7982 *Journal of Physiology* (1998), **513.2**, *pp.*399–410

The effect of extracellularly applied divalent cations on cytosolic Ca^{2+} in murine Leydig cells: evidence for a Ca^{2+}-sensing receptor

Olugbenga A. Adebanjo, Joseph Igietseme*, Christopher L.-H. Huang† and Mone Zaidi

*Center for Skeletal Aging and Osteoporosis, Veterans Affairs Medical Center and Medical College of Pennsylvania-Hahnemann School of Medicine, Allegheny University of the Health Sciences and University of Pennsylvania, Philadelphia, PA 19104, *Morehouse School of Medicine, Department of Microbiology and Immunology, Atlanta, GA 30310, USA and † The Physiological Laboratory, University of Cambridge, Cambridge CB2 3EG, UK*

(Received 6 March 1998; accepted after revision 3 September 1998)

1. The effect of extracellularly applied divalent cations upon cytosolic Ca^{2+} levels ($[Ca^{2+}]$) was investigated in fura-2-loaded mouse Leydig (TM3) cells.
2. The extracellular application of Ca^{2+} (2·5–15 mM) or Ni^{2+} (0·5–5 mM) elicited concentration-dependent elevations in cytosolic $[Ca^{2+}]$ that were followed by decays to baseline levels. Extracellular Mg^{2+} (0·8–15 mM) failed to influence cytosolic $[Ca^{2+}]$.
3. Conditioning applications of Ca^{2+} (2·5–10 mM), Mg^{2+} (2·5–15 mM) or Ni^{2+} (0·5–5 mM) all attenuated the cytosolic Ca^{2+} response to a subsequent test application of 5 mM $[Ni^{2+}]$.
4. The amplitude of Ni^{2+}-induced cytosolic Ca^{2+} signals remained constant in low-Ca^{2+} solutions. Such findings suggest a participation of Ca^{2+} release from intracellular stores. In parallel, depletion of Ca^{2+} stores by either ionomycin (5 μM, in low-Ca^{2+} solutions) or thapsigargin (4 μM) abolished or attenuated Ni^{2+}-induced Ca^{2+} transients.
5. Ionomycin (5 μM) elevated cytosolic $[Ca^{2+}]$ in Ca^{2+}-free solutions even after prior Ni^{2+} application, indicating the presence of Ni^{2+}-insensitive stores.
6. Caffeine (250 and 500 μM) elevated cytosolic $[Ca^{2+}]$ and attenuated Ni^{2+}-induced Ca^{2+} release. Furthermore, TM3 cells stained intensely with a specific anti-ryanodine receptor antiserum, Ab^{34}. These findings suggest that Ca^{2+} release is regulated by ryanodine receptors.
7. Both membrane depolarization and hyperpolarization, brought about by changes in extracellular $[K^+]$ ($[K^+]_e$) in the presence of valinomycin (5 μM), altered the waveform of the Ni^{2+}-induced cytosolic Ca^{2+} signal. Hyperpolarization, in addition, diminished the response magnitude. Such voltage-induced response modulation localizes the regulatory events to the Leydig cell plasma membrane.
8. We propose the existence of a cell surface divalent cation (Ca^{2+}) receptor in Leydig cells, the activation of which triggers Ca^{2+} fluxes through ryanodine receptors.

Leydig cells secrete the androgenic steroid testosterone that is vital both for male sexual development and the maintenance of skeletal integrity (Jackson, 1993). Testosterone secretion from Leydig cells in response to luteinizing hormone is modulated through changes in the intracellular levels of both Ca^{2+} and cyclic AMP (cAMP); the effect of cAMP is also exerted ultimately through a change in cytosolic $[Ca^{2+}]$ (Sullivan & Cooke, 1986). Furthermore, intracellular Ca^{2+} also influences steroidogenesis although the precise mechanism of this effect is unclear. It is believed that mitochondrial cholesterol transport and enzymatic side chain cleavage are both Ca^{2+}-sensitive (Sullivan & Cooke, 1986). In addition to intracellular $[Ca^{2+}]$ changes, changes in extracellular $[Ca^{2+}]$ ($[Ca^{2+}]_e$) also modulate testosterone secretion. The latter doubles when extracellular $[Ca^{2+}]$ is increased from 1 to 10 mM (Meikle *et al.* 1991). Again, the mechanism through which an elevated $[Ca^{2+}]_e$ is transduced into enhanced testosterone secretion is unknown. Additionally, we are also unclear about the altered or steady-state $[Ca^{2+}]_e$ experienced by Leydig cells *in vivo*.

Previous studies have shown that certain eukaryotic cells 'sense' changes in their extracellular [Ca^{2+}], a property that has been attributed to the existence of a variety of surface membrane Ca^{2+}-sensing receptors (Brown *et al.* 1995). These cells include parathyroid hormone-secreting chief cells (Brown, 1991; Brown *et al.* 1993), calcitonin-secreting thyroid C cells (Garrett *et al.* 1995), Ca^{2+}-absorbing gastric mucosal and intestinal cells (Pazianas *et al.* 1995; Cima *et al.* 1997; Gama *et al.* 1997), Ca^{2+}-reabsorbing renal medullary and cortical cells (Ricardi *et al.* 1995), cytotrophoblasts (Lundgren *et al.* 1994; Bax *et al.* 1994), neurones (Ruat *et al.* 1995; Quinn *et al.* 1997), bone-resorbing osteoclasts (Zaidi *et al.* 1989; Malgaroli *et al.* 1989) and bone-forming osteoblasts (Honda *et al.* 1995). Notably, the osteoclast Ca^{2+} receptor, which we believe is a functional component of a surface ryanodine receptor, responds to low-millimolar [Ca^{2+}] changes generated locally as a result of hydroxyapatite dissolution (Zaidi *et al.* 1995). Its activation results in the inhibition of bone resorption, possibly as part of a feedback mechanism of osteoclast control (Moonga *et al.* 1990).

All Ca^{2+}-sensing receptors are thought to trigger intracellular signals in response to changed $[Ca^{2+}]_e$. This, in turn, regulates cell function. Most commonly, this signal is a cytosolic [Ca^{2+}] change that results from both transmembrane Ca^{2+} influx and intracellular Ca^{2+} release (Brown, 1991; Zaidi *et al.* 1993*a*). The latter involves either ryanodine receptors or inositol trisphosphate (IP_3) receptors (Brown, 1991; Shankar *et al.* 1995*b*). In the osteoclast, however, the plasma membrane ryanodine receptor itself gates Ca^{2+} influx (Zaidi *et al.* 1995; Adebanjo *et al.* 1996).

This study has used methods employed previously to characterize Ca^{2+}-sensing in other cells (Brown, 1991; Zaidi *et al.* 1993*a*). Specifically, divalent cations have been used as surrogate Ca^{2+} agonists allowing us to distinguish Ca^{2+} release from Ca^{2+} influx (Nemeth, 1990; Shankar *et al.* 1993). Our results demonstrate a plasma membrane divalent cation (Ca^{2+}) receptor in Leydig cells, the activation of which triggers Ca^{2+} release from ryanodine receptor-gated intracellular Ca^{2+} stores.

METHODS

Reagents

Fura-2, fura-2 AM and ionomycin were purchased from Molecular Probes, Inc. (Eugene, OR, USA). Tissue culture materials, including Hepes and heat-inactivated fetal calf serum (FCS) were purchased from Gibco-BRL. EDTA, EGTA, Triton X-100 and trypsin were all obtained from Sigma Chemical Co. Measurements of cytosolic [Ca^{2+}] were carried out in either RPMI-1640 ([Ca^{2+}], 1·25 mM) (Gibco BRL) or modified Krebs (Ca^{2+}-free) medium. The latter comprised (mM): 130 NaCl, 5 KCl, 0·8 $MgCl_2$, 5 glucose, 10 Hepes and 1·2 EGTA (pH 7·4) ([Ca^{2+}] < 5 nM, by fura-2 measurements). An antibody, Ab^{34}, raised to the consensus calmodulin-binding sequence of the ryanodine receptor was kindly provided for us by Dr F. A. Lai (National Institute for Medical Research, London, UK). The antibody has been shown not to differentiate between the three known ryanodine receptor isoforms, types I, II and III. It also does not bind to any one of the known IP_3 receptor isoforms (Zaidi *et al.* 1995).

Culture of TM3 cells

Leydig cells (TM3, ATCC-CRL-1714, American Tissue Culture Collection, Riversville, MD, USA) derived from BALB/c mice have primary epithelial cell characteristics, are non-tumorigenic and express receptors for epidermal growth factor, luteinizing hormone, androgens, oestrogen and progesterone. The cells were grown in Hepes-buffered RPMI-1640 supplemented with FCS (10% v/v), glutamine (1% w/v), penicillin (50 kU l^{-1}) and streptomycin (50 mg l^{-1}). The cells were sub-cultured at confluence by washing in EDTA, followed by trypsin treatment (0·025% w/v) for 2 min, addition of RPMI-1640 before centrifugation, and resuspension in medium. The cells were maintained in tissue culture flasks (Fisher Scientific) at 37 °C and were harvested in their logarithmic growth phase.

Cytosolic [Ca^{2+}] measurements

A fluorescence method employing an inverted phase-contrast microscope (Diaphot, Nikon) was used to measure cytosolic [Ca^{2+}] in single TM3 cells using the Ca^{2+}-sensitive fluorochrome, fura-2 (Shankar *et al.* 1993). Glass coverslips containing dispersed cells were incubated with 10 μM fura-2 AM in serum-free RPMI-1640 for 30 min at 37 °C. They were then transferred to a Perspex bath on the microscope stage and exposed to agonists by pipetting solutions that were pre-warmed to 37 °C. The temperature of the solution was kept constant by a thermostatically controlled heating device. Its volume was maintained at 2 ml using a vacuum withdrawal of fluid rising beyond a constant bath level.

Fluorochrome-loaded TM3 cells were exposed alternately to excitation wavelengths of 340 and 380 nm approximately every second. This was achieved by using a microcomputer-driven wheel to which band-pass interference filters had been fitted. The emitted fluorescence was deflected to a dichroic mirror (400 nm), filtered at 510 nm, and directed to the microscope side-port fitted with a photomultiplier tube (PM28B, Thorn EMI). The photomultiplier tube produced single photon currents that were fed into a photon counter (Newcastle Photometric Systems, Newcastle-upon-Tyne, UK). Photon counts per second (c.p.s.) were recorded on an IBM microcomputer. The ratio of emitted fluorescence intensities due to excitation at 340 and 380 nm, F_{340}/F_{380}, was calculated and displayed.

The fura-2 signals were calibrated using a protocol for intracellular calibration described previously by Tsien & Pozzan (1989) and adopted by us (Shankar *et al.* 1993). Briefly, fura-2-loaded cells were bathed in a Ca^{2+}-free, EGTA-containing solution consisting of (mM): 130 NaCl, 5 KCl, 5 glucose, 0·8 $MgCl_2$, 10 Hepes and 0·1 EGTA. Ionomycin (5 μM) was first applied in order to obtain the minimum ratio due to lowest cytosolic [Ca^{2+}] (R_{min}) and the maximal fluorescent intensity at 380 nm (F_{max}). $CaCl_2$ (1 mM) was then applied together with 5 μM ionomycin in order to obtain values for the maximum ratio due to elevated cytosolic [Ca^{2+}] (R_{max}) and the minimal fluorescent intensity at 380 nm (F_{min}). Note that, at this concentration, ionomycin has been shown to equilibrate extracellular and intracellular Ca^{2+} pools in the osteoclast (Shankar *et al.* 1994). The dissociation constant (K_d) for Ca^{2+} and fura-2 at a temperature of 20 °C, an ionic strength of 0·1 M, and a pH of 6·85, is 224 nM (Tsien & Pozzan, 1989). The values, together with the experimental signal, R, were substituted into the equation:

$$\text{cytosolic } [Ca^{2+}] = K_d \times [(R - R_{min})/(R_{max} - R)] \times [(F_{max}/F_{min})].$$

The resulting cytosolic [Ca^{2+}] levels between treatment groups were compared by Analysis of Variance (ANOVA) with Bonferroni's correction for inequality.

Immunocytochemistry

Coverslips containing TM3 cells were fixed with glutaraldehyde (10% v/v) and permeabilized gently with Triton X-100 (0·1% v/v). They were then incubated with normal goat serum diluted in 10 mM phosphate-buffered saline (PBS; 1 in 10, pH 7.4) in multiwell dishes for 15 min. Excess serum was removed and replaced with Hanks' Balanced Salt Solution (HBSS). The cells were then incubated with non-immune rabbit serum (control) or Ab[34] (both diluted in HBSS, 1 in 100, v/v). After 1 h of incubation, the coverslips were rinsed gently with HBSS, drained, and re-incubated for a further hour with goat anti-rabbit FITC (1 in 20, diluted in HBSS). Finally, the coverslips were washed gently and drained. Cells were visualized on an epifluorescence microscope (Diaphot).

RESULTS

Effect of extracellular Ca^{2+} and Ni^{2+} on cytosolic $[Ca^{2+}]$ in TM3 cells

Application of RPMI-1640 containing added $CaCl_2$ extracellularly to TM3 cells at a $[Ca^{2+}]_e$ between 2·5 and 15 mM ($[Mg^{2+}]$, 0·8 mM) produced transient elevations of cytosolic $[Ca^{2+}]$ (Fig. 1). Each cytosolic Ca^{2+} response consisted of a rise over ~50 s followed by a decline towards basal levels (Fig. 1*A*). Figure 1*C* plots values of the magnitude (Δ) of the cytosolic $[Ca^{2+}]$ change (peak minus basal cytosolic $[Ca^{2+}]$: means + S.E.M.) *versus* the concentration of applied extracellular Ca^{2+}. An ANOVA with Bonferroni's correction for inequality revealed a significant difference in Δ cytosolic $[Ca^{2+}]$ when the response to an application of 2·5 mM $[Ca^{2+}]_e$ was compared with that to 10 mM $[Ca^{2+}]_e$ ($P = 0·018$), but not when a similar comparison was made between the response to 2·5 mM $[Ca^{2+}]_e$ and that to either 5 or 15 mM $[Ca^{2+}]_e$ ($P = 0·702$ and 0·490, respectively). In contrast, application of the related alkaline earth metal Mg^{2+} failed to elicit cytosolic Ca^{2+} signals at any concentration between 0·8 and 15 mM ($[Ca^{2+}]_e$, 1·25 mM).

In previous studies with osteoclasts, the transition metal cation Ni^{2+}, when used instead of Ca^{2+}, has allowed a clear distinction between processes attributable to intracellular Ca^{2+} release and those resulting from extracellular Ca^{2+} influx (Shankar *et al.* 1993). These studies went on to explore the effect of a range of divalent and trivalent metal ions and emerged with a rank order of potency of action, $Cd^{2+} > Ni^{2+} = La^{3+} > Al^{3+} > Ca^{2+} > Ba^{2+} = Sr^{2+} > Mg^{2+}$ (Zaidi *et al.* 1991; Shankar *et al.* 1992). The present study similarly assessed the effect of the transition metal cation Ni^{2+} on cytosolic $[Ca^{2+}]$ in cultured Leydig cells. Figure 2*A* demonstrates that Ni^{2+}, when applied to cells bathed in 1·25 mM $[Ca^{2+}]_e$ and 0·8 mM $[Mg^{2+}]_e$, typically triggered a transient elevation in cytosolic $[Ca^{2+}]$ at concentrations > 1·5 mM. The responses to Ni^{2+}, obtained over 60 and 120 s, typically consisted of a rapid elevation of cytosolic $[Ca^{2+}]$ to a peak followed by a decay to baseline that was more complex in waveform than that expected from a single exponential decline in some traces. Their maximum amplitude increased

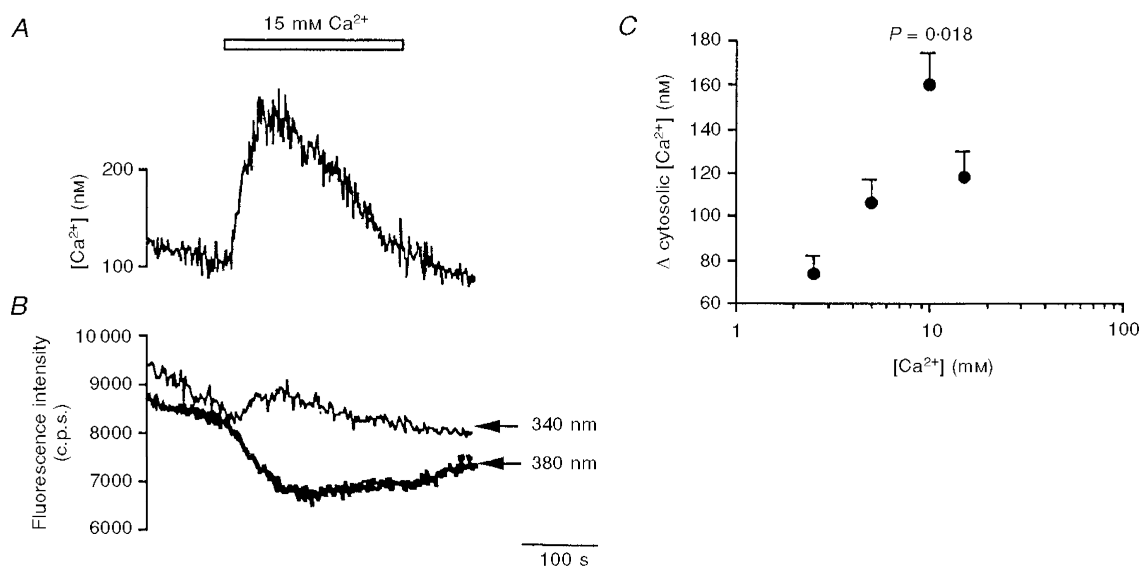

Figure 1. TM3 cell responses to elevations of extracellular $[Ca^{2+}]$

A and *B*, representative traces showing the effects of elevating extracellular $[Ca^{2+}]$ to 15 mM on cytosolic $[Ca^{2+}]$ (nM) (*A*) and fluorescence intensities (counts per second (c.p.s.)) at excitation wavelengths of 340 and 380 nm (*B*) in cultured TM3 cells (for details see Methods). *C*, effect of a range of extracellular $[Ca^{2+}]$ on the mean peak change (Δ) in cytosolic $[Ca^{2+}]$ (nM). The latter data points were derived by subtracting the basal from peak cytosolic $[Ca^{2+}]$. Each data point (mean + S.E.M.) was then compared with the response to 2·5 mM $[Ca^{2+}]$ (regarded as the control) by ANOVA with Bonferroni's correction for inequality. Except for 10 mM $[Ca^{2+}]$, the rest of the points were not significantly different ($P > 0·05$) from control ($n = 4$–6 for each point).

with [Ni^{2+}] up to 3 mM ($P = 0{\cdot}028$), but fell at 5 mM [Ni^{2+}], nevertheless remaining significantly higher ($P = 0{\cdot}009$) than the corresponding response to 0·5 mM [Ni^{2+}] (Fig. 2*B*). The larger responses to Ni^{2+} as compared with Ca^{2+} were consistent with the order of potency demonstrated previously with the osteoclast system (Zaidi *et al.* 1991; Shankar *et al.* 1992).

Conditioning cation applications inactivate the response to 5 mM [Ni^{2+}]

The effect of Ni^{2+} on cytosolic [Ca^{2+}] was inactivated by prior exposures to Ni^{2+}, Ca^{2+} or Mg^{2+}. In the first set of experiments, further cells were exposed to a range of Ni^{2+} concentrations (0·5–5 mM) that were themselves effective in triggering cytosolic Ca^{2+} transients (see above). When the cytosolic [Ca^{2+}] had returned to baseline, the cells were washed with serum-free medium, and then exposed to a second pulse of 5 mM [Ni^{2+}] within 1 or 2 min. Figure 3*A* and *B* displays traces of emitted fluorescence (F_{340} and F_{380}; photon c.p.s.) due to excitation at wavelengths of 340 and 380 nm, respectively (F_{340} and F_{380}; photon c.p.s.) below the resulting ratiometric (F_{340}/F_{380}) signals under two sets of experimental conditions. Figure 3*A* shows the results from a dye-loaded TM3 cell bathed in RPMI-1640 that was exposed to a conditioning application of 3 mM Ni^{2+} followed by a test application of 5 mM Ni^{2+} (horizontal open bars). Figure 3*B* displays the contrasting results of adding the 5·0 mM Ni^{2+}directly to free fura-2 (10 micromolar) in the bath solution.

A comparison of these results makes it unlikely that the traces in Fig. 3*A* primarily reflect a fura-2 reaction with Ni^{2+} that had permeated into the cytosol rather than a reflection of changes in cytoplasmic [Ca^{2+}]. First, Fig. 3*A* shows that the initial agonist application to the TM3 cells produced upward deflections in the F_{340} trace but downward deflections in the F_{380} traces. This is in contrast to the direct reaction between fura-2 and Ni^{2+} that produced downward deflections in both the F_{340} and the F_{380} signals (Fig. 3*B*). The relative deflections in the F_{340} and F_{380} traces in Fig. 3*A* thus do not fulfil the predictions of a direct reaction of dye with Ni^{2+}. Secondly, both (F_{340} and F_{380}) fluorescence traces

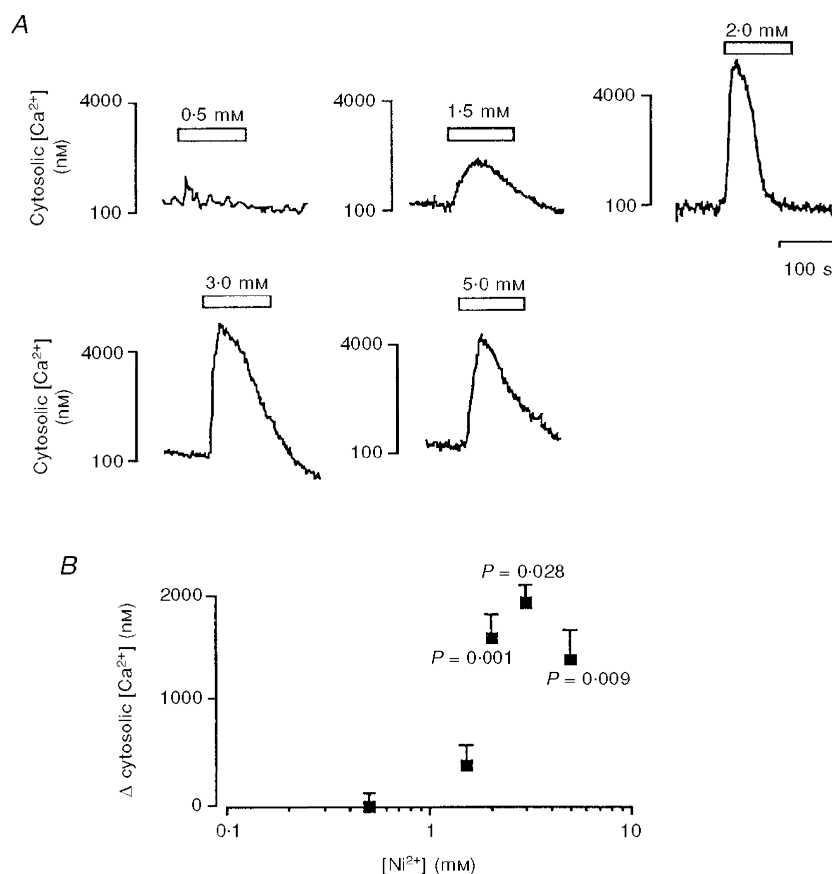

Figure 2. Cytosolic [Ca^{2+}] responses to extracellularly applied [Ni^{2+}]

A, representative traces showing the effect of extracellularly applied [Ni^{2+}] (0·5–5 mM) on cytosolic [Ca^{2+}] in cultured TM3 cells (for details, see Methods). *B*, effect of a range of extracellular [Ni^{2+}] (0·5–5 mM) on the mean peak change (Δ) in cytosolic [Ca^{2+}] (nM). The latter data points were derived by subtracting the basal from the peak cytosolic [Ca^{2+}]. Each data point was then compared with the response to 0·5 mM [Ni^{2+}] (regarded as the control) by ANOVA with Bonferroni's correction for inequality. *P* values are shown, $n = 4$–6 for each point.

from the TM3 cells eventually returned towards their previous stable baseline values (Fig. 3A). In contrast, the direct reaction between Ni^{2+} and free fura-2 produced a sustained deflection in both traces with no recovery whatsoever to the initial baseline (Fig. 3B). The latter finding would require a specific cellular mechanism for altering cytosolic Ni^{2+} in order to reproduce a trace of the form of Fig. 3A. Thirdly, the ratiometric (F_{340}/F_{380}) signal from the TM3 cells showed a corresponding deflection followed by return to baseline consistent with a net flux of Ca^{2+} into the cytosolic compartment followed by its net withdrawal. In contrast, Fig. 3B shows little significant alteration in the F_{340}/F_{380} ratio with the direct addition of Ni^{2+}. Finally, Fig. 3A shows that the subsequent test applications of Ni^{2+} to TM3 cells influenced neither the F_{340} and F_{380} traces nor their ratio, suggesting an inactivation of a process modifying cytosolic Ca^{2+} rather than a capacity for passive Ni^{2+} entry.

Figure 4 displays cytosolic Ca^{2+} responses resulting from a test application of 5 mM Ni^{2+} following conditioning applications of Ni^{2+} over a range of Ni^{2+} concentrations. Responses to the second Ni^{2+} pulse were diminished to an extent that depended upon the conditioning $[Ni^{2+}]$ (Fig. 4A and B). This reduction was significant at conditioning $[Ni^{2+}]$ of 4 and 5 mM ($P = 0.009$ and 0.027, respectively).

Figure 5 summarizes typical results from experiments that investigated whether extracellular Ca^{2+} or Mg^{2+} could inactivate the cytosolic Ca^{2+} response to 5 mM $[Ni^{2+}]$. In these experiments, the cells were exposed to a range of $[Ca^{2+}]_e$ (2·5–10 mM) or $[Mg^{2+}]_e$ (0·8–15 mM). Following recovery from any resulting cytosolic $[Ca^{2+}]$ change, as in the case of $[Ca^{2+}]_e$ elevation, the cells were washed with serum-free medium and a pulse of 5 mM $[Ni^{2+}]$ was applied within 1–2 min. Prior application of Ca^{2+} (panel A) or Mg^{2+} (panel B) resulted in a progressive concentration-dependent diminution of the cytosolic Ca^{2+} responses to Ni^{2+}. Each

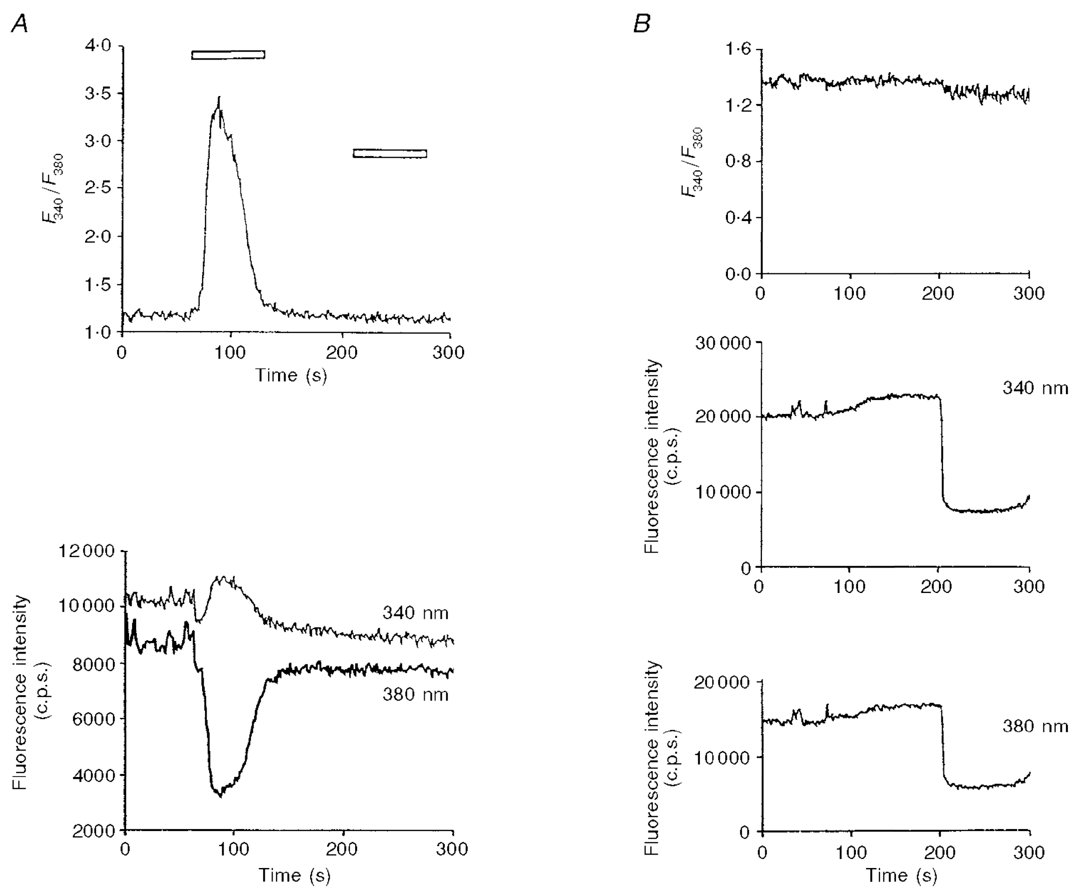

Figure 3. Fluorescence intensities at excitation wavelengths of 340 and 380 nm and the ratio F_{340}/F_{380} under two sets of experimental conditions

A, typical results from a dye-loaded cell bathed in RPMI-1640, exposed to a conditioning application of 3 mM Ni^{2+} followed by a test application of 5 mM Ni^{2+} (horizontal open bars). B, results of adding 5·0 mM Ni^{2+} directly to free fura-2 (10 micromolar) in the bath solution containing (mM): 130 NaCl, 5·0 KCl, 5 glucose and 10 Hepes. Note the downward deflections in both the F_{340} and the F_{380} signals after adding Ni^{2+} in B.

Table 1. Basal (pretreatment) and peak cytosolic [Ca^{2+}] from cytosolic [Ca^{2+}] responses of TM3 cells under different experimental conditions

External solution	Application	Pretreatment $[Ca^{2+}]_i$ (nM)	Peak $[Ca^{2+}]_i$ (nM)
$[Ca^{2+}]_e = 1·25$ mM	5 mM [Ni^{2+}]	111 ± 46·4	1482 ± 439 [a]
1·2 mM EGTA, $[Ca^{2+}]_e < 5$ nM	6 mM [Ni^{2+}]	78·9 ± 11·9	2216 ± 581 [a1]
	then 5 μM ionomycin	955 ± 23·1 [b]	1373 ± 39·0 [b1]
1·2 mM EGTA, $[Ca^{2+}]_e < 5$ nM	5 μM ionomycin	119 ± 18·1 [c]	6091 ± 690 [c1]
	then 6 mM [Ni^{2+}]	169 ± 4·06 [d]	61·8 ± 3·90 [d1]
1·2 mM EGTA, $[Ca^{2+}]_e < 5$ nM	4 μM thapsigargin	113 ± 26·0 [e]	206 ± 66·6 [e1]
	then 6 mM [Ni^{2+}]	145 ± 4·77	288 ± 122 [a2]

Statistics by ANOVA with Bonferroni's correction for inequality. [a] *vs.* [a1], $P = 0·418$; [b] *vs.* [b1], $P = 0·094$; [c] *vs.* [c1] and [d] *vs.* [d1], $P = 0·001$; [e] *vs.* [e1], $P = 0·222$; and [a] *vs.* [a2], $P = 0·033$ ($n = 3–6$ cells for each variable).

resulting transient increase in cytosolic [Ca^{2+}] was again followed by a decay that was often more complex in waveform than that which might be expected from a simple exponential decay. The attenuation was maximal at 10 mM [Ca^{2+}] or 10 mM [Mg^{2+}]; thus, at these concentrations, peak *versus* baseline cytosolic [Ca^{2+}] was not significantly different ($P = 0·1$ and 0·765, respectively). Note that when solutions without added Ca^{2+} and Mg^{2+} were used, the Ni^{2+}-induced peak cytosolic [Ca^{2+}] was not significantly different from control (cf. Fig. 2).

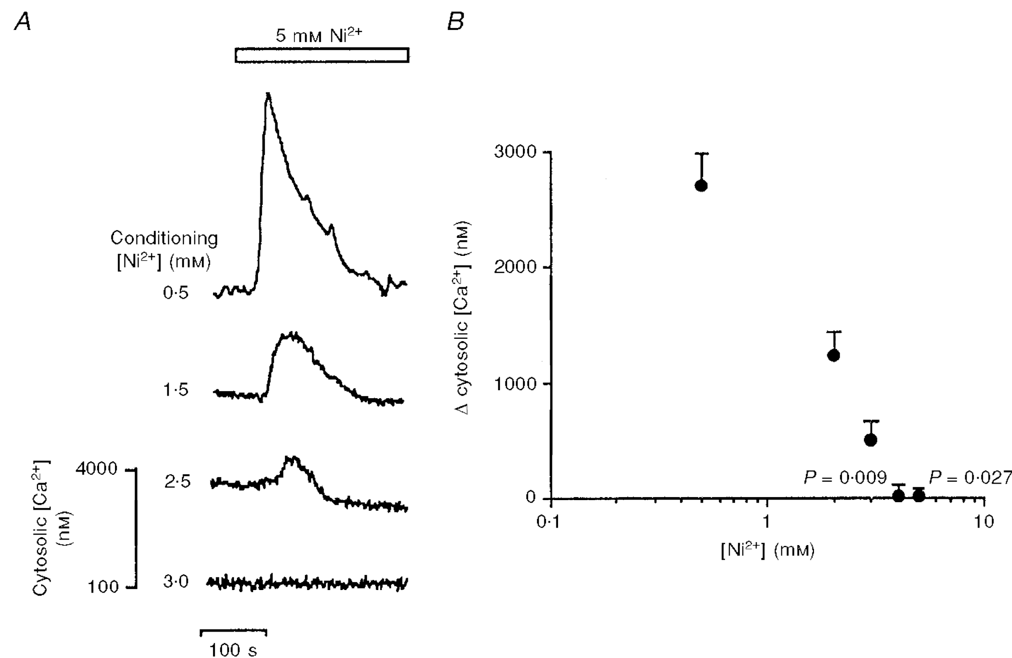

Figure 4. Conditioning applications of extracellular [Ni^{2+}] inactivate the cytosolic [Ca^{2+}] response to subsequent [Ni^{2+}] applications

A, representative traces showing the effect of conditioning with extracellular [Ni^{2+}] (0·5–3 mM) on the cytosolic [Ca^{2+}] change induced by the subsequent application of 5 mM [Ni^{2+}] (open bar) to cultured TM3 cells. The scale bar refers to changes in the levels of the cytosolic [Ca^{2+}] (nM) in the bottom trace. *B*, effect of a range of conditioning extracellular [Ni^{2+}] (0·5–5 mM) on the mean peak change (Δ) in cytosolic [Ca^{2+}] (nM) elicited by the subsequent application of 5 mM [Ni^{2+}]. The latter data points were derived by subtracting the basal from peak cytosolic [Ca^{2+}]. Each data point was then compared with the response to a conditioning 0·5 mM [Ni^{2+}] (regarded as control) by ANOVA with Bonferroni's correction for inequality. *P* values shown, $n = 4–6$ for each point.

Ni^{2+}-induced Ca^{2+} release persists despite reductions in extracellular [Ca^{2+}]

We next examined the extent to which the cytosolic Ca^{2+} signals observed following application of divalent cations might be attributed to the release of intracellularly stored Ca^{2+} as opposed to, but not excluding, transmembrane Ca^{2+} influx. The initial experiments investigated the effect of reducing the net inward electrochemical gradient on the movement of Ca^{2+} by employing Ni^{2+} as a surrogate Ca^{2+} agonist in cells bathed in modified Krebs solution containing 1·2 mM EGTA (see Methods). When added to non-esterified fura-2, the latter solutions caused a shift in baseline fluorescence ratio, F_{340}/F_{380}, consistent with a [Ca^{2+}] < 5 nM. In order to correct for the binding of Ni^{2+} to EGTA that would displace Ca^{2+} from the Ca^{2+}–EGTA complex, we used a calculated [Ni^{2+}] of 6 mM. This corresponded to an effective [Ni^{2+}] of 5 mM (Caputo, 1981) in view of the greater binding affinity of EGTA for Ni^{2+} as compared with Ca^{2+}. Under these conditions any contaminating Ca^{2+} would become free in solution. This would be at concentrations of the order of 1–10 μM that are considerably smaller (by around three orders of magnitude) than the Ca^{2+}-containing solution ([Ca^{2+}], 1·25 mM) used in the preceding controls. Finally, there was also no evidence for an effect of Ni^{2+} upon the fura-2 ratiometric signal. Thus Fig. 3*B* illustrates that the direct application of Ni^{2+} to non-esterified fura-2 failed significantly to shift the baseline fluorescence ratio, F_{340}/F_{380}. This indicated that fura-2 signals remained mostly unaffected by Ni^{2+}, although this does not exclude binding between the fluorochrome and Ni^{2+}.

Figure 6*A–D* illustrates further protocols wherein TM3 cells were exposed to Ni^{2+} either in the presence of Ca^{2+} (1·25 mM, panel *A*) or in Ca^{2+}-free, EGTA-containing medium ([Ca^{2+}] < 5 nM, panel *B*). Comparison of Fig. 6*A* and *B* confirms that exposure of TM3 cells to Ni^{2+} in EGTA-containing solution as opposed to a 1·25 mM [Ca^{2+}]$_e$ did not result in a measurable difference in the overall magnitude of the resulting cytosolic [Ca^{2+}] signal. The decay phases of the cytosolic [Ca^{2+}] responses in the cells that were exposed to normal levels of extracellular [Ca^{2+}] did appear in some cases to be slightly more prolonged than responses from cells where extracellular [Ca^{2+}] was reduced. Table 1

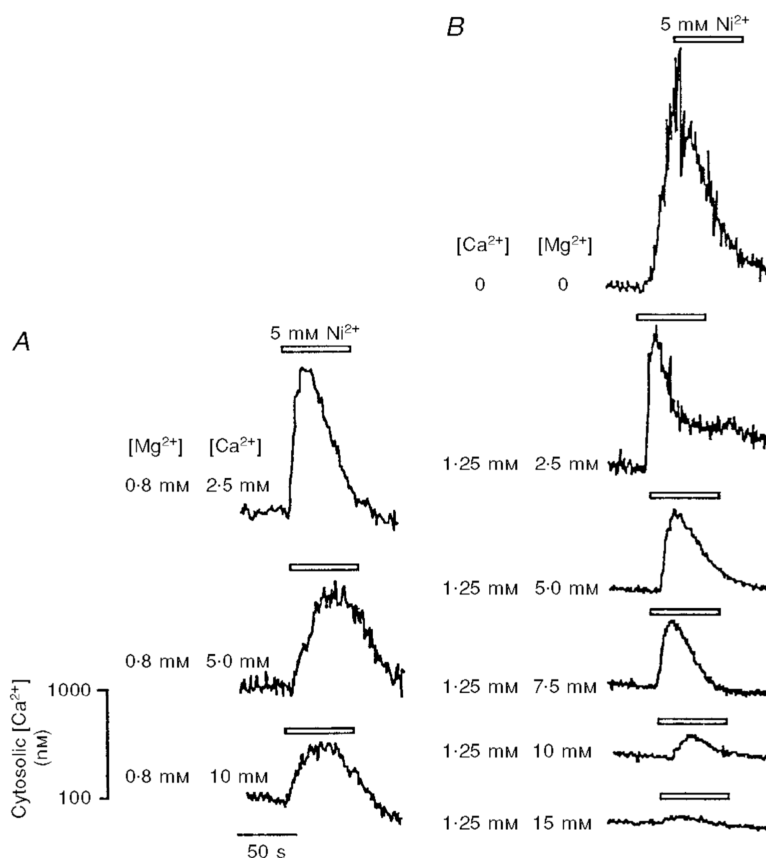

Figure 5. Conditioning applications of Ca^{2+} or Mg^{2+} inactivate the cytosolic [Ca^{2+}] response to extracellularly applied Ni^{2+}

Representative traces showing the effect of extracellular [Ni^{2+}] (5 mM) (open bars) on the cytosolic [Ca^{2+}] (nM) of cultured TM3 cells, in various protocols wherein the cells were bathed in media with different [Ca^{2+}] and [Mg^{2+}] values, as indicated. Prior application of Ca^{2+} (*A*) or Mg^{2+} (*B*) resulted in a progressive concentration-dependent diminution of the cytosolic Ca^{2+} responses to Ni^{2+}. The vertical scale bar refers to the bottom trace, which was obtained under conditions of 0·8 mM Mg^{2+} and 10 mM Ca^{2+}.

Table 2. Peak change (Δ) cytosolic [Ca^{2+}] of TM3 cells under different experimental conditions

Protocol	Concentration	$\Delta[Ca^{2+}]_i$ (nM)
Ni^{2+} (control)	5 mM	1371 ± 393
Caffeine	250 μM	66·8 ± 13·6 [1]
	500 μM	46·4 ± 10·5 [2]
Ni^{2+} after caffeine	250 μM	136 ± 44·0 [3]
	500 μM	401 ± 216 [4]
db-cAMP	200 μM	0
Ni^{2+} after db-cAMP	200 μM	2443 ± 685 [5]

Statistics by ANOVA with Bonferroni's correction for inequality. Basal compared with peak cytosolic [Ca^{2+}]: [1] $P = 0·004$; [2] $P = 0·010$. Peak $\Delta [Ca^{2+}]_i$ of treatment compared with Ni^{2+} alone (control): [3] $P = 0·006$; [4] $P = 0·057$; and [5] $P = 0·274$ ($n = 3–6$ cells for each variable). db-cAMP, dibutyryl cyclic AMP.

shows that the Ni^{2+}-induced peak Δ cytosolic [Ca^{2+}] in the two situations was not significantly different ($P = 0·418$). When the cytosolic Ca^{2+} transient returned to baseline, the cells bathed in Ca^{2+}-free, EGTA-containing medium were exposed to 5 μM ionomycin to deplete any remaining intracellular Ca^{2+} stores. There was still some rise ($P = 0·094$) in cytosolic [Ca^{2+}], suggesting that a single application of Ni^{2+} did not deplete all the intracellular Ca^{2+} stores.

Ni^{2+}-induced Ca^{2+} release depends on intracellular Ca^{2+} stores

Figure 6*C* and *D* represents typical results from two complementary experiments that sought to investigate the effect of depleting intracellular Ca^{2+} stores on the Ni^{2+} effect. First, cells were treated with 5 μM ionomycin in Ca^{2+}-free medium. As expected, this produced a highly significant ($P < 0·001$), transient rise in cytosolic [Ca^{2+}]; a subsequent Ni^{2+} application then failed to elevate cytosolic [Ca^{2+}]. Instead there was a small, but significant ($P < 0·001$) decrement in basal cytosolic [Ca^{2+}] (Fig. 6*C* and Table 1). Parallel experiments used thapsigargin, a microsomal Ca^{2+}-ATPase inhibitor. In Ca^{2+}-free medium, thapsigargin elicited a small rise in cytosolic [Ca^{2+}], indicating store depletion (Table 1). The subsequent application of Ni^{2+} triggered a cytosolic Ca^{2+} signal that was significantly attenuated compared with that elicited in the absence of thapsigargin, either in the presence (cf. Fig. 6*A*, $P = 0·041$) or absence (cf. Fig. 6*B*, $P = 0·033$) of extracellular Ca^{2+}. These results confirmed a participation of Ca^{2+} release from intracellular stores in the Leydig cell response to extracellular applications of divalent cations.

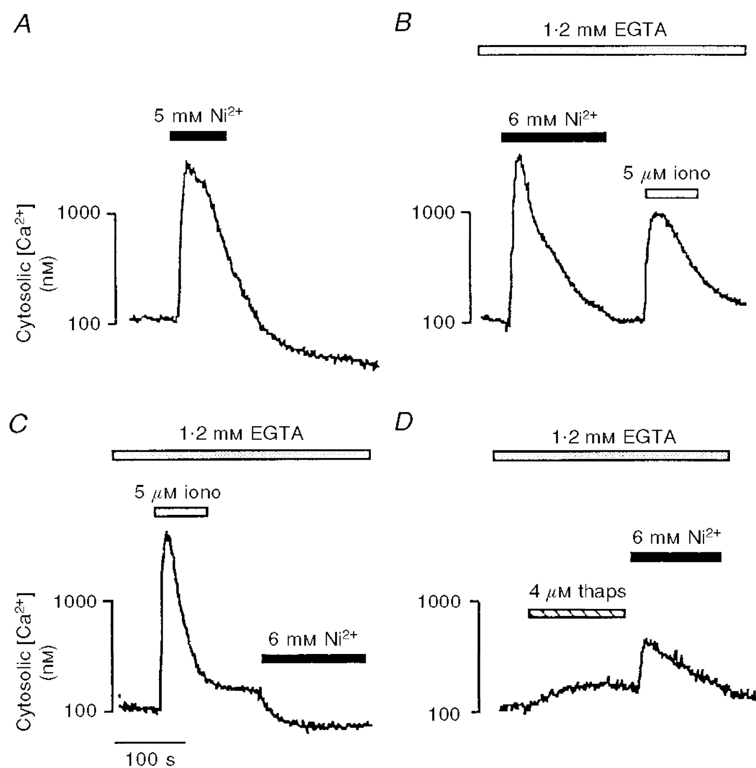

Figure 6. Ni^{2+}-induced cytosolic [Ca^{2+}] elevations utilize intracellular Ca^{2+} stores

Representative traces showing the effects of extracellular [Ni^{2+}] (5 mM; filled bars), ionomycin (iono, 5 μM; open bars) and thapsigargin (thaps, 4 μM; shaded bars), in various protocols, on the cytosolic [Ca^{2+}] of cultured TM3 cells bathed either in medium containing 1·25 mM Ca^{2+} and 0·8 mM Mg^{2+} (*A*) or in modified Krebs solution containing 1·2 mM EGTA (*B–D*). The statistical analysis is given in Table 1.

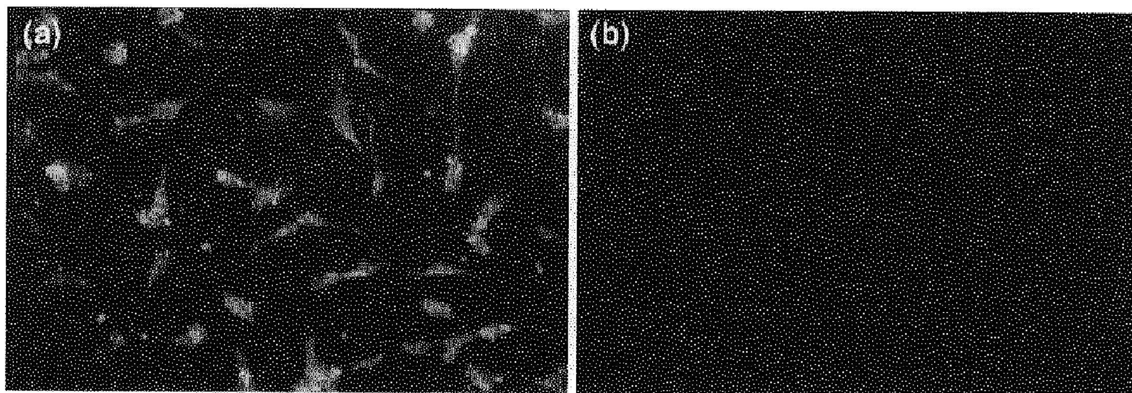

Figure 7. Presence of ryanodine receptors in TM3 cells

Immunofluorescent micrographs of TM3 cells incubated with either antiserum Ab[34] (a) or with non-immune rabbit serum (b; negative control). Field of view, 375 μm × 255 μm.

Ni^{2+}-induced Ca^{2+} release may involve ryanodine receptor-gated Ca^{2+} stores

We next investigated the sensitivity of the intracellular Ca^{2+} stores to caffeine, a known ryanodine receptor agonist. Caffeine itself elevated cytosolic [Ca^{2+}] when applied at concentrations of 250 and 500 μM ($P = 0{\cdot}004$ and 0·01, respectively). More importantly, at both concentrations caffeine also inhibited the cytosolic Ca^{2+} response to Ni^{2+} significantly ($P = 0{\cdot}006$ and 0·06, respectively) (Table 2). That ryanodine receptors were present in TM3 cells was next confirmed immunocytochemically. Notably, permeabilized TM3 cells stained strongly with the antiserum Ab[34]. Cells incubated with non-immune rabbit serum, instead of the antiserum, did not stain (Fig. 7). Taken together, the data suggest that (a) caffeine-sensitive, ryanodine receptor-gated Ca^{2+} stores are present in TM3 cells, and (b) these stores appear to be involved in Ni^{2+}-induced cytosolic Ca^{2+} release.

Note that caffeine is also a phosphodiesterase inhibitor at the concentrations tested and hence is expected to increase cellular cAMP levels. To exclude the latter as a mechanism of caffeine action, we tested the effect of a cell-permeant cAMP analogue, dibutyryl cAMP, on Ni^{2+}-induced Ca^{2+} release. Dibutyryl cAMP (200 μM) neither elevated cytosolic [Ca^{2+}] nor inhibited Ni^{2+}-induced Ca^{2+} release ($P = 0{\cdot}274$) (Table 2).

Membrane potential modulates Ni^{2+}-induced cytosolic Ca^{2+} transients

We finally sought to investigate the effect of changing the cell membrane potential on Ni^{2+}-induced Ca^{2+} release. This was achieved by using 5 μM valinomycin, a K^+ ionophore, in the presence of either 5 or 100 mM [K^+]$_e$. In the presence of valinomycin, 5 mM [K^+]$_e$ is known to shift the membrane potential in the negative direction, whereas 100 mM [K^+]$_e$ causes membrane depolarization (Shankar *et al.* 1995*a*). The

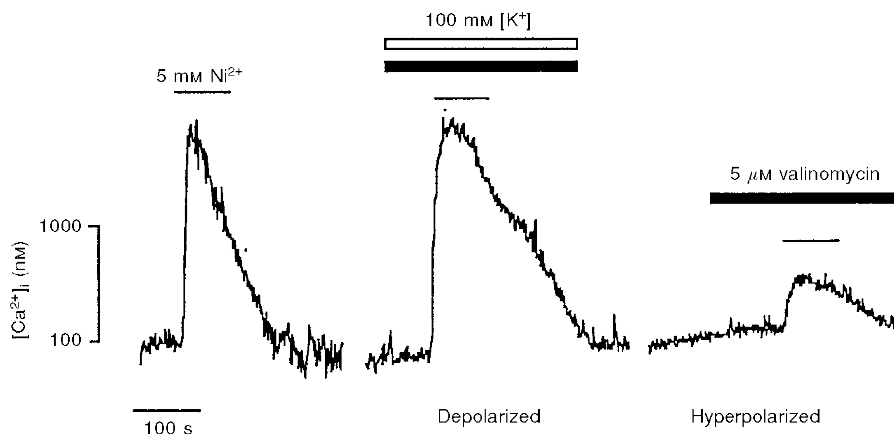

Figure 8. Membrane potential modulates Ni^{2+}-induced cytosolic Ca^{2+} transients

Representative traces showing the effect of extracellular [Ni^{2+}] (5 mM; black lines) on the cytosolic [Ca^{2+}] of cultured TM3 cells, in various protocols whereby the membrane potential was altered by using the K^+ ionophore, valinomycin (5 μM; filled bars), in the presence of either 5 mM (hyperpolarized) or 100 mM [K^+]$_e$ (open bar) (depolarized).

latter manoeuvre slowed the decline of the Ni^{2+}-induced cytosolic Ca^{2+} signal in the absence of any effect on peak Δ cytosolic $[Ca^{2+}]$ (Fig. 8). Conversely, hyperpolarization attenuated the magnitude of the cytosolic Ca^{2+} signal in addition to slowing its decay phase (Fig. 8). These effects of membrane voltage change are consistent with regulatory events that are localized to the Leydig cell membrane.

DISCUSSION

The present study was prompted by the observation that extracellular $[Ca^{2+}]$, when elevated from 1 to 10 mM, doubles Leydig cell testosterone secretion (Meikle *et al.* 1991). Here we show, for the first time, that such elevated extracellular Ca^{2+} levels elicit sharp increases in cytosolic $[Ca^{2+}]$. In addition, by employing experimental strategies used to study extracellular Ca^{2+} sensing in other cells (for reviews see Brown, 1991; Zaidi *et al.* 1993*a*; Brown *et al.* 1995), we have obtained new insights into transduction mechanisms that Leydig cells use to trigger Ca^{2+} release in response to changes in $[Ca^{2+}]_e$.

The transition metal cation, Ni^{2+}, was used in our earlier studies to demonstrate and to characterize a Ca^{2+}-sensing receptor on the osteoclast (Bax *et al.* 1993; Shankar *et al.* 1993; Zaidi *et al.* 1993*a*). Here we provide evidence that Ni^{2+} acts at the Leydig cell surface consistent with the existence of a specific receptor activated by Ca^{2+} and Ni^{2+}. Firstly, Ni^{2+} is thought not to permeate cells; instead it blocks plasma membrane Ca^{2+} channels (Caputo, 1981; Huang, 1988). Secondly, by monitoring the fluorescence separately at each excitation wavelength, 340 and 380 nm, we did not observe classical quenching of the fura-2 signals at any Ni^{2+} concentration tested. Thirdly, it is unlikely that the observed fluorescence changes would have resulted from a fura–Ni^{2+} interaction intracellularly. If so, we should have seen a non-decaying signal as there is no known mechanism of Ni^{2+} efflux from cells. Furthermore, we have confirmed that in the same experimental system, the direct exposure of non-esterified (free) fura-2 to Ni^{2+} did not appreciably alter the ratio of the emission, F_{340}/F_{380}. Thus, it is unlikely that the Ni^{2+}-induced changes in the fluorescence ratio F_{340}/F_{380} could have resulted primarily from the binding of permeated Ni^{2+} to fura-2 within the cells.

Further evidence implicates a cell surface site for Ni^{2+} action more directly. Such an activation site should extend across the cell surface membrane, and hence be exposed to the transmembrane electric field. Thus, we find that a change in cell membrane voltage alters the magnitude and waveform of the Ni^{2+}-induced Ca^{2+} signal (Shankar *et al.* 1995*a*). In the presence of the K^+ ionophore valinomycin, 5 mM $[K^+]_e$ was found to attenuate and prolong the Ni^{2+}-induced cytosolic Ca^{2+} signal. Likewise, 100 mM $[K^+]_e$ prolonged inactivation. The receptor for Ni^{2+} may therefore well be an integral protein that is either itself sensitive to the transmembrane electric field, or whose binding with charged ligands is voltage dependent.

In previous studies with the osteoclast, we have used Ni^{2+} as a substitute for Ca^{2+} to examine changes in cytosolic $[Ca^{2+}]$ in the absence of transmembrane Ca^{2+} influx (Bax *et al.* 1993; Shankar *et al.* 1993; Zaidi *et al.* 1993*a*). Here, we demonstrate a major role for intracellular Ca^{2+} release in the generation of the Ni^{2+}-induced cytosolic Ca^{2+} signal. Thus, a marked reduction of Ca^{2+} in the extracellular solution was found to conserve the magnitude of the Ca^{2+} signal. These results should be treated with caution, as the binding of Ni^{2+} to EGTA and the consequent displacement of Ca^{2+} into the solution would not reverse the cellular electrochemical gradient of Ca^{2+}. There is therefore the possibility that even in EGTA-containing solutions, Ni^{2+} may trigger Ca^{2+} influx; the latter may, in turn, contribute to the Ni^{2+}-induced cytosolic Ca^{2+} signal. However, depletion of releasable Ca^{2+} stores using either a Ca^{2+} ionophore, ionomycin (in Ca^{2+}-free medium) or, more specifically, a microsomal membrane Ca^{2+}-ATPase inhibitor, thapsigargin (in Ca^{2+}-free medium), abolished the Ni^{2+} response (Zaidi *et al.* 1993*b*; Shankar *et al.* 1994). These results with thapsigargin provide more direct evidence for the release of Ca^{2+} from intracellular stores and are reminiscent of hormone effects on membrane receptors or, indeed, cation effects on cell surface Ca^{2+}-sensing receptors (Brown, 1991; Berridge, 1993; Zaidi *et al.* 1993*a*; Brown *et al.* 1995).

The latter studies prompted us to examine whether such Ni^{2+}-induced Ca^{2+} release involved a participation of caffeine-sensitive ryanodine receptors. Note that apart from their classical location in microsomal membranes, ryanodine receptors are also present on the plasma membranes of osteoclasts (Zaidi *et al.* 1995). We thus tested the effect of caffeine on basal cytosolic Ca^{2+} levels and on Ni^{2+}-induced Ca^{2+} release. Caffeine, applied at 250 and 500 μM, itself triggered cytosolic Ca^{2+} signals. At the same concentrations, caffeine significantly inhibited Ni^{2+}-induced cytosolic Ca^{2+} release. The concentrations appear somewhat lower than those used in skeletal muscle (1–10 mM), but are similar to those effective in the osteoclast (50–500 μM) (Shankar *et al.* 1995*b*). In contrast, dibutyryl cAMP did not elevate cytosolic $[Ca^{2+}]$ or attenuate Ni^{2+}-induced Ca^{2+} release. This excludes an effect of caffeine through its inhibition of phosphodiesterase, and a consequent elevation in cellular cAMP. In parallel experiments, an anti-ryanodine receptor antiserum, Ab^{34}, stained Leydig cells strongly and specifically, confirming the expression of ryanodine receptors. Taken together, the results argue strongly for the involvement of ryanodine receptors in Ni^{2+} action on Leydig cells, without ruling out the participation of IP_3 receptors.

Finally, we investigated the interaction between the three cations, Ca^{2+}, Mg^{2+} and Ni^{2+}. Mg^{2+} itself was found not to elevate cytosolic $[Ca^{2+}]$. This is similar to the cation's action on the osteoclast (Zaidi *et al.* 1991), but contrasts with its potent activating action in parathyroid cells (Brown, 1991). However, both Mg^{2+} and Ca^{2+} inhibited Ni^{2+}-induced Ca^{2+} release in a concentration-dependent manner. This inhibition is unlikely to be due to empty Ca^{2+} stores, as Mg^{2+} itself did

not trigger Ca^{2+} release from these stores. It is likely that Mg^{2+} and Ca^{2+} compete with, or else displace Ni^{2+} from its cell surface binding site. Hypothetically, this could result from differences in the physicochemical properties of the cations, such as their crystal ionic radii (0·099 nm for Ca^{2+} *versus* 0·069 nm for Ni^{2+}).

In conclusion, the results provide strong evidence that a divalent cation (Ca^{2+}) receptor is present on the Leydig cell surface. The receptor appears to be coupled to Ca^{2+} release from ryanodine receptor-gated intracellular Ca^{2+} stores. Currently, we have no structural information on this putative entity. Its molecular characterization may nevertheless have significant therapeutic implications. Notably, testosterone and its analogues are being currently investigated for use in preventing muscle and bone loss in ageing men. Furthermore, in men with prostate cancer, testosterone levels must be reduced. Hence it is of interest to modulate endogenous testosterone secretion *in vivo*, potentially by a molecule that could activate or inhibit the Leydig cell Ca^{2+}-sensing receptor. The latter strategy has been used to develop a novel 'calcimimetic', a potent inhibitor of parathyroid hormone secretion for use in humans (Silverberg *et al.* 1997).

Adebanjo, O. A., Shankar, V. S., Pazianas, M., Simon, B., Lai, F. A., Huang, C. L.-H. & Zaidi, M. (1996). Extracellularly applied ruthenium red and cyclic ADP-ribose elevate cytosolic Ca^{2+} in isolated rat osteoclasts. *American Journal of Physiology* **270**, F469–475.

Bax, B. E., Shankar, V. S., Bax, C. M. R., Alam, A. S. M. T., Zara, S. J., Pazianas, M., Huang, C. L.-H. & Zaidi, M. (1993). Functional consequences of the interaction of Ni^{2+} with the osteoclast Ca^{2+} receptor. *Experimental Physiology* **78**, 517–529.

Bax, C. M. R., Bax, B. E., Bain, M. & Zaidi, M. (1994). Ca^{2+} channels in human term trophoblastic cells. A study using the Ca^{2+}-sensitive dye, fura-2. *Trophoblast Research* **8**, 573–580.

Berridge, M. J. (1993). Inositol trisphosphate and calcium signaling. *Nature* **361**, 315–325.

Brown, E. M. (1991). Extracellular Ca^{2+} sensing, regulation of parathyroid cell function, and role of Ca^{2+} and other ions as extracellular (first) messengers. *Physiological Reviews* **71**, 371–411.

Brown, E. M., Gamba, G., Ricardi, D., Lombardi, M., Butters, R., Kifor, O., Sun, A., Hediger, M. A., Lytton, J. & Hebert, S. C. (1993). Cloning and characterization of an extracellular calcium sensing receptor from bovine parathyroid. *Nature* **366**, 575–580.

Brown, E. M., Pollak, M., Seidman, C. E., Seidman, J. G., Chou, Y.-H. W., Ricardi, D. & Hebert, S. C. (1995). Calcium-ion-sensing cell surface receptors. *New England Journal of Medicine* **333**, 234–240.

Caputo, C. (1981). Nickel substitution for calcium and the timecourse of potassium conductances for single muscle fibres. *Journal of Muscle Research and Cell Motility* **2**, 167–182.

Cima, R. R., Cheng, I., Klingensmith, M. E., Chattopadhyay, N., Kifor, O., Hebert, S. C., Brown, E. M. & Soybel, D. (1997). Identification and functional assay of an extracellular Ca^{2+} receptor in *Necturus* gastric mucosa. *American Journal of Physiology* **273**, G1051–1060.

Gama. L., Baxendale-Cox, L. M. & Breitwieser, G. E. (1997). Ca^{2+} sensing receptors in intestinal epithelium. *American Journal of Physiology* **273**, C1168–1175.

Garrett, J. E., Tamir, H., Kifor, O., Simin, R. T., Rogers, K. V., Mithal, A., Gagel, R. F. & Brown, E. M. (1995). Calcitonin-secreting cells of the thyroid express an extracellular calcium receptor gene. *Endocrinology* **136**, 5202–5211.

Honda, Y., Fitzsimmons, R. J., Baylink, D. J. & Mohan, S. (1995). Effects of extracellular calcium on insulin-like growth factor II in human bone cells. *Journal of Bone and Mineral Research* **10**, 1660–1665.

Huang, C. L.-H. (1988). Intramembrane charge movements in skeletal muscle. *Physiological Reviews* **68**, 1197–1247.

Jackson, J. A. (1993). Osteoporosis in men. In *Primer on Metabolic Bone Diseases and Disorders of Mineral Metabolism*, ed. Favus, M. J., pp. 255–257. Lippincott Raven, Philadelphia.

Lundgren, S., Hjalm, G. & Hellman, P. (1994). A protein involved in calcium sensing of the human parathyroid and placental cytotrophoblast cells belongs to the LDL-receptor protein superfamily. *Experimental Cell Research* **212**, 344–350.

Malgaroli, A., Meldolesi, J., Zambonin-Zallone, A. & Teti, A. (1989). Control of cytosolic free calcium in rat and chicken osteoclasts. The role of extracellular calcium and calcitonin. *Journal of Biological Chemistry* **264**, 14342–14347.

Meikle, A. W., Liu, X.-A. & Stringham, J. D. (1991). Extracellular calcium and luteinizing hormone effects on 22-hydroxycholesterol used for testosterone production in mouse Leydig cells. *Journal of Andrology* **12**, 148–151.

Moonga, B. S., Moss, D. W., Patchell, A. & Zaidi, M. (1990). Intracellular regulation of enzyme secretion from rat osteoclasts and evidence for a functional role in bone resorption. *Journal of Physiology* **429**, 29–45.

Nemeth, E. F. (1990). Regulation of cytosolic calcium by extracellular divalent cations in C-cells and parathyroid cells. *Cell Calcium* **11**, 323–327.

Pazianas, M., Adebanjo, O. A., Shankar, V. S., James, S. V., Colston, K. W., Maxwell, J. D. & Zaidi, M. (1995). Extracellular Ca^{2+} sensing by the enterocyte. Prediction of a novel divalent cation sensor. *Biochemical and Biophysical Research Communications* **210**, 448–453.

Quinn, S. J., Ye, C.-P., Diaz, R., Kifor, O., Bai, M., Vassilev, P. & Brown, E. (1997). The Ca^{2+}-sensing receptor: a target for polyamines. *American Journal of Physiology* **273**, C1315–1323.

Ricardi, D., Pak, J., Lee, W.-S., Gamba, G., Brown, E. M. & Hebert, S. C. (1995). Cloning and functional expression of a rat kidney extracellular calcium/polyvalent cation-sensing receptor. *Proceedings of the National Academy of Sciences of the USA* **9**, 131–135.

Ruat, M., Molliver, M. E., Snowman, A. M. & Snyder, S. H. (1995). Calcium sensing receptor: molecular cloning and localization to nerve terminals. *Proceedings of the National Academy of Sciences of the USA* **92**, 3161–3165.

Shankar, V. S., Bax, C. M. R., Alam, A. S. M. T., Bax, B. E., Huang, C. L.-H. & Zaidi, M. (1992). The osteoclast Ca^{2+} receptor is highly sensitive to activation by transition metal cations. *Biophysical and Biochemical Research Communications* **187**, 913–918.

Shankar, V. S., Bax, C. M. R., Bax, B. E., Alam, A. S. M. T., Simon, B., Pazianas, M., Moonga, B. S., Huang, C. L.-H. & Zaidi, M. (1993). Activation of the Ca^{2+} 'receptor' on the osteoclast by Ni^{2+} elicits cytosolic Ca^{2+} signals: evidence for receptor activation and inactivation, intracellular Ca^{2+} redistribution and divalent cation modulation. *Journal of Cellular Physiology* **155**, 120–129.

Shankar, V. S., Huang, C. L.-H., Adebanjo, O. A., Pazianas, M. & Zaidi, M. (1994). Calcium influx and redistribution in isolated rat osteoclasts. *Experimental Physiology* **79**, 537–545.

Shankar, V. S., Huang, C. L.-H., Adebanjo, O. A., Simon, B. J., Alam, A. S. M. T., Moonga, B. S., Pazianas, M., Scott, R. H. & Zaidi, M. (1995*a*). The effect of membrane potential on surface Ca^{2+} receptor activation in rat osteoclasts. *Journal of Cellular Physiology* **162**, 1–8.

Shankar, V. S., Pazianas, M., Huang, C. L.-H., Simon, B., Adebanjo, O. & Zaidi, M. (1995*b*). Caffeine modulates Ca^{2+} receptor activation in isolated rat osteoclasts and induces intracellular Ca^{2+} release. *American Journal of Physiology* **268**, F447–454.

Silverberg, S. J., Bone, H. G. III, Marriott, T. B., Locker, F. G., Thys-Jacobs, S., Dziem, G., Kaatz, S., Sanguinetti, E. L. & Bilezikian, J. P. (1997). Short-term inhibition of parathyroid hormone secretion with calcium receptor agonist in patients with primary hyperparathyroidism. *New England Journal of Medicine* **337**, 1506–1510.

Sullivan, M. & Cooke, B. A. (1986). The role for Ca^{2+} in steroidogenesis in Leydig cells. *Biochemical Journal* **236**, 45–51.

Tsien, R. Y. & Pozzan, T. (1989). Measurement of cytosolic free Ca^{2+} with quin 2. *Methods in Enzymology* **172**, 232–262.

Zaidi, M., Alam, A. S. M. T., Huang, C. L.-H., Pazianas, M., Bax, C. M. R., Bax, B. E., Moonga, B. S., Bevis, P. J. R. & Shankar, V. S. (1993*a*). Extracellular Ca^{2+} sensing by the osteoclast. *Cell Calcium* **14**, 271–277.

Zaidi, M., Datta, H. K., Patchell, A., Moonga, B. S. & MacIntyre, I. (1989). ‘Calcium-activated’ intracellular calcium elevation: a novel mechanism of osteoclast regulation. *Biochemical and Biophysical Research Communications* **163**, 1461–1465.

Zaidi, M., Kerby, J., Huang, C. L.-H., Alam, A. S. M. T., Rathod, H., Chambers, T. J. & Moonga, B. S. (1991). Divalent cations mimic the inhibitory effects of extracellular ionized calcium on bone resorption by isolated rat osteoclasts: further evidence for a ‘calcium receptor’. *Journal of Cellular Physiology* **149**, 422–427.

Zaidi, M., Shankar, V. S., Bax, C. M. R., Bax, B. E., Bevis, P. J. R., Pazianas, M., Alam, A. S. M. T. & Huang, C. L.-H. (1993*b*). Linkage of extracellular and intracellular control of cytosolic Ca^{2+} in rat osteoclasts in the presence of thapsigargin. *Journal of Bone and Mineral Research* **8**, 961–967.

Zaidi, M., Shankar, V. S., Tunwell, R. E., Adebanjo, O. A., McKrill, J., Pazianas, M., O’Connell, D., Simon, B., Rifkin, B. R., Venkitaraman, A., Huang, C. L.-H. & Lai, F. A. (1995). A ryanodine receptor-like molecule expressed in the osteoclast plasma membrane functions in extracellular Ca^{2+} sensing. *Journal of Clinical Investigation* **96**, 1582–1590.

Acknowledgements

This study was supported by grants to M.Z. from the National Institute on Aging (NIH RO1 AG 14917-02), the Department of Veterans Affairs (Merit Review Award) and the Amgen Corporation, Inc., Thousand Oaks, CA, USA. C.L.-H.H. acknowledges the support of the Leverhulme Trust (UK) and the Biotechnology and Biological Research Council (BBSRC) of the UK. J.I. acknowledges the support of grants AI41231 and RR03034 from the NIH.

Corresponding author

C. L.-H. Huang: The Physiological Laboratory, University of Cambridge, Downing Street, Cambridge CB2 3EG, UK.

Email: clh11@cus.cam.ac.uk

Reprint requests

M. Zaidi: Centre for Osteoporosis and Skeletal Aging, Geriatrics and Extended Care Service, Veterans Affairs Medical Center, University and Woodland Avenues, Philadelphia, PA 19104, USA.

Email: zaidim@auhs.edu