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Abstract
Abnormal dietary deficiency in Mg as well as abnormalities in Mg metabolism appear to play important roles as risk factors for ischemic heart disease and acute myocardial infarction, namely in hypertensive vascular disease, diabetic vascular disease, insulin resistance, atherosclerosis and vasospasm. Experimental, epidemiological as well as clinical evidence that supports a role for Mg in these risk factors are reviewed. Extracellular Mg ions ($[Mg^{2+}]_o$) exert important actions upon divalent cation metabolism, transport and intracellular release of $[Ca^{2+}]_i$ and intracellular free Mg ($[Mg^{2+}]_i$) in both vascular smooth muscle and endothelial cells. Digital imaging microscopy, using molecular fluorescent probes, clearly indicates that both intracellular free Ca^{2+} and intracellular free Mg^{2+} are compartmented in both vascular smooth muscle cells and endothelial cells. $[Mg^{2+}]_o$ appears to exert important effects on the precise subcellular location and concentration of both $[Ca^{2+}]_i$ and $[Mg^{2+}]_i$. Use of specific ion-selective electrodes for $[Mg^{2+}]_o$ has revealed that $[Mg^{2+}]_o$ can change more rapidly than heretofore believed in cardiovascular pathophysiologic states. The latter new findings therefore suggest that the ionized level of $[Mg^{2+}]_o$ is an important determinant of vascular tone, contractility and reactivity.

Introduction

Over the past dacade, a considerable number of experimental, epidemiological and clinical studies have been published which point to an important role for magnesium ions (Mg^{2+}) in the etiology of cardiovascular pathophysiology [for recent reviews, see 1–10]. Abnormal dietary deficiency in Mg as well as abnormalities in Mg metabolism appear to play important roles in ischemic heart disease, congestive heart failure, sudden cardiac death, atherosclerosis, a number of cardiac arrhythmias, vascular complications in diabetes mellitus (DM) and hypertension. Below, we attempt to review the experimental, epidemiological and clinical evidence that supports a role for Mg in hypertension and vascular complications in DM. In addition, since much of the problem in hypertension and DM vascular complications is related to alterations in vascular tone, vascular reactivity, and

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atherogenesis, the effects Mg^{2+} exert on blood vessels and the atherosclerotic process are reviewed. Inasmuch as Mg^{2+} is known to impact upon divalent cation metabolism and transport in vascular smooth muscle (VSM) and endothelial cells [e.g., see 1–3, 5, 6, 11–13], we have also chosen to review some of this data and to examine some recent information on cellular compartmentation of these cations.

Evidence for Role of Mg in Hypertensive Vascular Disease

Experimental Evidence

With respect to a link between Mg and experimental hypertension, several reports are available utilizing various animal models. For example, experiments have been carried out using dietary deficiency of Mg in rats, spontaneously hypertensive rats (SHRs), audiogenic stress in rats, DOCA-salt-induced hypertension in rats and pulmonary hypertension in rats induced with monocrotaline or hypoxia. In 1984, it was reported that 12 weeks of mild dietary deficiency of Mg could induce elevation of blood pressure and constriction of arterioles, venules and precapillary sphincters in the microcirculation [14]; the milder the dietary depletion of Mg, the less the elevation in arterial blood pressure and the less the peripheral vasoconstriction. Using the SHR, it has been demonstrated that it has lower red blood cell and plasma Mg levels than either Wistar controls or WKY rats [15–19], (e.g. table 1). From a number of these studies, it is also clear that SHRs exhibit elevated serum Ca/Mg ratios [e.g., 15, 16, 20]. The latter would tend to elevate arteriolar tone, increase peripheral vascular reactivity, and increase peripheral vascular resistance. More rencently, Ng et al. [21], using the fluorescent probe mag-fura-2 to measure intracellular free Mg ($[Mg^{2+}]_i$) reported that aortic smooth muscle cells as well as striated muscle cells in these SHRs exhibited lowered levels of $[Mg^{2+}]_i$.

In 1981, it was first suggested by Altura and Altura [22] that VSM cells from SHR would have decreased membrane permeability to extracellular Mg^{2+} ($[Mg^{2+}]_o$), lowered $[Mg^{2+}]_i$ and altered Ca^{2+}-Mg^{2+} exchange and membrane-binding sites [22]. At that time, we also noted that elevation of $[Mg^{2+}]_o$ exerted very little in the way of a relaxant action on SHR blood vessels, indicating an underlying defect in vascular Mg^{2+} metabolism and calcium handling at the plasma and intracellular membranes [22]. Furthermore, we suggested that these defects are probably inherited [14]. More recently, this hypothesis has received support from three different types of studies in human subjects, one study done in intact humans with mild hypertension [23] and two done on plasma membranes of red blood cells obtained from patients with high blood pressure [24, 74]. Fujita et al. [23] clearly found an attenuated vasodilator response to administration of Mg in subjects with borderline hypertension compared to controls, while Mattingly et al. [24] noted that RBC plasma membranes from hypertensive subjects did not handle Mg^{2+} as did similar RBC membranes from control subjects. Palisso et al. [74] reported that the eryhtrocyte membranes of essential hypertensive patients exhibit decreased permeability to $[Mg^{2+}]_o$.

Table 1. Serum magnesium levels in SHR

Ref. No.	Serum Mg, m*M*		Age weeks
	Normotensive	SHR	
15	1.24 ± 0.057 (34)	0.92 ± 0.24** (29)	14–16
16	0.88 ± 0.04 (12)	0.78 ± 0.03* (12)	11
17	0.86 ± 0.02 (8)	0.79 ± 0.01* (8)	8
18	2.02 ± 0.07	1.08 ± 0.05** (30)	19–21

Serum Mg values represent means ± SEM. Number of animals is given in parentheses [from 20]. *$p < 0.05$; **$p < 0.001$.

If hypertension is, in part, due to an imbalance in transport of Mg^{2+} across plasma membranes, one should see alterations in either its transport, per se, and/or alterations in cellular content and subcellular distribution of this divalent cation in blood vessels and the myocardium. In 1986, Wallach and Verch [25] reported that several tissues from SHRs demonstrated significantly lowered levels of Mg (e.g., heart, lungs, kidney, bone). More recently, others using rats stressed with noise, to induce high blood pressure, found that both the heart and blood vessels exhibited significantly lowered levels of Mg concomitant with elevations in Ca content [26, 27]. The work of others on isolated RBC membranes, obtained from hypertensive subjects, suggests rather strongly that Mg^{2+} is not bound (or probably transported) in the same manner as in normal RBC membranes [24]. However, to our knowledge good experimental data on Mg^{2+} transport across either vascular smooth muscle or cardiac myocyte plasma membranes from hypertensive subjects or animals are not available. No data are available on subcellular transport of $[Mg^{2+}]_i$ in tissues of hypertensive animals or humans.

If Mg is important in the etiology and pathophysiol-

ogy of hypertensive vascular disease, one might expect less than normal Mg intake to exacerbate a preexisting hypertensive state and excess Mg intake to lower arterial blood pressure. As expected, SHRs as well as DOCA-salt-maintained rats and audiogenic-stressed rats fed diets low in Mg clearly exhibit an exacerbation of arterial blood pressure [26–29]. Considerable evidence indicates that oral and parenteral administration of Mg^{2+} can lower blood pressure in SHRs, audiogenic-stressed animals and DOCA-salt-maintained hypertensive animals [6, 10, 20, 26–28, 30, 31]. In addition, it has also been demonstrated that Mg can attenuate elevations in arterial blood pressure induced by nonspecific stressors [32].

Is there evidence to indicate that there is a genetic predisposition to hypertension which is Mg- and sex-linked? In the past 5 years, a group in Japan has begun to publish several reports which suggest that male children of parents with a genetic history of familial hypertension exhibit significant deficits in red blood cell Mg content [72, 73]. In these studies of junior high school students there was an inverse relationship between arterial blood pressure and red blood cell intracellular Mg content. These are the first studies to support the suggestion we made in 1984 [14] that there probably is a close relationship between genetic control of tissue $[Mg^{2+}]_i$ and a predilection for high blood pressure.

It is of considerable interest to point out, here, that rats made pulmonary-hypertensive either by feeding a plant extract, monocrotaline, or by subjection to experimental hypoxia, exhibit significant attenuation of pulmonary hypertension and right ventricular hypertrophy when fed Mg salts [33–38]. In addition, in a series of experiments, we have demonstrated that the pulmonary vascular muscle and endothelial cell hyperplasia, characteristic of pulmonary hypertension, can be almost completely prevented by feeding rats higher than normal Mg intake in their diets [34–36]. Such findings, which remain to be demonstrated in human subjects with pulmonary hypertension, suggest that Mg^{2+} can exert profound effects on collagen and elastin deposition as well as the enzymes and growth factors involved in the hyperplastic process observed in pulmonary hypertension.

Very recently, Laurant et al. [31] using rats with DOCA-salt hypertension found that Mg-enriched diets not only reduced mean arterial blood pressure but increased the generation of prostacyclin (PGI_2) in their aortas. This was not seen in control animals. An increased synthesis of PGI_2 in peripheral blood vessels and tissues would, in itself, tend to lower arterial blood pressure, as it is a powerful vasodilator substance and acts to prevent platelet aggregation. Such an observation lends support to the hypothesis suggested a few years ago by Nadler et al. [39], in hypertensive human subjects, that prostanoids may be the target site for Mg^{2+}. They reported that administration of the cyclooxygenase inhibitor, indomethacin, to these subjects prevented the beneficial blood pressure-lowering action of Mg. It is of interest to point out, here, that the potency of the vasodilator action of PGI_2 and eicosanoids on peripheral blood vessels is controlled by the concentration of Mg^{2+} [40, 41].

Whether or not the primary effects of Mg deficiency are on Ca^{2+} influx and intracellular Ca^{2+} release and on membrane binding in vascular smooth muscle and cardiac myocytes, and the secondary actions are on modulation of synthesis of vasodilator (and/or vasoconstrictor) substances is not clear. However, it is clear that in those cases where electrolyte contents and fluxes have been examined, Ca is always also altered. But, data on other electrolytes are beginning to point towards the importance of other ions like phosphate in Mg deficiency-induced hypertension [42, 43]. We have demonstrated that rats that develop severe hypertension, as a consequence of implanting large doses of DOCA (and feeding NaCl), exhibit serum hypomagnesemia and hypophosphatemia concomitant with elevation in serum Ca [10, 20]. Such animals fed Mg (in the form of Mg aspartate HCl) show marked attenuation of the high blood pressure and a restoration of both the serum Mg and phosphate levels to normal. Since hypophosphatemia, in itself, has been shown to cause hypertension [42, 43], one must raise the possibility that alterations in phosphate metabolism may play an important role in certain forms of Mg deficiency-induced hypertension. In any event, the experimental findings, gathered from numerous laboratories over the past 10 years, support the hypothesis offered more than 15 years ago that defects in Mg metabolism and deficits in dietary intake of Mg are probably important contributing factors to several forms of experimental hypertension [22, 44].

Clinical Evidence

Up to the present, there are at least 28 independent studies which show that patients with hypertension of diverse etiologies can exhibit hypomagnesemia either in serum and/or tissue [see 20, for reviews and references]. On average, patients with long-term essential hypertension appear to exhibit at least a 15% deficit in serum Mg [20]. Reports by Petersen et al. 45], Resnick et al. [46], Dyckner and Wester [47], Touyz et al. [48–50] and

Paolisso et al. [51] clearly demonstrate inverse correlations between total serum (or tissue) Mg concentrations and arterial blood pressures. Other studies provide evidence, in our opinion, for the idea that certain hypertensive patients exhibit reduced urinary excretion of Mg which is inversely correlated to diastolic blood pressure [for a review, see 20].

The average 15% deficit in total Mg mentioned above, which we calculated from 24 different studies in the literature [20], would represent a loss of about 125 μM from the serum. If this represented a deficit of free, ionized [Mg^{2+}], it would be enough to produce potent constriction of arterioles in the splanchnic and skeletal muscle vasculatures [14, 20, 26]; 125 μM $[Mg^{2+}]_o$ are known to induce potent vasodilation of arterioles in these microvasculatures, at least in the rat [52–54].

A number of epidemiological studies support a rather strong inverse relationship between dietary intake of Mg (or vegetables high in Mg) and systolic and diastolic arterial blood pressures [1, 2, 4, 20, 22, 55–61]. However, at least one epidemiological study carried out in Belgium did not find an inverse relationship between dietary Mg intake and systemic arterial blood pressure [62]. Although several studies found that Mg treatment of hypertensives lowered arterial blood pressure significantly [e.g., 47, 63–66], several studies could not confirm a beneficial effect of Mg supplementation, even in mild, untreated hypertension subjects [67–70]. A number of confounding factors could, however, account for these differences in efficacy. In some beneficial studies, Mg oxide was used with great effect [66]; in others where no significant effects was observed, Mg aspartate was usually the salt used. In addition, in a number of the studies, in which no significant effects on arterial blood pressure was observed, the Mg supplementation failed to either alter the serum level of Mg or failed to increase significantly the urinary output of Mg; this might signify differences in basal Mg balance and/or metabolism among the groups of subjects. Moreover, there was often no change in either red blood cell Mg or plasma renin activity [e.g., 69], suggesting possibly that the dose of Mg given either was too small (e.g., only 10 mmol/day) or not given long enough (e.g., only 8 weeks). An attenuated vasodilator response, first noticed in SHR rats [22] and seen clinically recently in some subjects with borderline hypertension [23], supports the latter. It must also be entertained that since all of the previous studies assessed total Mg (TMg), but not ionized Mg (IMg^{2+}), the former parameter to guide therapeutic efficacy may not be reliable. Recently, we have found that even borderline hypertensives usually exhibit significantly depressed serum ionized, but not TMg levels [Altura and Altura, unpubl. findings]. Moreover, pregnant women with transient hypertension during labor clearly exhibit deficits in IMg^{2+}, but not TMg, which is related to the incidence of hypertension [71]. In addition, it is clear from the positive results obtained, so far, that oral Mg supplementation seems to be of particular benefit in hypertensive subjects who are on traditional nonpotassium-sparing diuretics [47, 64, 65] and that irrespective of the therapy used to treat hypertensive subjects, an alleviation of the high blood pressure is always associated with elevation in the levels of RBC intracellular free Mg [46].

Evidence for Role of Mg in Diabetic Vascular Disease

Experimental Evidence

With respect to experimental DM, its vascular effects and Mg, several studies were published during the past decade [2, 6, 10, 15, 20, 22, 74]. Streptozotocin-induced DM in rats is clearly associated with an intense magnesuria, glycosuria and polyuria [136]. The increase in urinary Mg concentration parallels the degree of glycosuria. This supports and extends numerous previous older findings that DM can result in hypomagnesemia [see references in 2, 4, 6, 9, 20, 22, 75–79]. Basal tension of aortas and portal veins excised from rats administered alloxan steadily increased from the 1st through 8th weeks as the degree of diabetes (assessed by serum glucose, triglycerides, cholesterol, creatinine) became progressively worse [15, 80]. These studies also demonstrated alterations in vascular reactivity associated with elevated serum Na/Ca and Ca/Mg ratios. Twenty to 25% of the latter alloxan-diabetic rats exhibited significant elevations in arterial blood pressure by the 8th week after treatment [15]. This is particularly interesting since long-term DM in humans results in hypertension in 40–80% of patients [see reviews, 20, 77]. It is of particular interest to note that elevation of $[Mg^{2+}]_o$ failed to relax the diabetic rat aortas; diabetic venous smooth muscle also demonstrated little response to high concentrations of Mg^{2+} [20, 22, 80]. Most importantly, the diabetic vessels of these rats showed marked elevations in total exchangeable and membrane-bound calcium [81]. Such data, overall, lend support to our concept that the vascular membranes in diabetic subjects probably have undergone alterations in their Mg-Ca exchange sites and permeability to $[Mg^{2+}]_o$ [22]. But, are these divalent cation

permeability and membrane alterations a result of the diabetic state or are they linked to the etiology of the syndrome? In this context, it is of particular interest to note that Nelson and Boquist [82] and Boquist [83] have found that alloxan and streptozotocin can produce direct alterations in membrane permeability of mouse liver mitochondria in vitro. High concentrations of alloxan induced efflux of endogenous Mg^{2+}, K^+ and adenine nucleotides, efflux of accumulated Ca^+, K^+ uptake inhibition, loss of membrane potential, and swelling. We were particularly interested to learn from these studies that the loss of Mg^{2+} preceded the release of accumulated Ca^{2+}, which paralleled the efflux of K^+ and swelling. This, thus, resembles very closely what has been observed for Mg-K-Ca interactions and control of vascular homeostasis [2, 6, 84, 85]. In addition, animal studies have indicated that there is increased glucagon stimulation [86], decreased insulin secretion [87] and reduced insulin uptake in low $[Mg^{2+}]_o$ [88]. It will be extremely important to extend such studies with alloxan and streptozotocin to isolated blood vessels and electrolyte transport across the vascular walls.

Clinical Evidence

With respect to clinical observations in DM, there appears to be a strong growing association between it, hypertension and abnormal glucose tolerance [51, 75, 89]. A considerable amount of data has accumulated to indicate that control of DM is inversely related to Mg deficiency [74, 76, 77, 90–92]. Diabetic retinopathy is clearly associated with a state of Mg deficiency [20, 90, 93]. Interestingly, insulin-dependent as well as noninsulin-dependent diabetes mellitus (NIDDM) are associated with reduced total serum and intracellular levels of Mg [20, 77, 94–99] as well as increased urinary loss of Mg [20, 77, 100]. Even though all of the subjects investigated did not always exhibit simultaneous reduction in serum TMg and intracellular free Mg, oral Mg treatment improved control of both insulin-dependent [97] and NIDDM [51, 98].

We have utilized a new ion-selective electrode (ISE) for IMg^{2+} to measure serum IMg^{2+} in fasting subjects with and without NIDDM, and compared these values to levels of serum TMg and intracellular free Mg^{2+} analyzed by ^{31}P-NMR spectroscopy in RBCs [101]. Both IMg^{2+} and intracellular free Mg^{2+}, but not TMg, were significantly reduced in NIDDM compared with nondiabetic control subjects. A close relationship was noted between serum IMg^{2+} and intracellular free Mg^{2+} ($r = 0.73$; $p < 0.001$). We, thus, hypothesize that Mg deficiency, both extracellular and intracellular, is a characteristic of chronic stable mild NIDDM, and may predispose subjects to the excess cardiovascular morbidity of the diabetic state. Moreover, by adequately reflecting cellular Mg metabolism rather than serum TMg levels, IMg^{2+} measurements may provide a more readily available tool than has heretofore been available to analyze Mg metabolism in a variety of vascular-related diseases.

Mg and Atherogenesis – Important Link between Vascular Disease, Hypertension and Ischemic Heart Disease

Hypercholesterolemia has been widely accepted as a high risk factor for development of atherosclerosis, hypertension and ischemic heart disease [3, 10, 102], particularly since cholesterol-rich diets lead to deposition of lipids in blood vessel walls and an atherosclerotic-like state in experimental animals [for a review, see 103]. Increased blood levels of lipoproteins are thought to lead eventually to endothelial cell injury or denudation with concomitant uptake of the former molecules [103–105]. It is, however, not clear how the lipoproteins and Ca^{2+} gain access to the normally and relatively impermeable arterial walls.

Approximately 35 years ago, it was suggested that use of intramuscular $MgSO_4$ in patients with coronary insufficiency might lower serum B-lipoproteins and result in improvement of the clinical condition [106]. It has been known for sometime that Mg deficiency is associated with a number of dyslipidemias [3, 10, 78, 107–112]. We have recently found that dietary deficiency of Mg (compatible with the reduced dietary intake of Mg seen in the adult population of the western world) in rabbits exacerbates atherogenesis, and lipid deposition in arterial muscle, and stimulates (or activates) macrophages of the reticuloendothelial system [113, 114]. In addition, we demonstrated that pretreatment of animals with orally administered Mg aspartate HCl: (1) attenuated the atherosclerotic process markedly, (2) lowered serum cholesterol and triglycerides in normal as well as atherosclerotic animals, and (3) elevated Ca in the arterial wall. In addition, in these studies, we noted that the extent of the atherogenic lesions was, in fact, poorly correlated with the level of serum cholesterol and highly dependent on the level of dietary Mg. It would appear from these new, quantitative data that dietary Mg intake plays an important modulatory role in controlling lipid metabolism in the arterial wall. Many of these results have

been confirmed recently by others [115]. In recent studies, we have noted that oral Mg administration to rabbits with developing atherosclerosis, in the arterial walls, can reverse a large part of the atherogenesis [116]. Such new experiments lend support to the hypothesis that early intervention with higher than normal dietary intake of Mg could greatly attenuate the incidence of atherogenesis, hypertension and ischemic heart disease. It is also of additional interest to point out, here, that a number of ischemic heart disease risk factors (i.e., atherosclerosis, hypertension, coronary vasospasm, hyperreactivity of coronary arteries, insulin resistance) may have as a common denominator either a Mg-deficient state or dysfunctional Mg metabolism.

Mg Ions, Vascular Contractility and Basal Vascular Tone

Magnesium ions have been shown to directly alter baseline tension or tone of blood vessels [2, 5, 6, 14, 20, 22, 40, 44, 52, 54, 78, 85]. Decrements in $[Mg^{2+}]_o$ result, in a concentration-dependent manner, in rapid elevations in contractile tension development in a variety of mammalian arteries and arterioles. Elevation in $[Mg^{2+}]_o$ concentration, on the other hand, above the physiological level inhibits spontaneous mechanical activity and lowers baseline tension; none of these findings can be attributed to changes in osmolarity or the release or synthesis of any known vasoactive substance.

We have shown, previously, that these elevations in mechanical activity are lowered markedly when external Ca^{2+} ions are lowered or chelated [22, 44, 78, 134]. Such findings suggest that some influx of extracellular Ca^{2+} ions are necessary for these contractile responses. In addition, a variety of neurohumoral agents which induce a contraction in VSM exhibit heightened contractile responses as external Mg^{2+} is lowered, and depressed or attenuated contractile activity is observed as $[Mg^{2+}]_o$ is elevated [2, 5, 6, 22, 40, 44, 53, 78, 84, 85, 134]. How are these actions of $[Mg^{2+}]_o$ brought about?

Mg^{2+} and Ca Channels

It has been suggested that Mg^{2+} can regulate Ca^{2+} flux across the vascular smooth muscle cell membranes as well as its release from intracellular storage sites [2, 5, 6, 13, 22, 40, 44, 78, 85, 117, 118, 127, 134–136]. Since a change in cytosolic free Ca^{2+} concentration is a very important second messenger in facilitation of contraction or relaxation, it may be physiologically relevant that Mg^{2+} regulates the activity of vascular smooth muscle cells by competing with Ca^{2+} and modulating the level of cytosolic free intracellular Ca^{2+}.

Acute administration of Mg in animals and human subjects induces hypotension and peripheral vasodilation [1, 2, 5, 6]. This vasodilator effect has been attributed, at least in part, to its Ca^{2+} channel-blocking property as well as modulation of intracellular free Ca^{2+} in VSM cells [2, 3, 22, 44, 78, 117, 118].

Influence of $[Mg^{2+}]_o$ on $[Ca^{2+}]_i$ and $[Mg^{2+}]_i$

$[Mg^{2+}]_i$ is a cofactor for numerous enzymes and signal-transduction proteins and regulates bioenergetics and ion transport. Thus, it seems highly desirable to know whether or not changes in external Mg concentration can alter intracellular free Mg^{2+} in vascular smooth muscle cells. It seems also desirable to know whether or not changes in external Mg^{2+} concentration can also alter the subcellular distribution of both intracellular compartmentalized Ca^{2+} and Mg^{2+}.

During the past 10 years, it has become quite apparent that the endothelial cell layer plays an active and important role in modulating vascular tone by synthesizing and releasing a variety of endothelial-derived relaxant factors. Several different groups have now shown that these endothelial-derived relaxant factors are modulated by the extracellular concentration of Mg^{2+} [11, 12, 119–121]. A rise of intracellular free Ca^{2+} concentration is known to be closely coupled to the synthesis and release of PGI_2, endothelial-derived relaxation factors, and cell contraction in response to a number of agonists or physical stimuli. It is now well-established that intracellular Ca^{2+} release in endothelial cells can be mediated by activation of plasma membrane receptors coupled to phospholipase C and generation of 1,4,5-inositol-triphosphate or IP_3. However, the contribution of Ca^{2+} release via non-IP_3-dependent or Ca^{2+} release mechanisms in endothelial cells remains controversial. It would thus be of importance to define the role, if any, of Mg^{2+} in Ca^{2+}-induced Ca^{2+} release in endothelial cells.

With these points in mind, we have undertaken a series of studies using cultured rat aortic and canine cerebral VSM cells as well as human endothelial cells to define the role of external Mg in modulation of intracellular Ca^{2+} and intracellular Mg^{2+}, and their compartmentation, if any, in vascular muscle and endothelial cells. For these ongoing studies, we are directly measuring intracellular $[Ca^{2+}]$, and intracellular $[Mg^{2+}]$, and mapping the intracellular free calcium and Mg distribution via Ca^{2+} and Mg^{2+} imaging in single cells, similar to

imaging techniques recently described [118, 122]. The distribution of intracellular free calcium and magnesium ions was determined using a digital imaging microscopy system [118]. Aortic smooth muscle cells from rats as well as canine cerebral VSM cells were isolated and cultured in Dulbecco's modified Eagle's medium at 37 °C in an humidified atmosphere composed of 95% air-5% CO_2. The intima of the blood vessels were rubbed gently with wire to rub off the endothelial cells. Experiments were done to compare primary cultured cells to cells passed several times to determine whether this made a difference. In addition, a human aortic endothelial cell line obtained from the NIH was utilized [13].

Immunohistochemical staining with a monoclonal antibody recognizing exclusively α-smooth muscle actinin indicates that over 96% of our cultures are pure VSM cells.

Changes in intracellular free Ca^{2+} were measured with the Ca^{2+} fluorescence indicator fura-2 [122]. This molecular probe has a high affinity for Ca^{2+} over Mg^{2+} and other divalent cations. A ratiometric technique was used to estimate intracellular free Ca^{2+} [118]. All cells were loaded with 2 μ*M* fura-2 acetoxymethyl ester, the cell-permeant form of fura-2 in the culture media for 60 min. After loading of the molecular fluorescent probe, the cells were perfused with media containing different concentrations of free Mg^{2+} and Ca^{2+}. Both ionic extracellular Mg^{2+} and Ca^{2+} concentrations were measured with ISE [123].

Background fluorescence for both excitation wavelengths was acquired from blanks for each experiment and subtracted from each pair of images separately before ratioing [128]. Fluorescence ratios were obtained by dividing the 340-nm image by the 380-nm image. Intracellular free Ca^{2+} was calculated according to Grynkiewicz et al. [124]. Particular care was taken to minimize photobleaching of the probe. Experiments were undertaken in total darkness and exposure to excitation light was less than 2 min in all experiments.

Aortic Smooth Muscle. Most of the VSM cells in primary cultures appeared spindle-shaped in 1.2 m*M* normal external Mg^{2+} concentration. The resting distribution of intracellular free Ca^{2+} appeared heterogeneous within the cells by ratio-image analysis. The perinuclear region, arbitrarily chosen as the central ellipsoid area in the widest part of the cell, revealed the most brightness, reflecting a relatively higher Ca^{2+} than the peripheral cytoplasmic area. This observation of a $[Ca^{2+}]_i$ gradient in the vascular muscle cell suggests that the plasma membrane and the discrete localized process, within the perinuclear area, may regulate intracellular free Ca^{2+} activity subcellularly (see below).

In order to probe the effects of $[Mg^{2+}]_o$ on intracellular free Ca^{2+}, the $[Mg^{2+}]_o$ concentrations in the media were altered. Somewhat to our surprise, removal of extracellular Mg after only 5 min of washing had dramatic effects on cell fluorescence and geometry [118]. Removal of extracellular Mg^{2+} resulted in a dramatic increase in free intracellular Ca^{2+} concomitant with a rounding-up or contraction of the cells. Reduction in $[Mg^{2+}]_o$ to 0 m*M* resulted in about a 6-fold increment in $[Ca^{2+}]_i$ [118]. To examine the changes produced under physiological conditions, $[Mg^{2+}]_o$ was lowered to 0.3 m*M*, the lowest ionized physiological level seen clinically [123]. This maneuver raised $[Ca^{2+}]_i$ about 3.5-fold. However, a heterogeneous distribution of $[Ca^{2+}]_i$ was still evident when $[Mg^{2+}]_o$ was lowered to 0.3 m*M*, even though $[Ca^{2+}]_i$ in the peripheral cytoplasmic region was increased. Although the changes of cell geometry induced by contraction could influence the accuracy of $[Ca^{2+}]_i$ measurements, the ratioed image reflects an absolute increase in $[Ca^{2+}]_i$.

In contrast to lowering $[Mg^{2+}]_o$, elevation of Mg^{2+} to 4.8 m*M* (i.e., 4 times normal) was found to decrease $[Ca^{2+}]_i$ to about 72 n*M* showing relatively less of an influence on $[Ca^{2+}]_i$ compared to $[Mg^{2+}]_o$ removal [118].

These observations support our previous findings in intact and isolated blood vessels which indicate that reduction in $[Mg^{2+}]_o$ results in spasms of blood vessels, elevation of basal tone and increments in spontaneous mechanical activity. The intracellular free Ca^{2+} changes brought about by varying $[Mg^{2+}]_o$ clearly indicate the dependence of $[Ca^{2+}]_i$ and basal $[Ca^{2+}]_i$ upon $[Mg^{2+}]_o$.

To obtain further insights into the existence and nature of these regulatory processes, aortic cells were exposed to media in which both $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ were omitted [118]. It was anticipated that such a maneuver would reveal the steady-state distribution of intracellular free Ca^{2+} across the sarcolemma and intracellular organelle membranes. A strong dependence of the $[Mg^{2+}]_o$-induced increment of $[Ca^{2+}]_i$ upon $[Ca^{2+}]_o$ was observed as exemplified by the marked lowering of $[Ca^{2+}]_i$ from approximately 92 to about 3 n*M*. This represents about a 200-fold decrement compared with cells treated by $[Mg^{2+}]_o$ withdrawal alone. The $[Ca^{2+}]_i$ gradient thus completely disappears in the absence of $[Ca^{2+}]_o$. Although removal of both $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ could be interpreted as resulting in dye leakage from the cells, these data also suggest that both divalent cations are

needed for maintaining membrane permeability and integrity of these cells.

Cerebral VSM. Turning to cultured canine cerebral VSM cells, the mean basal $[Ca^{2+}]_i$ is about 90 n*M*, similar to that obtained in rat aortic smooth muscle cells. But we found the range of $[Ca^{2+}]_i$ in these cells to be from 10 to 270 n*M* [125, unpubl. findings]. Since these cells are primary cells, not from a cell line, different smooth muscle cell phenotypes or different stages of the cell cycle and/or chronological age could account for such variations in $[Ca^{2+}]_i$.

Since we have demonstrated that basal tone of cerebral VSM cells are exquisitely sensitive to external $[Mg^{2+}]_o$ [126, 127], it was of great interest to determine if small physiological decrements in $[Mg^{2+}]_o$ would alter $[Ca^{2+}]_i$ incrementally.

Our data indicate that as external Mg^{2+} is lowered from the brain physiologic level of 1.3 m*M* stepwise to 0.48 m*M* Mg^{2+}, the intensity of the fluorescence increases dramatically suggesting that the $[Ca^{2+}]_i$ levels are rising incrementally. It is also apparent that the intensity of the fluorescence varies from one part of the cell to another with the perinuclear area showing the greatest intensity.

Our data show that stepwise decrements of $[Mg^{2+}]_o$, via perfusion of the cultured cells, with different $[Mg^{2+}]_o$-deficient solutions, resulted in stepwise elevations in $[Ca^{2+}]_i$ in a concentration-dependent manner. These findings, when viewed in light of those obtained on the rat aortic cells [118], suggest that regulation of $[Ca^{2+}]_i$ by $[Mg^{2+}]_o$ may be a general cellular property of vascular smooth muscle cells. Somewhat to our surprise, however, a relatively small change of $[Mg^{2+}]_o$ from 1.36 to 1.07 m*M*, which can be seen in normal human CSF [128] elevated $[Ca^{2+}]_i$ significantly to about 215 n*M*, an approximately 2.5-fold increase over basal levels. Further decreases in $[Mg^{2+}]_o$ resulted in larger increments in $[Ca^{2+}]_i$ in as little as 2 min of exposure. Compared to rat aortic smooth muscle cells, canine cerebral VSM cells are clearly more sensitive to alteration of $[Mg^{2+}]_o$.

In other experiments [125, unpubl. findings], we found that a decrease in $[Mg^{2+}]_o$ from 1.3 to 0.48 m*M* doubled the basal value of $[Ca^{2+}]_i$ within 2 min, and elevation of $[Ca^{2+}]_i$ occurred much quicker compared to that observed with smaller, stepwise lowering of $[Mg^{2+}]_o$. But, similar to the previous results, noted above, $[Ca^{2+}]_i$ *did not* seem to remain in a steady state, and an elevated $[Ca^{2+}]_i$ was maintained for at least 22 min of observation. Such findings suggest two important things: (1) a dependence of the time course of $[Ca^{2+}]_i$ rise upon $[Mg^{2+}]_o$, and (2) a role for Mg^{2+} as a regulatory ligand which could regulate cellular processes within a few minutes, much more rapidly than thought heretofore.

Intracellular Free Mg^{2+} Measurements

For measurements of intracellular free Mg^{2+}, we used the fluorescent probe, mag-fura-2 after the work of Raju et al. [129] published in 1989. As with fura-2, 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 were obtained by dividing the 335 n*M* image by the 370 n*M* image, $[Mg^{2+}]_i$ was then calculated according to Raju et al. [129]. Particular care was taken here to minimize photobleaching of the probe.

Aortic Smooth Muscle. The fluorescent intensity in a single VSM cell could be recorded accurately with high resolution [130]. Like that seen for fura-2, there was a clear heterogeneous distribution of the probe, again with the perinuclear area showing the greatest fluorescent intensity. When the cells were exposed to elevated $[Mg^{2+}]_o$ for only 2–5 min, the mag-fura-2 fluorescence intensity increased considerably, and once again displayed a clear heterogeneous distribution with the perinuclear areas exhibiting the greatest brightness.

The calculated intracellular free Mg^{2+}, i.e., 0.63 ± 0.09 m*M*, indicate that the basal level of ionized Mg in these cells is about 600 μ*M*. However, when the external Mg^{2+} is elevated 4-fold, the basal level of $[Mg^{2+}]_i$ rises about 2.5-fold to about 1.63 ± 0.08 m*M*. The $[Mg^{2+}]_i$ to $[Mg^{2+}]_o$ ratio *falls* significantly from 0.5 to about 0.3. These rises of $[Mg^{2+}]_i$ are maintained for at least 60 min.

Since we have shown clearly (see above) that elevation of $[Mg^{2+}]_o$ to 4.8 m*M* decreased intracellular free Ca^{2+} to about 70 n*M*, overestimation of $[Mg^{2+}]_i$ caused by interference with excess Ca^{2+} seems very unlikely. The dissociation of the Mg-fura-2 complex is about 65 μ*M*, which is clearly much higher than the nanomolar levels of $[Ca^{2+}]_i$ we have observed.

Thus, the increases in $[Mg^{2+}]_i$, recently reported, may be of significant physiological relevance, because $[Mg^{2+}]_i$ in the millimolar range is known to fit Michaelis-Menton K_m values for many cellular enzyme systems, and clearly exerts potent inhibition on Ca channel activity. We propose that increased $[Mg^{2+}]_i$ maintains low $[Ca^{2+}]_i$, thus preventing peripheral vasoconstriction (or vasospasm) and hypertension.

Since Mg^{2+} affects binding of Ca^{2+} to other probes like aequorine, a widely used bioluminescent indicator,

an exact knowledge of $[Mg^{2+}]_i$ would be required for aequorine experiments measuring $[Ca^{2+}]_i$.

Compartmentation of Cellular $[Ca^{2+}]_i$ and $[Mg^{2+}]_i$

Subcellular Distribution of $[Ca^{2+}]_i$ in Low $[Mg^{2+}]_o$

About 1 decade ago, Popescu et al. [131] using X-ray microprobe analysis in skeletal muscle suggested that Mg was compartmented in skeletal muscle with the nuclei and mitochondria accounting for 70% of the Mg. Using differential ultracentrifugation and atomic absorption spectrophotometry, Gunther [132] suggested that most of the Mg in liver cells resides in the microsome fraction with only about 10% each in the nuclear and cytosolic fractions. Both of these studies could assess only total Mg contents in subcellular fractions, not the ionized levels and did not assess Ca^{2+} in the same cellular compartments.

Using molecular fluorescent probes and digital image microscopy analysis, we have been attempting to determine the basal ionized Ca and Mg levels in subcellular compartments as well as what happens when cells are exposed to different levels of $[Mg^{2+}]_o$. For purposes of analysis, we chose to examine three areas in the cells, namely peripheral, perinuclear, and nuclear regions. The perinuclear area contains most of the endoplasmic reticulum, sarcoplasmic reticulum (SR) and Golgi apparatus.

Examination of canine cerebral VSM cells exposed to lowered $[Mg^{2+}]_o$ (i.e., 0.48 m*M*) revealed $[Ca^{2+}]_i$ gradients among the nucleus, perinuclear region and peripheral areas, with the perinuclear region showing over a 4- to 14-fold greater level of ionized free Ca^{2+} when compared to either the peripheral or nuclear areas, respectively (fig. 1). Such a spatial inhomogeneity of ionized Ca^{2+} in these cells suggests that the plasma membrane of VSM cells, the nuclear membrane and discrete localized processes regulate Ca^{2+} activity at the subcellular level.

Simultaneously with the alteration of $[Ca^{2+}]_i$, the heterogeneous distribution of $[Ca^{2+}]_i$ was still evident, and subcellular pools of Ca^{2+} increased dramatically (i.e., within 2–5 min) when $[Mg^{2+}]_o$ was lowered to 0.48 m*M*. The 5-fold increase of perinuclear $[Ca^{2+}]_i$, observed when $[Mg^{2+}]_o$ was lowered from 1.36 to 0.48 m*M*, clearly indicates that more $[Ca^{2+}]_i$ is in and/or around the SR and other cellular organelles. On the other hand, it is possible that the 11-fold elevation of peripheral $[Ca^{2+}]_i$ may result from Ca^{2+} influx.

Compared to the perinuclear and peripheral $[Ca^{2+}]_i$ the nucleus contains the lowest $[Ca^{2+}]_i$ in the region in

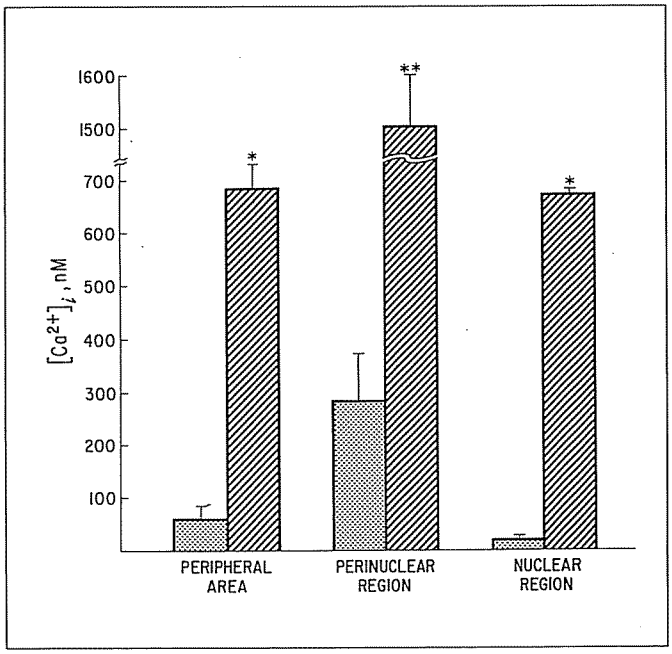

Fig. 1. Effects of reduction in $[Mg^{2+}]_o$ on subcellular pools of $[Ca^{2+}]_i$ in perinuclear, peripheral and nuclear subcellular regions of canine cultured cerebral VSM cells. Cells were imaged using fura-2. ▨ = Normal 1.36 m*M* $[Mg^{2+}]_o$; ▨ = 0.48 m*M* $[Mg^{2+}]_o$. *$p < 0.001$, vs. controls, 1.36 m*M* $[Mg^{2+}]_o$, **$p < 0.01$, vs. other values. Extracellular free $[Mg^{2+}]$ was measured using ISE [123]. Adapted from Zhang et al. [125], note change in ordinate.

cells exposed to 1.36 m*M* $[Mg^{2+}]_o$, suggesting that Ca^{2+} may not passively transport across the nuclear membrane. However, nuclear $[Ca^{2+}]_i$ clearly followed increases of perinuclear $[Ca^{2+}]_i$ when cells were exposed to low $[Mg^{2+}]_o$, and a level which was 33 times higher than values obtained in normal 1.36 m*M* $[Mg^{2+}]_o$ but was *not* different from peripheral $[Ca^{2+}]_i$. The reasons for such changes in nuclear $[Ca^{2+}]_i$ are not known. A lowering of $[Mg^{2+}]_o$ could result in an increase in nuclear membrane permeability to Ca^{2+} and/or Ca^{2+} flux from the SR producing elevated Ca^{2+} content in the nucleus.

Subcellular Distribution of $[Mg^{2+}]_i$ in High $[Mg^{2+}]_o$

Turning to experiments with mag-fura-2, we noted a slightly different pattern of subcellular distribution of ionized free Mg in VSM cells [133, unpubl. findings]. Here, we noted that although the perinuclear area, like for $[Ca^{2+}]_i$, also contains the highest concentration of free Mg^{2+}, the nuclear area contains a higher concentration of Mg^{2+} than that of the peripheral area (fig. 2). The latter is just the opposite of that seen for $[Ca^{2+}]_i$ (see above; fig. 1). When external $[Mg^{2+}]_o$ is elevated 4-fold,

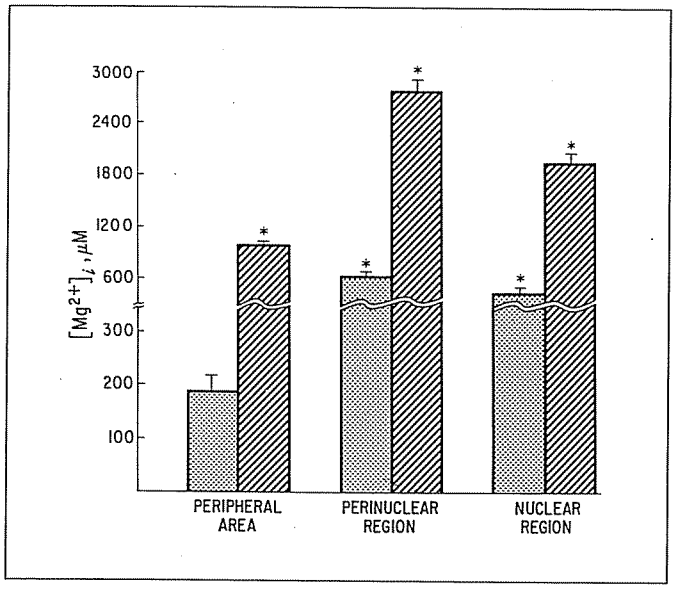

Fig. 2. Effects of elevation in $[Mg^{2+}]_o$ on subcellular compartmentation of $[Mg^{2+}]_i$ in cultured rat aortic smooth muscle cells. Cells were imaged using mag-fura-2. ▨ = Normal 1.2 m*M* $[Mg^{2+}]_o$; ▨ = 4.8 m*M* $[Mg^{2+}]_o$. *$p < 0.01$, vs. all other values [adapted from 130 and 133, note change in ordinate].

there is also a 4-fold increase in ionized Mg in the perinuclear and nuclear areas as well as a 5-fold elevation in free $[Mg^{2+}]_i$ in the peripheral area.

The normally high $[Mg^{2+}]_i$ in the perinuclear regions could represent Mg^{2+} release from Mg binding, and uptake elements there (probably SR), which limit Mg^{2+} diffusion. It also suggests that the Mg^{2+} permeability of the SR could be higher in VSM cells than that in skeletal muscle cells.

Our new data demonstrate that $[Mg^{2+}]_i$ in cultured aortic and cerebral vascular muscle can be elevated by increasing $[Mg^{2+}]_o$ within a relatively short time, which agrees well with the time course of relaxation, and the Ca^{2+} decrement induced by elevation of $[Mg^{2+}]_o$ [126, 127, 134–136]. Since these changes in $[Mg^{2+}]_i$ occur rather quickly, it is suggested that the plasma membranes of VSM cells may be more permeable to Mg^{2+} than thought heretofore.

Concomitant and parallel increases of both perinuclear $[Mg^{2+}]_i$ and peripheral $[Mg^{2+}]_i$ suggest that the SR can affect cytoplasmic $[Mg^{2+}]_i$ by buffering Mg^{2+} influx across the plasma membrane. Compared to the perinuclear region, the nuclear $[Mg^{2+}]_i$ was lower, but higher than that in the periphery when exposed to either 1.2 or 4.8 m*M* $[Mg^{2+}]_o$.

Exactly how elevation in $[Mg^{2+}]_o$ produces rapid rises in cellular $[Mg^{2+}]_i$ is not clear. However, irrespective of the exact underlying molecular mechanisms, our findings of a fine regulation of $[Ca^{2+}]_i$ as well as $[Mg^{2+}]_i$ by $[Mg^{2+}]_o$ suggest that the role of Mg^{2+} as a cellular regulator in VSM cells may be physiologically relevant.

The fine responses in $[Ca^{2+}]_i$ and its subcellular distribution suggest that changes in $[Mg^{2+}]_o$ could induce substantial potentiation or inhibition of vascular contraction, and thus regulate regional blood flow and its distribution much more finely than believed up to now.

$[Mg^{2+}]_o$ and Intracellular Free Calcium in Endothelial Cells

Magnesium ions play a well-known inhibitory role in caffeine-mediated Ca^{2+} release from the SR in muscle cells in general, but little is known regarding the effects of Mg^{2+} in endothelial cells. Recent studies, including our own, indicate that Mg^{2+} may be involved in regulation of Ca^{2+} homeostasis in endothelium since changes of extracellular $[Mg^{2+}]_o$ affect release of endothelial-derived relaxant factors and nitric oxide from endothelium [11–13, 119–121]. It would thus be important 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. For such experiments, we utilized a human aortic endothelial cell line obtained from the NIH [13]. To avoid synchronized activities of endothelial cells, coverslips containing cells in near confluent density were used. The cells were loaded with fura-2 for 60 min as for the VSM cells. The cells were superfused with media containing 1.2 or 0.3 m*M* $[Mg^{2+}]_o$ in the presence and absence of 10 m*M* caffeine.

It seems clear from our new studies that, unlike VSM cells, endothelial cells do not exhibit enhanced Ca^{2+} influx as $[Mg^{2+}]_o$ is lowered. Moreover, high concentrations of caffeine, an ER-SR releaser of Ca^{2+}, exerts no effects [13]. However, if Mg was lowered simultaneously with the addition of 10 m*M* caffeine, there was a considerable increase in fluorescence.

Our results indicate that the basal level of intracellular free Ca^{2+} in human endothelial cells is similar to that seen in VSMs, i.e. about 75 n*M*. Lowering $[Mg^{2+}]_o$ or adding 10 m*M* caffeine caused insignificant changes in basal $[Ca^{2+}]_i$ [13]. However, treatment with 10 m*M* caffeine in 0.3 m*M* $[Mg^{2+}]_o$ resulted in a significant elevation of Ca^{2+}, or about 7-fold increment. Such a large magnitude in elevation of $[Ca^{2+}]_i$ suggests to us that the caffeine-sensitive pool is large compared to the intracel-

lular Ca^{2+} content in endothelial cells. Since lowering of $[Mg^{2+}]_o$ alone failed to alter basal $[Ca^{2+}]_i$ in endothelial cells, 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 $[Mg^{2+}]_o$ was lowered. Pretreatment of the endothelial cells with 1 m*M* Ni^{2+} partially inhibited the caffeine-induced Ca^{2+} release in EC exposed to 0.3 m*M* Mg^{2+}, suggesting that filling or emptying of caffeine-sensitive intracellular pools may be associated with Ca^{2+} entry [13]. Such Ca^{2+} movements across the plasma membrane of endothelial cells may thus be regulated by Mg^{2+}.

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 extracellular ionized Mg^{2+} concentration, which have been noted recently in human subjects [123], could induce substantial potentiation or inhibition of endothelial functions by such mechanisms.

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