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SPECIAL REPORT

Mg^{2+} and caffeine-induced intracellular Ca^{2+} release in human vascular endothelial cells

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Interaction of ionized magnesium ($[Mg^{2+}]_o$) and caffeine in regulation of intracellular free calcium concentration ($[Ca^{2+}]_i$) in human aortic endothelial cells was studied using fura-2 and digital imaging microscopy. In 1.2 mM $[Mg^{2+}]_o$, basal $[Ca^{2+}]_i$ was 73.7 ± 22.4 nM, with a heterogeneous distribution within the cells. No significant changes of basal $[Ca^{2+}]_i$ were found either when cells were treated with 10 mM caffeine or when $[Mg^{2+}]_o$ was lowered from 1.2 mM to 0.3 mM. However, a combined superfusion of the cells with 0.3 mM $[Mg^{2+}]_o$ and 10 mM caffeine resulted in a significant elevation of $[Ca^{2+}]_i$ to 382.8 ± 57.1 nM, probably by release of Ca^{2+} from internal stores, which was attenuated by $NiCl_2$ (1 mM). These results suggest that a Ca^{2+}-induced Ca^{2+} release mechanism is involved in regulation of $[Ca^{2+}]_i$ in endothelial cells, which may be either regulated or modulated by Mg^{2+}.

Keywords: Magnesium; calcium; caffeine; endothelial cells; fura-2

Introduction A rise of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) is known to be closely coupled to important functional properties of the vascular endothelial cells, such as synthesis/release of prostacyclin (PGI_2) or endothelial-derived relaxing factors (EDRFs) and cell contraction, in response to a number of agonists or physical stimuli (Furchgott & Vanhoutte, 1989; Moncada *et al.*, 1991). The elevated $[Ca^{2+}]_i$ in endothelial cells is due to an influx of extracellular Ca^{2+} and release of intracellular Ca^{2+} (Schilling & Elliott, 1992). It has been well-established that intracellular Ca^{2+} release can be mediated by activation of plasma membrane receptors coupled to phospholipase C and generation of inositol 1,4,5-trisphosphate(IP_3) (Schilling & Elliot, 1992). However, the contribution of Ca^{2+} release via non-IP_3-dependent or Ca^{2+}-induced Ca^{2+} release mechanism(s) in endothelial cells remains controversial. For example, opposite effects of caffeine, a marker for Ca^{2+}-induced Ca^{2+} release, on $[Ca^{2+}]_i$ have been found (Buchan & Martin, 1991; Schilling & Elliott, 1992).

Magnesium ions (Mg^{2+}) play an inhibitory role in caffeine-mediated Ca^{2+} release (Karaki & Weiss, 1988) and on $[Ca^{2+}]_i$ regulation in vascular smooth muscle cells (Zhang *et al.*, 1992), but little is known regarding the effects of Mg^{2+} in endothelial cells. Recent reports indicate that Mg^{2+} may be involved in regulation of Ca^{2+} homeostasis in endothelium since changes of extracellular Mg^{2+} ($[Mg^{2+}]_o$) affect release of EDRFs from endothelium in isolated blood vessels (Altura & Altura, 1987). It would thus be of importance to define the interaction of Mg^{2+} and caffeine in the regulation of $[Ca^{2+}]_i$ in endothelium and thus elucidate the mechanism(s) of intracellular Ca^{2+} release in endothelial cells.

Methods Experiments were carried out on a human aortic endothelial cell line (No. AG09799A, 20 passages) obtained from the NIA Aging Cell Repository (Camden, NJ, U.S.A.). Endothelial cells were cultured in 199 media (Sigma Chem. Co., St Louis, MO, U.S.A.) with 15% FSA at 37°C in a humidified atmosphere composed of 95% air:5% CO_2. To avoid synchronized activities of endothelial cells (Sage *et al.*, 1989), coverslips containing cells in near confluent density were used. The cells were loaded with fura-2 (Molecular Probes, Eugene, OR, U.S.A.) by incubating them with 2 μM fura-2/AM in the culture media for 60 min under 95% air:5% CO_2. Then, the coverslips were placed in a chamber on a thermostat-regulated stage of a Nikon fluorescence microscope and superfused with 2.2 mM Ca^{2+}-HEPES buffer solutions (pH 7.4, 37°C) containing 0.3 mM or 1.2 mM Mg^{2+}, respectively, followed by treatment with 10 mM caffeine. In other experiments, effects of a calcium channel influx blocker, $NiCl_2$ (1 mM), on $[Ca^{2+}]_i$ was tested in 0.3 mM Mg^{2+} solution. The ionic activities of Mg^{2+} and Ca^{2+} in HEPES buffer solutions were monitored by ion selective electrodes (NOVA Biomedical Corp, Waltham, MA, U.S.A.) and adjusted with $MgSO_4$ or $CaCl_2$ (Altura *et al.*, 1992). The HEPES buffer solutions also contained (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, HEPES 5, and glucose 10. $[Ca^{2+}]_i$ was measured with a TN8500 FluorPlex III Image Analyzer (Tracor Northern, Madison, WN, U.S.A.). Images of fura-2 fluorescence at 510 nm emission were obtained with 340 and 380 nm excitation wavelengths with a silicon intensified target (SIT) camera. The time interval of switching between these two wavelengths was 5 s. Background fluorescence for both excitation wavelengths were acquired from blanks for each experiment and subtracted from each pair of images separately before ratioing. Fluorescence ratios ($R_{340/380}$) were obtained by dividing the 340 nm image by the 380 nm image. No image misalignments occurred when these two ratiometric images were superimposed.

An *in vitro* calibration method was used to calculate $[Ca^{2+}]_i$ of single endothelial cells. Fura-2 pentapotassium salt (5 μM) was dissolved in small volumes of EGTA-buffered standard solutions (pH 7.1, 37°C) containing either 2.51 mM (max) or 0 mM-Ca^{2+} (min), to generate the maximum (R_{max}) and minimum (R_{min}) fluorescence ratios at the 340 nm and 380 nm wavelength, *i.e.* 5.0550 and 0.3512, respectively. $[Ca^{2+}]_i$ was calculated according to the following equation:

$$[Ca^{2+}]_i = K_d \times B \times (R-R_{min})/(R_{max}-R)$$

A K_d of 224 nM was used for the fura-2/Ca^{2+} complex (Zhang *et al.*, 1992). B is the ratio of fluorescence intensity of fura-2 to the Ca:fura-2 complex excited at 380 nm.

Results Table 1 shows effects of caffeine on $[Ca^{2+}]_i$ in human cultured vascular endothelial cells at 1.2 mM and 0.3 mM extracellular Mg^{2+} concentrations ($[Mg^{2+}]_o$). With 1.2 mM $[Mg^{2+}]_o$, the basal level of $[Ca^{2+}]_i$ in cultured endothelial cells, estimated from the ratio (F340/F380), was 74 ± 22 nM. The values are comparable with basal values found by others (Sage *et al.*, 1989; Buchan & Martin, 1991). The distribution of $[Ca^{2+}]_i$ appeared heterogeneous within the

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Table 1 Effects of extracellular magnesium and caffeine on intracellular free calcium ion in human aortic endothelial cells

$[Mg^{2+}]_o$ (mM)	n	$[Ca^{2+}]_i$ (nM) *Basal*	$[Ca^{2+}]_i$ (nM) *Caffeine* (10 mM) *treatment*
1.2	5	73.7 ± 22.4	76.8 ± 11.9
0.3	7	56.2 ± 14.1	382.8 ± 57.1*
0.3 + $NiCl_2$ 1 mM	12	79.9 ± 10.7	114.1 ± 9.3*

n = the number of cells; Values (means ± s.e.mean) represent $[Ca^{2+}]_i$ in the same group of endothelial cells before and 10 min after caffeine.
*Significantly different from all other values by Student's t test ($P<0.05$).

cells, *i.e.* there were areas of higher intracellular free calcium concentrations, referred to as brightness spots which were scattered among the cells, and around these brightness spots were areas with very low $[Ca^{2+}]_i$ concentration. The brightness spots most probably represent Ca^{2+} release from the extensive Ca^{2+} binding and uptake elements of internal storage sites which strongly limit Ca^{2+}diffusion. Subsequent treatment (10 min, which should permit a steady state to develop; Buchan & Martin, 1991) of 10 mM caffeine, exhibited little effects on $[Ca^{2+}]_i$ (76 ± 14 nM) and its subcellular distribution in all cells tested.

Lowering $[Mg^{2+}]_o$ to 0.3 mM Mg^{2+} caused insignificant changes in $[Ca^{2+}]_i$ (57 ± 14 nM, $P>0.05$) in the endothelial cells. However, treatment (10 min) with 10 mM caffeine (in 0.3 mM Mg^{2+}) resulted in significant elevation of $[Ca^{2+}]_i$, *i.e.* to 383 ± 57 nM, or about a 6.8 fold increment. Concomitant with the $[Ca^{2+}]_i$ elevation, the number and intensity of brightness spots were increased within endothelial cells, indicating that more Ca^{2+} ions were released from the internal stores. With 1 mM $NiCl_2$ in 0.3 mM $[Mg^{2+}]_o$ solution, caffeine still induced an increase of $[Ca^{2+}]_i$, but to a lesser degree compared to that without Ni^{2+} (Table 1).

Discussion In contrast to bovine aortic endothelial cells in which caffeine consistently elevated $[Ca^{2+}]_i$ with 1 mM $[Mg^{2+}]_o$ present in the medium (Buchan & Martin, 1991), human aortic endothelial cells responded to caffeine only when placed in low (0.3 mM) $[Mg^{2+}]_o$. These findings suggest that the elevated $[Ca^{2+}]_i$ resulted from a release of Ca^{2+} from internal stores, since caffeine is known to increase the sensitivity for Ca^{2+} release, at least in muscular tissues (Karaki & Weiss, 1988). Ni^{2+} ions, calcium channel blockers, partially inhibited such caffeine-induced $[Ca^{2+}]_i$ rises, supporting this tenet. Such a large elevation of $[Ca^{2+}]_i$ (about a 6.8 fold increment) also suggests that the caffeine-sensitive Ca^{2+} pool is large compared to the intracellular Ca^{2+} content in endothelial cells.

Our results with different $[Mg^{2+}]_o$ suggest that Mg^{2+} may regulate caffeine-mediated Ca^{2+} release in human aortic endothelial cells. Since lowering of $[Mg^{2+}]_o$ (0.3 mM) alone failed to affect basal $[Ca^{2+}]_i$ in endothelial cells (Table 1), it is unlikely that potentiation of Ca^{2+} release was brought about by an increase in the Ca^{2+} concentration in the vicinity of the internal Ca stores when $[Mg^{2+}]$ was lowered. The synergistic effects of caffeine and low $[Mg^{2+}]_o$ may be due to a release of inhibition by Mg^{2+}, as proposed for muscle cells (Karaki & Weiss, 1988). Since Ca^{2+} released by caffeine might activate phospholipase C to generate IP_3, or a decrease in hydrolysis of IP_3 by inhibition of inositol trisphosphate phosphomonoesterase might occur as a result of removing the enzyme cofactor Mg^{2+} (Downes *et al.*, 1982), an activation of an IP_3-induced Ca^{2+} release mechanism may co-exist.

Pretreatment with a calcium channel blocker, Ni^{2+}, partially inhibited caffeine-induced Ca^{2+} release in endothelial cells exposed to 0.3 mM $[Mg^{2+}]_o$, suggesting that filling or emptying of caffeine-sensitive intracellular Ca pools may be associated with $[Ca^{2+}]_o$ entry (Schilling & Elliott, 1992). Such Ca^{2+} movements across the plasma membrane in endothelial cells may be regulated by $[Mg^{2+}]_o$. However, irrespective of the precise pathway(s) of the signal transduction, the elevated $[Ca^{2+}]_i$ clearly suggests that a caffeine-mediated (or Ca^{2+}-induced) Ca^{2+} release participates in regulation of $[Ca^{2+}]_i$ in endothelial cells, which appears to be modulated by Mg^{2+}. It is thus possible that changes in $[Mg^{2+}]_o$, which have been noted in human subjects (Altura *et al.*, 1992), could induce substantial potentiation or inhibition of endothelial functions by the mechanisms described here.

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