinoside, and after a further 2 days in culture the cells were used for experiments. In some experiments using fura 2, freshly isolated hepatocytes were used after a 60 min incubation at 37 °C in supplemented William's medium E to allow attachment to coverslips.

For fura 2 loading, primary cultures of hepatocytes were preincubated (10 min) in cold (4 °C) extracellular medium (EM) (NaCl, 135 mM; KCl, 5.9 mM; MgCl_2 , 1.2 mM; CaCl_2 , 1.5 mM; Hepes, 11.6 mM; glucose, 11.5 mM, pH 7.3) and then loaded with fura 2 by incubation at 20 °C in EM supplemented with fura 2/AM (fura 2 acetoxymethyl ester) (2 μM), BSA (1 mg/ml) and Pluronic F-127 (200 $\mu\text{g}/\text{ml}$) for 2 h. After loading, the hepatocytes were washed and incubated for a further 1–3 h at 20 °C to allow complete hydrolysis of the intracellular fura 2/AM. The intracellular distribution of the fura 2 was established by recording fluorescence at the isosbestic wavelength [λ_{ex} (excitation wavelength) 360 nm] while continuously perfusing the cells with EM containing saponin at a concentration (30 $\mu\text{g}/\text{ml}$) previously shown to permeabilize the plasma membrane without damaging intracellular Ca^{2+} stores [18]. The results demonstrate that, in cultured hepatocytes, $80 \pm 1\%$ ($n = 8$) of the fura 2 was cytosolic. The same methods were used to load freshly isolated hepatocytes with fura 2, and although we did not quantify the fura 2 distribution in these cells, they did respond to $[\text{Arg}^8]\text{vasopressin}$ (AVP) with an increase in $R_{340/380}$ (the ratio of fluorescence recorded at 340 and 380 nm) (see below), suggesting that much of the indicator was cytosolic. Our methods (1) optimized conditions for culture of primary hepatocytes while minimizing contamination with other cell types, (2) allowed hepatocytes to be reliably loaded with cytosolic fura 2, and (3) provided cells that responded to a range of agonists (AVP, ATP, angiotensin II) and thapsigargin with an increase in $[\text{Ca}^{2+}]_i$ that resulted from both mobilization of intracellular Ca^{2+} stores and stimulated Ca^{2+} entry.

Measurements of fura 2 fluorescence

Coverslips to which cultured hepatocytes were attached were mounted vertically in a poly(methyl acrylate) cuvette in the light-path of either an Hitachi F-4500 or a Perkin-Elmer LS-50B fluorescence spectrofluorimeter [19]. The cells were continuously perfused (8.5 ml/min) at 20 °C with EM or Ca^{2+} -free EM.

Although the latter medium was not supplemented with EGTA, which would chelate heavy-metal ions, we observed indistinguishable responses to AVP in it and Ca^{2+} -free EM supplemented with EGTA (not shown), indicating that either medium effectively prevented Ca^{2+} entry. The perfusion system allowed the medium bathing the cells to be changed with a half-time of ≈ 9 s. Rapid switching (0.8–1 s/ratio) of the excitation wavelengths (340–380 nm) was by means of either a rotating filter wheel or monochromator. Emitted light (λ_{em} is emission wavelength) was collected at 510 nm. All recordings were corrected for autofluorescence by addition of ionomycin (10 μM) and MnCl_2 (5 mM) at the end of each recording.

Even when EM was prepared with the purest reagents, it was contaminated with heavy-metal ions, such that fura 2 fluorescence was quenched by as much as 96% ($\lambda_{\text{ex}} = 340\text{--}380$ nm) and then reversed by addition of *N,N,N',N'*-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN) or diethylenetriaminepenta-acetic acid (DTPA), chelators of heavy-metal ions [20]. Although using Teflon FEP bottles and limiting storage of media to < 24 h diminished the problem, it was not eliminated; TPEN (20 μM) was therefore included in most media. Because TPEN binds heavy-metal ions with very high affinity (less than nanomolar) [20], we report the extracellular concentration of heavy-metal ions as their total concentration less the 20 μM bound to TPEN. Under conditions identical with those used for experiments, TPEN (20 μM for 2–3.5 h) had no effect on hepatocyte viability, whether assessed by Trypan Blue exclusion ($104 \pm 8\%$), cell morphology, the ability of cells to retain lactate dehydrogenase ($95 \pm 6\%$, $n = 3$) or the ability of the cells to respond reversibly to Ca^{2+} -mobilizing stimuli.

Effect of Ni^{2+} on fura 2 fluorescence ratios

Because Ni^{2+} binds with higher affinity than Ca^{2+} to Ca^{2+} buffers and we cannot prepare media in which the total $[\text{Ca}^{2+}]$ is ≈ 3 μM , we examined the effects of Ni^{2+} on fura 2 fluorescence in cytosol-like medium (CLM) containing 1 mM CaCl_2 . This procedure prevents any significant change in medium free $[\text{Ca}^{2+}]$ after Ni^{2+} addition, but the fluorescence signal recorded at $\lambda_{\text{ex}} = 380$ nm is too small to be reliably used for fluorescence ratios (Figure 1). The results demonstrate that Ni^{2+} quenched fura 2 fluorescence at all excitation wavelengths and that concentrations of Ni^{2+} that quenched $\leq 76\%$ of the fluorescence ($\lambda_{\text{ex}} = 360$ nm) did not significantly affect the $R_{340/360}$ fluorescence ratio (Figure 1). Since even the highest concentration of NiCl_2 used to stimulate intact cells (10 mM) quenched only $8.5 \pm 0.6\%$ ($n = 3$) of the fura 2 fluorescence ($\lambda_{\text{ex}} = 360$ nm) during the time taken for maximal changes in fluorescence ratios (see Figure 3 below), the $R_{340/360}$ ratio is used in most experiments to provide a measure of $[\text{Ca}^{2+}]_i$ that is unaffected by cytosolic Ni^{2+} .

Estimates of free $[\text{Ni}^{2+}]$

To quantitatively estimate the cytosolic free $[\text{Ni}^{2+}]$ from the quenching of fura 2 fluorescence, we first established the affinity of fura 2 for Ni^{2+} . Fura 2 fluorescence ($\lambda_{\text{ex}} = 360$ nm) was recorded at 20 °C in CLM containing 1 mM CaCl_2 while adding increasing concentrations of NiCl_2 . By assuming that Ca^{2+} and Ni^{2+} compete for the same binding site on fura 2 and that its K_D^{Ca} is 135 nM at 20 °C [21], we estimated K_D^{Ni} to be 24 nM. Using the same method for Mn^{2+} , we obtained a K_D^{Mn} value of 3.4 nM, which is similar to that previously reported (3.2 nM) [21], confirming the validity of the approach. From the affinities of fura 2 for Ca^{2+} and Ni^{2+} , the resting $[\text{Ca}^{2+}]_i$ derived by calibrating $R_{340/380}$ signals of unstimulated hepatocytes (77 ± 6 nM, $n = 31$) and the extent to which fura 2 fluorescence ($\lambda_{\text{ex}} = 360$ nm) was

Figure 1 Ni²⁺ quenches fura 2 fluorescence without affecting $R_{340/360}$

Increasing concentrations of NiCl₂ (0, 0.1, 1, 10, 100, 125, 172, 188, 250, 500, 1000 and 5000 μ M) were added to CLM ($[Ca^{2+}] = 1$ mM) containing 100 nM fura 2 and fluorescence ($\lambda_{em} = 510$ nm) was recorded at each of the indicated excitation wavelengths (i). Despite quenching of $\leq 76\%$ of the fluorescence signal ($\lambda_{ex} = 360$ nm), the fluorescence ratio ($R_{340/360}$) was unaffected by Ni²⁺ (≤ 500 μ M) (ii).

quenched after addition of extracellular NiCl₂ ($0.4 \pm 0.2\%$, $n = 3$), we estimated the free $[Ni^{2+}]_i$. Our results, suggest that, in the interval between addition of a maximal concentration of NiCl₂ (280 μ M) and the onset of the increase in $[Ca^{2+}]_i$, the free $[Ni^{2+}]_i$ increased to < 0.2 nM. Even during prolonged exposure (2.4 ± 0.7 min, $n = 3$) to extracellular Ni²⁺ (280 μ M), during which the resulting increase in $[Ca^{2+}]_i$ peaked, $[Ni^{2+}]_i$ did not exceed ≈ 4.8 nM.

To quantitatively compare responses to Ni²⁺ in the presence and absence of extracellular Ca²⁺ (Figure 4 below), the effects of Ca²⁺ on the free $[Ni^{2+}]_i$ need to be considered. TPEN binds Ca²⁺ with low affinity ($K_D^{Ca} 40$ μ M) [20]; its apparent affinity for Ni²⁺ in EM ($[Ca^{2+}] 1.5$ mM) will therefore be reduced by ≈ 37 -fold relative to its affinity in Ca²⁺-free EM. Because the K_D^{Ni} of TPEN is likely to be less than a nanomolar value, even a 37-fold decrease in its apparent affinity would not appreciably affect our estimates of the free $[Ni^{2+}]_i$ within the micromolar range over which it stimulates Ca²⁺ signals.

Materials

Reagents for the preparation of hepatocytes were from suppliers listed previously [18]. Cell culture media, with the exception of serum (Advanced Protein Products, Brockmoor, West Midlands, U.K.), were from Gibco-BRL (Paisley, Renfrewshire, Scotland, U.K.). Fura 2/AM and Pluronic F-127 were from Molecular Probes Europe BV (Leiden, The Netherlands). Percoll was from Pharmacia (Uppsala, Sweden). The chloride salts of heavy-metal ions (Sigma) were of analytical grade. All other reagents, including cytosine arabinoside and TPEN, were from Sigma. All solutions, which were stored for no more than 24 h in Teflon FEP bottles (Nalgene), were prepared with deionized water purified using a Milli-Q Plus 185 system (Millipore, Watford, U.K.).

RESULTS

Effects of Ni²⁺ on the fura 2 fluorescence of hepatocytes

Addition of NiCl₂ (1 mM) to fura 2-loaded hepatocytes caused changes in fura 2 fluorescence ($R_{340/380}$) that were similar in magnitude to those evoked by maximally effective concentrations of the Ca²⁺-mobilizing hormones, ATP (1 mM) (Figure 2, upper panel) or AVP (100 nM) (results not shown). Ni²⁺ had no significant effect on the autofluorescence of hepatocytes. The effects of Ni²⁺ on $R_{340/380}$ were completely reversible (Figure 2, lower panel).

These effects of NiCl₂ are consistent with an ability to increase $[Ca^{2+}]_i$, but we were concerned that Ni²⁺ might enter hepatocytes; differential quenching of fura 2 fluorescence at different excitation wavelengths might then cause changes in $R_{340/380}$. Because the $R_{340/360}$ fluorescence ratio is unaffected by Ni²⁺ (Figure 1 and the Materials and methods section), it allows an unambiguous measurement of $[Ca^{2+}]_i$ and was therefore used in subsequent experiments with heavy-metal ions.

Addition of NiCl₂ (1 mM) to fura 2-loaded hepatocytes caused an increase in $R_{340/360}$ that was similar in amplitude to that evoked by AVP (100 nM) (Figure 3, and Figure 5 below). In common with previously reported effects of Ca²⁺-mobilizing

Figure 2 Ni²⁺ and Ca²⁺-mobilizing hormones cause similar reversible changes in the fura 2 fluorescence of cultured hepatocytes

Upper panel: fura 2-loaded hepatocytes were incubated in EM (without TPEN) and either NiCl₂ (1 mM) (i) or ATP (1 mM); similar results were obtained with AVP (ii) for the period shown by the thick horizontal bar. The results indicate that Ni²⁺ and Ca²⁺-mobilizing hormones cause similar changes in fura 2 fluorescence ratio ($R_{340/380}$). Lower panel: hepatocytes were exposed to NiCl₂ (10 mM) for the periods shown by the thick horizontal bars. The results show that Ni²⁺ causes reversible increases in $R_{340/380}$.

Figure 3 Ni^{2+} increases $[\text{Ca}^{2+}]_i$ in cultured hepatocytes

Cultured hepatocytes loaded with fura 2 were incubated in EM containing TPEN ($20 \mu\text{M}$) and NiCl_2 (1 mM) was added for the period shown by the solid horizontal bar. Fluorescence at each excitation wavelength (i) and the fluorescence ratios $R_{340/380}$ (ii) and $R_{340/360}$ (iii) are shown. Since Ni^{2+} does not itself affect $R_{340/360}$ (Figure 1), the results, typical of three similar experiments, demonstrate that it evokes an increase in $[\text{Ca}^{2+}]_i$. The inset to panel (ii), drawn to the same scale as the main panel, shows the lack of effect of NiCl_2 (1 mM , solid horizontal bar) on the $[\text{Ca}^{2+}]_i$ of A7r5 (a) or BC₃H1 (b) smooth-muscle cells, or calf pulmonary-artery endothelial cells (c). Results typical of at least three independent experiments are shown.

hormones on $[\text{Ca}^{2+}]_i$ [22], the effects of Ni^{2+} occurred after a latency. In the presence of extracellular Ca^{2+} , the latency decreased from $64 \pm 9 \text{ s}$ ($n = 3$) with $\approx 10 \mu\text{M}$ free Ni^{2+} to $19 \pm 2 \text{ s}$ ($n = 3$) with 10 mM Ni^{2+} ; the latter delay is largely attributable to the $\approx 9 \text{ s}$ mixing time of the perfusion system. In the presence of extracellular Ca^{2+} and $20 \mu\text{M}$ TPEN, the maximal and half-maximal (EC_{50}) effects occurred with free $[\text{Ni}^{2+}]$ of $\approx 280 \mu\text{M}$ and $\approx 28 \pm 6 \mu\text{M}$ [Hill coefficient (h) = 2.8 ± 0.1 ; $n = 3$], respectively (Figure 4). Similar effects of NiCl_2 were observed in the absence of TPEN, but the maximal responses and EC_{50} values varied between experiments and the concentration-effect relationships were shallow, presumably reflecting variable contamination of media with heavy-metal ions.

Effects of Ni^{2+} on intracellular Ca^{2+} stores

The maximal effect of Ni^{2+} and the EC_{50} for the effect of free Ni^{2+} on $R_{340/360}$ were similar in the presence (maximal increase = 0.58 ± 0.05 ; $\text{EC}_{50} \approx 28 \pm 6 \mu\text{M}$, $n = 3$) and absence (maximal increase = 0.51 ± 0.03 ; $\text{EC}_{50} \approx 24 \pm 9 \mu\text{M}$, $n = 3$) of extracellular Ca^{2+} (Figure 4). In the absence of extracellular Ca^{2+} , Ni^{2+}

Figure 4 Concentration-dependent effects of Ni^{2+} on $R_{340/360}$ in the presence and absence of extracellular Ca^{2+}

Cultured hepatocytes loaded with fura 2 were incubated in EM containing TPEN ($20 \mu\text{M}$). The peak $R_{340/360}$ signals evoked by the addition of the indicated total $[\text{Ni}^{2+}]$ were recorded in the presence (●) or absence (○) of extracellular Ca^{2+} . Results are means $\pm$ S.E.M. for three independent experiments.

Figure 5 Ni^{2+} and AVP mobilize the same intracellular Ca^{2+} stores

Cultured hepatocytes loaded were exposed to AVP (100 nM) or NiCl_2 (1 mM) for the periods shown. All media contained $20 \mu\text{M}$ TPEN, but Ca^{2+} (1.5 mM) was present for only the periods shown by solid horizontal bars. Insets show results from experiments in which the intracellular stores were allowed to refill with Ca^{2+} between challenges. The results, each typical of three independent experiments, show the $R_{340/360}$ signals.

(1 mM) and AVP (100 nM) evoked similar transient increases in $[\text{Ca}^{2+}]_i$ and prior exposure to either agonist substantially attenuated the subsequent response to the other (Figure 5). Ni^{2+} abolished the subsequent $R_{340/360}$ response to AVP, which decreased from 0.56 ± 0.05 to 0 ($n = 3-6$), and AVP decreased the

Table 1 Changes in [Ni²⁺]_i are unlikely to be the cause of increased [Ca²⁺]_i

Cultured hepatocytes were incubated in EM containing TPEN (20 μ M) before addition of extracellular NiCl₂ at the indicated concentrations. The extent to which addition of Ni²⁺ quenched the fluorescence of cytosolic fura 2 in the interval between addition of extracellular NiCl₂ and the increase in [Ca²⁺]_i is shown. The results are means $\pm$ S.E.M. for three independent experiments. Note that in this Table, in contrast to the results presented in the text, the total extracellular [Ni²⁺] is shown.

Total extracellular [Ni ²⁺]	Evoked increase in $R_{340/360}$	Quench of fluorescence ($\lambda_{ex} = 360$) (%)
30 μ M	0.14 $\pm$ 0.02	1 $\pm$ 0
100 μ M	0.53 $\pm$ 0.01	0.1 $\pm$ 0
300 μ M	0.58 $\pm$ 0.05	0.4 $\pm$ 0.2
1 mM	0.55 $\pm$ 0.01	0.6 $\pm$ 0.2
3 mM	0.60 $\pm$ 0.03	1 $\pm$ 0.4
10 mM	0.65 $\pm$ 0.04	1.6 $\pm$ 0.2

subsequent response to Ni²⁺ from 0.60 $\pm$ 0.03 to 0.23 $\pm$ 0.04 ($n = 3$ –6) (Figure 5). When the experiments were repeated with a recovery interval (20 min) in Ca²⁺-containing EM, neither agonist significantly affected the subsequent response to the other (Figure 5 insets); after AVP, the response to Ni²⁺ was 110 % (0.66 $\pm$ 0.04, $n = 3$) of its initial value, and after Ni²⁺, the response to AVP was 100 % (0.56 $\pm$ 0.03, $n = 3$) of its initial value. Although AVP and Ni²⁺ released the same intracellular Ca²⁺ stores (Figure 5), the increase in [Ca²⁺]_i evoked by AVP (or ATP) decayed more rapidly ($t_{1/2} = 64 \pm 5$ s, $n = 6$) than that evoked by Ni²⁺ ($t_{1/2} = 139 \pm 5$ s, $n = 6$) (Figure 5), suggesting that Ni²⁺ may lack the ability of AVP and other Ca²⁺-mobilizing hormones to activate Ca²⁺ extrusion from hepatocytes [23].

The effects of Ni²⁺ in the presence of extracellular Ca²⁺ were complicated by its ability both to stimulate Ca²⁺ mobilization (Figure 5) and to inhibit Ca²⁺ entry through the capacitative pathway [24]. In the presence of extracellular Ca²⁺, the increase in [Ca²⁺]_i evoked by low concentrations of Ni²⁺ (< 1 mM) was sustained for as long as both extracellular Ca²⁺ and Ni²⁺ were present. The responses to higher concentrations of Ni²⁺ (1–10 mM) were transient in both the presence and absence of extracellular Ca²⁺ (results not shown). These results are consistent with Ni²⁺-evoked Ca²⁺ mobilization being more sensitive to Ni²⁺ (EC₅₀ $\approx$ 24 μ M; Figure 4) than is blockade of Ca²⁺ entry (IC₅₀ = 200–220 μ M) [24,25].

Although addition of Ni²⁺ to fura 2-loaded hepatocytes did cause quenching of fura 2 fluorescence, the effect was neither substantial (typically < 1 % quenching at $\lambda_{ex} = 360$ nm, corresponding to [Ni²⁺]_i < 5 nM at the peak of the Ca²⁺ signal) nor correlated with the amplitude of the [Ca²⁺]_i signals evoked (Table 1).

In EM, both freshly isolated and cultured hepatocytes responded to AVP (100 nM) with an increase in [Ca²⁺]_i, although the amplitude of the increase in $R_{340/360}$ was greater in cultured (0.59 $\pm$ 0.06, $n = 3$) than in fresh cells (0.23 $\pm$ 0.01, $n = 3$). Only two of three preparations of fresh cells responded to Ni²⁺ (10 mM), and the $R_{340/360}$ changes it evoked were < 20 % of those observed in cultured cells (results not shown). The estimated [Ni²⁺]_i, derived from measurement of fura 2 quenching, was similar in fresh and cultured hepatocytes (results not shown).

Ni²⁺ (1 mM) had no significant effect on the [Ca²⁺]_i of A7r5 or BC₃H1 smooth-muscle cell lines, of a calf pulmonary endothelial cell line (Figure 3ii, inset) or of pituitary cells derived from ovine pars tuberalis in primary culture, although each cell type re-

Figure 6 Zn²⁺ and Ni²⁺ mobilize the same intracellular Ca²⁺ stores

Cultured hepatocytes loaded with fura 2 were incubated in EM and exposed to ZnCl₂ (100 μ M) or NiCl₂ (1 mM) for the periods shown. Ca²⁺ (1.5 mM) was present in EM for only the periods shown by solid horizontal bars; TPEN (20 μ M) was present throughout. The results, representative of those from three similar experiments, show the changes in $R_{340/360}$.

sponded with an increase in [Ca²⁺]_i when stimulated with an appropriate hormone.

Effects of other ions

Neither increasing [Mg²⁺] from 1.2 to 5 mM nor decreasing extracellular pH from 7.3 to 6.0 affected the [Ca²⁺]_i of cultured hepatocytes. Addition of MnCl₂ (5 mM) or CoCl₂ (1 mM) caused quenching of the intracellular fura 2 fluorescence at all excitation wavelengths without significantly affecting $R_{340/360}$ (results not shown). CdCl₂ (1 mM), which has similar effects to Ca²⁺ on fura 2 fluorescence [21], caused a slow sustained increase in fluorescence at $\lambda_{ex} = 340$ nm and a decrease at $\lambda_{ex} = 380$ nm, but never triggered the rapid transient fluorescence changes evoked by AVP or Ni²⁺. Addition of either CuCl₂ (100 μ M; free [Cu²⁺] $\approx$ 80 μ M) (results not shown) or ZnCl₂ (100 μ M; free [Zn²⁺] $\approx$ 80 μ M) (Figure 6) to cultured hepatocytes in Ca²⁺-free EM evoked transient reciprocal changes in fluorescence at $\lambda_{ex} = 340$ and 380 nm and transient increases in $R_{340/360}$. The effect of Zn²⁺ (80 μ M) was similar both in the shape of the response (Figure 6) and in the maximal amplitude of the increase in $R_{340/360}$ (0.52 $\pm$ 0.03, $n = 4$) to that evoked by a maximally effective concentration of Ni²⁺ (1 mM; 0.59 $\pm$ 0.04, $n = 3$). Furthermore, hepatocytes pre-treated with ZnCl₂ (80 μ M) in Ca²⁺-free EM scarcely responded when subsequently exposed to NiCl₂ (1 mM): the increase in $R_{340/360}$ decreased from 0.59 $\pm$ 0.04 in control cells to 0.11 $\pm$ 0.02 ($n = 3$) in those pre-treated with Zn²⁺ (Figure 6). The hepatocytes fully recovered their sensitivity to Ni²⁺ when the incubations with the two ions were separated by a recovery period in normal EM (Figure 6).

Low concentrations of La³⁺ (μ M) block Ca²⁺ entry in a variety of cells and might therefore prevent Ni²⁺ entry to reveal whether its effects on [Ca²⁺]_i require interaction with an intracellular

Figure 7 High concentrations of La^{3+} evoke a sustained increase in $[\text{Ca}^{2+}]_i$

Throughout the trace, hepatocytes were incubated in Ca^{2+} -free EM containing TPEN ($20 \mu\text{M}$) and then stimulated with LaCl_3 (5 mM) or NiCl_2 (1 mM) for the periods shown.

target. LaCl_3 ($480 \mu\text{M}$) had no effect on the fura 2 fluorescence of hepatocytes at any excitation wavelength and nor did it prevent Ni^{2+} entry; it did, however, cause the increase in $[\text{Ca}^{2+}]_i$ evoked by NiCl_2 ($80 \mu\text{M}$) to be sustained for as long as La^{3+} remained, even in the absence of extracellular Ca^{2+} (results not shown). A higher concentration of LaCl_3 (5 mM) mimicked the effects of Ni^{2+} on $R_{340/360}$ (Figure 7) by mobilizing the same intracellular Ca^{2+} stores as Ni^{2+} and AVP (results not shown). However, whereas the responses to Ni^{2+} and AVP were transient in Ca^{2+} -free EM (Figures 5 and 6), those evoked by La^{3+} were sustained (Figure 7). Furthermore, when hepatocytes were pre-treated with La^{3+} (5 mM , 11 min) in the absence of extracellular Ca^{2+} , they were later able to respond to Ni^{2+} despite the continued absence of extracellular Ca^{2+} (Figure 7). These results suggest that, as the extracellular $[\text{La}^{3+}]$ falls, it causes sequential reversal of the Ca^{2+} mobilization and then of the inhibition of the plasma-membrane Ca^{2+} -ATPase, thereby allowing intracellular Ca^{2+} stores to refill and again respond to Ni^{2+} .

DISCUSSION

Extracellular Ni^{2+} stimulates mobilization of Ca^{2+} stores in hepatocytes

Addition of Ni^{2+} to hepatocytes evoked reversible (Figure 2) changes in fura 2 fluorescence that were characteristic of an increase in $[\text{Ca}^{2+}]_i$ and entirely different from the direct effects of Ni^{2+} on fura 2 fluorescence (Figure 1). The ability of Ni^{2+} to increase $[\text{Ca}^{2+}]_i$ results from release of intracellular Ca^{2+} stores. The effect of Ni^{2+} on $[\text{Ca}^{2+}]_i$ was transient (Figures 2, 3, 5 and 6) and only minimally affected by removal of extracellular Ca^{2+} (Figures 4, 5 and 6). The EC_{50} for the Ni^{2+} -stimulated increase in $[\text{Ca}^{2+}]_i$ was unaffected by extracellular Ca^{2+} (Figure 4). Furthermore, Ni^{2+} had no effect on $R_{340/360}$ in hepatocytes in which the intracellular Ca^{2+} stores had been emptied by thapsigargin (results not shown). Ni^{2+} , ATP and AVP had similar effects on $[\text{Ca}^{2+}]_i$ (Figures 2 and 5), and each released the same pool of intracellular Ca^{2+} (Figure 5).

Why should Ni^{2+} mimic hormones in primary cultures of hepatocytes, but have little effect on freshly isolated cells? The discrepancy is not a consequence of contamination of the primary cultures, because most cells were morphologically identifiable as hepatocytes and they responded to appropriate Ca^{2+} -mobilizing hormones (AVP, ATP, angiotensin II), but not to bradykinin, which evokes an increase in $[\text{Ca}^{2+}]_i$ in fibroblasts. Plasma-membrane receptors damaged during enzymic isolation of hepatocytes are known to be resynthesized and regain their appropriate distribution during primary culture [26]. Our results,

in which AVP responses were also ≈ 3 -fold larger in primary cultures, might therefore be explained by suggesting that a putative plasma membrane receptor for heavy-metal ions (and to a lesser extent that for AVP) is damaged or re-distributed during isolation of hepatocytes and regains its function in primary culture. The same explanation may account for the observation that Ni^{2+} ($\approx 200 \mu\text{M}$) stimulates Ca^{2+} influx and potentiates AVP-evoked bile secretion [25] from intact liver, while most [27,28], though not all [29], studies have failed to detect an effect of Ni^{2+} on $[\text{Ca}^{2+}]_i$ in freshly isolated hepatocytes.

By interacting with thiol groups, heavy-metal ions can inhibit InsP_3 5-phosphatase activity [30], both inhibit nuclear Ca^{2+} uptake and promote Ca^{2+} release [31], inhibit SERCA ('sarco/endoplasmic reticulum Ca^{2+} -ATPase')-type Ca^{2+} -ATPases [32], and stimulate ryanodine receptors [33]. The effects of heavy-metal ions on $[\text{Ca}^{2+}]_i$ in hepatocytes were invariably accompanied by changes in fura 2 fluorescence ($\lambda_{\text{ex}} = 360 \text{ nm}$; increase for Zn^{2+} , decreases for Ni^{2+} , Cu^{2+} , La^{3+}) that suggested the metal ions had reached the cytosol. Interactions between heavy-metal ions and the thiol groups of any of the intracellular proteins involved in Ca^{2+} regulation could, therefore, underlie the effects of Ni^{2+} on $[\text{Ca}^{2+}]_i$, but it is more likely that the heavy metals exert their effects from the extracellular surface of the cell. First, while increases in extracellular Ni^{2+} evoked concentration-dependent increases in $[\text{Ca}^{2+}]_i$, there was no consistent relationship between $[\text{Ni}^{2+}]_i$ and the increase in $[\text{Ca}^{2+}]_i$ (Table 1). Secondly, our estimate of $[\text{Ni}^{2+}]_i$ ($< 5 \text{ nM}$) during maximal responses to extracellular Ni^{2+} was far lower than the concentrations (several micromolar) of heavy-metal ions that affect the intracellular proteins involved in Ca^{2+} regulation [30–33]. Thirdly, the specificity of heavy-metal ions in evoking increases in $[\text{Ca}^{2+}]_i$ in hepatocytes ($\text{Ni}^{2+}/\text{Zn}^{2+} > \text{Cu}^{2+} > \text{La}^{3+}$; Co^{2+} , Cd^{2+} and Mn^{2+} ineffective) is different from their relative affinities for thiol groups ($\text{Cd} > \text{Zn} > \text{Co}$) [34], their effects on intracellular Ca^{2+} transport ($\text{Cd}^{2+} > \text{Zn}^{2+}$) [32], and from their abilities to displace Zn^{2+} from metallothionein and evoke increases in $[\text{Zn}^{2+}]_i$ and glycogenolysis ($\text{Cd}^{2+} > \text{Cu}^{2+} > \text{Zn}^{2+}$; Ni^{2+} and Co^{2+} ineffective) [35]. Finally, the lack of effect of Ni^{2+} in freshly isolated hepatocytes, which do respond to Ca^{2+} -mobilizing stimuli, is most readily explained by suggesting that plasma-membrane receptors damaged during cell isolation recover only after a period in culture.

We cannot yet establish whether the site through which heavy-metal ions exert their effects responds solely to heavy-metal ions or whether they modify residues of other Ca^{2+} -mobilizing receptors to cause their activation. The complete and rapid reversibility of the effects of Ni^{2+} (Figure 2, lower panel) and the specificity of the site in recognizing only some heavy-metal ions, argue in favour of a receptor that specifically recognizes metal ions. Furthermore, under physiological conditions, hepatocytes are both more sensitive to Ni^{2+} ($\text{EC}_{50} < 30 \mu\text{M}$) (Figure 4) than are other cation receptors ($\text{EC}_{50} > 100 \mu\text{M}$) [13] and as sensitive to Ni^{2+} as the parathyroid Ca^{2+} receptor is to its most potent agonist, Gd^{3+} ($\text{EC}_{50} \approx 35 \mu\text{M}$) [5].

The site in hepatocytes differs in its recognition properties from all other proposed receptors for extracellular cations. It is unlike the receptor cloned from parathyroid cells [3,5,36] because it is neither activated by changes in extracellular $[\text{Ca}^{2+}]$ or $[\text{Mg}^{2+}]$, nor do changes in extracellular $[\text{Ca}^{2+}]$ affect the sensitivity of hepatocytes to Ni^{2+} (Figure 4). The receptors, suggested to be present in fibroblasts, endothelial cells, neuroblastoma cells and arterial smooth muscle, are activated by a decrease in extracellular pH [37]. Cd^{2+} is a more potent stimulus than Ni^{2+} , and both Zn^{2+} and Cu^{2+} are competitive antagonists [11,12]. None of these characteristics are shared with hepatocytes. The inability of Cd^{2+}

to evoke a Ca²⁺ signal in hepatocytes argues that the putative receptor is also different from those in osteoclasts [13] and *Xenopus* oocytes [16]. We conclude that the sites through which heavy-metal ions stimulate Ca²⁺ mobilization in hepatocytes is distinct from those that mediate responses of other tissues to extracellular cations.

Physiological implications

The putative cation receptor of hepatocytes is not an extracellular Ca²⁺ receptor (Figure 4) and nor is the mRNA for the parathyroid receptor or its homologues expressed in liver [5,6]. Nor is Ni²⁺ likely to be an endogenous ligand because Ni²⁺ appears to fulfil biological functions only in anaerobic prokaryotes [38], although in mammals it is the most common cause of allergic reactions to metals, and Ni²⁺ toxicity is associated with loss of hepatic glycogen [39]. The liver, by excreting Cu²⁺ in bile, is an important regulator of plasma Cu²⁺ levels, and numerous hepatic disorders follow Cu²⁺ toxicosis [17]. The liver also provides the major control of plasma Zn²⁺, and there are antagonistic effects between Cu²⁺ and Zn²⁺ [17]. The putative receptor might therefore be involved in plasma Zn²⁺/Cu²⁺ regulation. Although total plasma [Zn²⁺] ($\approx 150 \mu\text{M}$) and [Cu²⁺] ($\approx 20 \mu\text{M}$) are high in humans [40], their free concentrations ($2 \times 10^{-10} \text{ M}$ for Zn²⁺) [41] are much lower because they bind to albumin. However, even if the free plasma [Zn²⁺] and [Cu²⁺] are too low in the general circulation to stimulate hepatic receptors, their free concentrations in the portal circulation or, for Cu²⁺, in bile may be sufficient to activate hepatocytes. Alternatively, Zn²⁺ may function as a local hormone or neurotransmitter. Zn²⁺ is present at high concentrations within vesicles of many nerve terminals [42] and it is accumulated with insulin in the secretory vesicles of pancreatic β -cells. Since insulin (and Zn²⁺) are released directly into the portal circulation, the liver is exposed to higher concentrations of each than are other tissues: the endogenous ligand of the putative heavy-metal ion receptor in liver may therefore be Zn²⁺, which might then play some part in co-ordinating metabolic activities of the liver.

We conclude that, in hepatocytes, though not in other cell types, micromolar concentrations of certain extracellular heavy-metal ions evoke a reversible release of Ca²⁺ from hormone-sensitive stores. We suggest that a novel heavy-metal ion receptor may be responsible and that it may stimulate InsP₃ formation, although the low density of the primary cultures of hepatocytes has so far prevented reliable measurements of the formation of [³H]inositol phosphates. This putative receptor may allow hepatocytes to respond to Zn²⁺ released into the portal circulation with insulin, it may provide a site through which heavy-metal ions exert toxic effects, and its presence limits the utility of certain heavy-metal ions as pharmacological tools in the analysis of Ca²⁺ signalling pathways. The latter is a particular problem for Ni²⁺ because it cannot be used to inhibit Ca²⁺ entry ($\text{IC}_{50} \approx 200 \mu\text{M}$) [24,25] without also stimulating Ca²⁺ mobilization ($\text{EC}_{50} \approx 30 \mu\text{M}$).

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