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Low extracellular Mg^{2+} contraction of arterial muscle: role of protein kinase C and protein tyrosine phosphorylation

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Abstract

The effects of extracellular Mg^{2+} ion ($[Mg^{2+}]_0$) deficiency on basal tension of isolated rat aortae and rat aortic smooth muscle cell Ca^{2+} metabolism were investigated in the present study. The contractions of rat aortae induced by diverse concentrations of low $[Mg^{2+}]_0$ were potentiated, greatly, by removal of the endothelium or pre-incubation of intact rat aortic rings with L-N^G-monomethyl-arginine (L-NMMA). $[Mg^{2+}]_0$ deficiency-induced contractions were inhibited, to different degrees, by pre-treatment of the vessels with low concentrations of Gö6976, bisindolymaleimide I, genistein or a combination of bisindolymaleimide I with genistein. IC_{50} levels found for these three agents were found to be not too different from K_i values for these drugs. Pre-treatment of rat aortic smooth muscle cells with Gö6976, bisindolymaleimide I, genistein or a combination of bisindolymaleimide I with genistein suppressed, significantly, or almost eliminated both the rapid and stable increments in $[Ca^{2+}]_i$ induced by Mg^{2+}-free medium. The present findings suggest that both protein kinase C and protein tyrosine phosphorylation appear to play important roles in Mg^{2+} deficiency-induced contractions of isolated rat aortic smooth muscle, most likely via phosphorylation of L-type Ca^{2+} channels. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Aortic ring, rat; Mg^{2+} deficiency; Ca^{2+}; Signal transduction pathway; Endothelium; Nitric oxide (NO)

1. Introduction

Both animal and human studies indicate a statistical relationship between reduced dietary intake of magnesium and cardiovascular disease (Sjögren et al., 1989; Eisenberg, 1992; Rasmussen, 1993; Altura and Altura, 1995). Mg^{2+} has been demonstrated to modulate basal tone, myogenic tone and contractile responsiveness of vascular smooth muscle cells to various physiological and pharmacological stimuli, in part, by modulating Ca^{2+} concentration, binding and transport (Altura and Altura, 1978, 1995; Sjögren and Edvinsson, 1988; Zhang et al., 1992a). Low $[Mg^{2+}]_0$ results in elevation of contraction in a variety of mammalian arteries and arterioles (Altura and Altura, 1978, 1980, 1995; Sjögren et al., 1989).

It is now clear that multiple and complex signaling pathways participate in mechanisms of peripheral vasoconstriction (Malarkey et al., 1996). $[Ca^{2+}]_i$ is a major determinant of contractile force in all types of muscles. Protein kinase C and the thin filament-associated proteins have been considered to contribute in several ways to contractile regulation in vascular smooth muscle (Lee and Severson, 1994; Malarkey et al., 1996), including a possible regulation of $[Ca^{2+}]_i$ and Ca^{2+} channels. Protein tyrosine phosphorylation plays vital roles in several important cellular processes. It has been suggested that phosphorylation of proteins on tyrosine residues may be an important mechanism for regulating $[Ca^{2+}]_i$ in vascular smooth muscle cells (Di Salvo et al., 1993; Semenchuk and Di Salvo, 1995; Malarkey et al., 1996).

During the past 5 to 6 years, using specific Mg^{2+}-ion-selective electrodes, pioneered in our laboratory, we have demonstrated that patients with severe ischemic heart disease, untreated essential hypertension and stroke exhibit lowered levels of serum ionized Mg^{2+} but usually no change in total serum Mg levels (Altura and Altura, 1992; Altura et al., 1997a,b). We have recently found that such low, defined serum extracellular ionized Mg^{2+} ($[Mg^{2+}]_0$)

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levels can result in a rapid concentration-dependent rise in cytosolic intracellular free calcium ions ($[Ca^{2+}]_i$) in cultured aortic, coronary and cerebral arterial smooth muscle concomitant with contraction of these isolated primary vascular smooth muscle cells (Altura et al., 1997b; Zhang et al., 1992a). However, the precise mechanism whereby low $[Mg^{2+}]_0$ raises $[Ca^{2+}]_i$ and alters arterial vascular tone is not clear.

During the past decade, it has been shown that the endothelial cell lining may also be important in the vascular actions of $[Mg^{2+}]_0$ (Altura and Altura, 1987; Zhang et al., 1992b). Whether the spasmogenic vascular actions of Mg^{2+} deficiency are associated with a specific intracellular signal transduction pathway(s), e.g., protein kinase C and protein tyrosine phosphorylation, remains to be elucidated. The objective of the present study was to investigate the possible roles of protein kinase C and protein tyrosine phosphorylation on $[Ca^{2+}]_i$ and the contractile effects of low $[Mg^{2+}]_0$ on isolated rings of rat aorta (with and without endothelium) and rat aortic smooth muscle cells.

2. Materials and methods

2.1. General procedures

Male adult Wistar rats (350–450 g) were sacrificed by stunning and subsequent decapitation (Altura and Altura, 1974). The thoracic aortae were removed carefully and immediately placed in normal Krebs–Ringer bicarbonate solution containing (in mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, dextrose 10 and $NaHCO_3$ 25 (Altura and Altura, 1974; Zhang et al., 1992b). These segments were mounted on stainless-steel pins under 2 g resting tension in organ baths, attached to force transducers (Grass Model FT 03) and connected to Grass Model 7 polygraphs. The organ baths, containing normal Krebs–Ringer bicarbonate solution, were gassed continuously with 95% O_2 and 5% CO_2 and warmed to 37°C (pH 7.4). Tissues were allowed to equilibrate for at least 90 min before data collection. Incubation media were routinely changed every 15 min as a precaution against interfering metabolites (Altura and Altura, 1970). Stimulation of rings with 80 mM KCl was repeated every 30–45 min, two to three times, until vascular responses were stable. The successful removal of endothelium was assessed by showing that acetylcholine (10^{-8} to 10^{-6} M) failed to relax segments pre-contracted by 0.2 μM phenylephrine, while such concentrations of acetylcholine did relax the pre-contracted, endothelium-intact segments (Zhang et al., 1992b; Zheng et al., 1994).

To achieve similar levels of vessel tone, in intact aortic rings, treated with L-NMMA, the segments were pre-tensioned initially with a smaller physical force (1.6 g) rather than that (2.0 g) of untreated rings, since L-NMMA potentiates vessel tone by blocking the synthesis of nitric oxide (NO).

Ionization of magnesium in low Mg^{2+}, or Mg^{2+}-free modified Krebs–Ringer bicarbonate solution, was monitored by NOVA Biomedical ion-selective electrodes (Altura and Altura, 1992). For extracellular low Mg^{2+} or Mg^{2+}-free experiments (after incubation in normal Krebs–Ringer bicarbonate solution for 45 min), the rings were exposed to either low Mg^{2+} or Mg^{2+}-free modified Krebs–Ringer bicarbonate solution, and the data were obtained. Responses to low Mg^{2+} or Mg^{2+}-free solutions and other drugs were expressed as a percentage of the stable level of contraction induced by 80 mM KCl. All of the animal experimental procedures were approved by our institutional animal care and use committee.

2.2. Intracellular Ca^{2+} measurement

Primary smooth muscle cells from rat aorta (after endothelial denudation) were isolated and cultured in Dulbecco's modified Eagle's medium at 37°C in a humidified atmosphere composed of 95% O_2 and 5% CO_2 according to previously established methods (Zhang et al., 1992a). Cells for image analysis experiments were seeded on glass coverslips (12 mm diameter; about 1×10^4 cells/coverslip) and used 2–3 days postseeding. Monolayers of the smooth muscle cells grown on the coverslips were loaded with 2.0 μM of the acetoxymethyl ester of 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy)-2-(2′-amino-5′methyl-phenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid

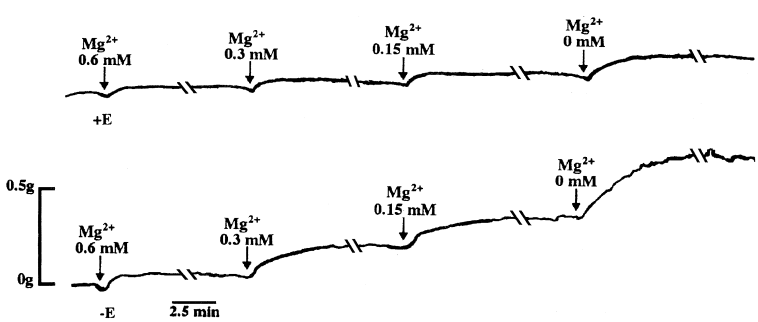

Fig. 1. Contractile responses to extracellular Mg^{2+} deficiency in isolated, intact (+E) and endothelium-denuded (−E) rat aortic rings. Vertical bar: tension in g; horizontal bar: time in min.

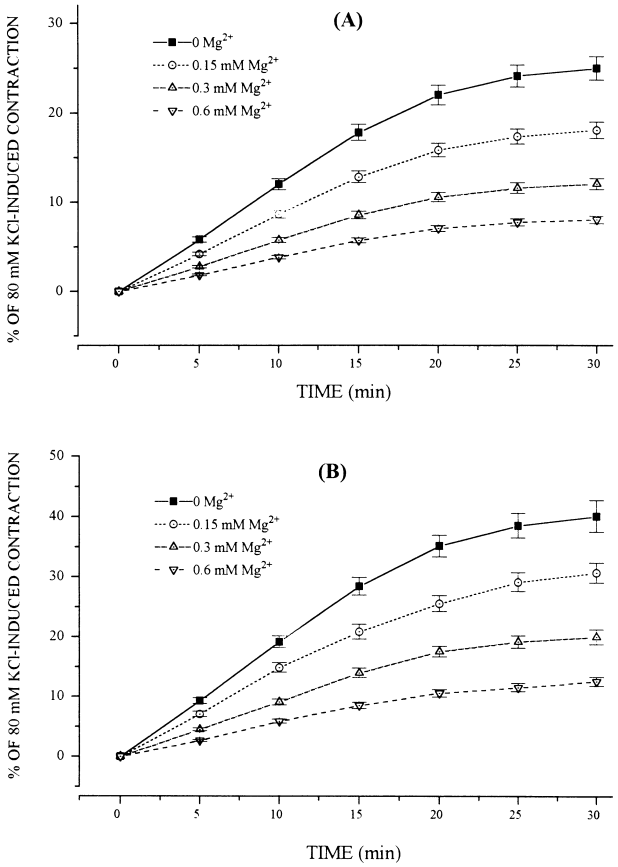

Fig. 2. Concentration-dependent contractile effects of Mg^{2+}-free medium and extracellular Mg^{2+} deficiency on isolated rat aortic rings with (A) and without endothelium (B). Each point represents the mean ± S.E. expressed as percentage of the tension developed by 80 mM KCl. The number of experiments is six each.

(fura-2-AM) and 0.12% pluronic acid F-127 (60 min, 37°C). The monolayers were washed two to three times with phosphate-buffered saline and 20 mM HEPES (pH 7.4) and incubated with this buffer (Zhang et al., 1992a) at room temperature until ready to use. The monolayers were inserted in a leakproof coverslip holder. Buffer was added to the monolayer on the coverslip. The coverslip holder was mounted onto the stage of a temperature-controlled Nikon TMS inverted microscope with a long working distance Nikon Fluor objective (n.a. 0.5), attached to a 300 Xenon light source and the CCD camera for image acquisition.

The cultured smooth muscle cell monolayers, preloaded with fura-2-AM, were excited alternatively, at 340 and 380 nm, and the emission intensity was recorded at 510 nm, using a silicon intensified target camera. Background autofluorescence for both excitation wavelengths was acquired from blanks for each experiment and subtracted from each pair of images separately before ratioing. Fluorescence ratios (R) were obtained by dividing the 340-nm image by the 380-nm image. No image misalignments occurred when those two ratiometric images were superimposed. The resulting images were then used to calculate $[Ca^{2+}]_i$ in

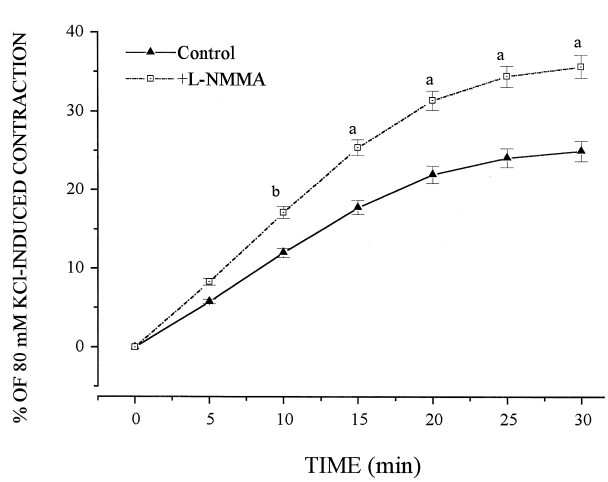

Fig. 3. Contractile responses to Mg^{2+}-free medium, obtained in the intact rat aortic rings and the intact rings treated with 150 μM L-NMMA. Each point represents the mean ± S.E. expressed as percentage of the tension developed by 80 mM KCl. (a) denotes $P < 0.01$ and (b) denotes $P < 0.05$ compared to control. $N = 6$ each.

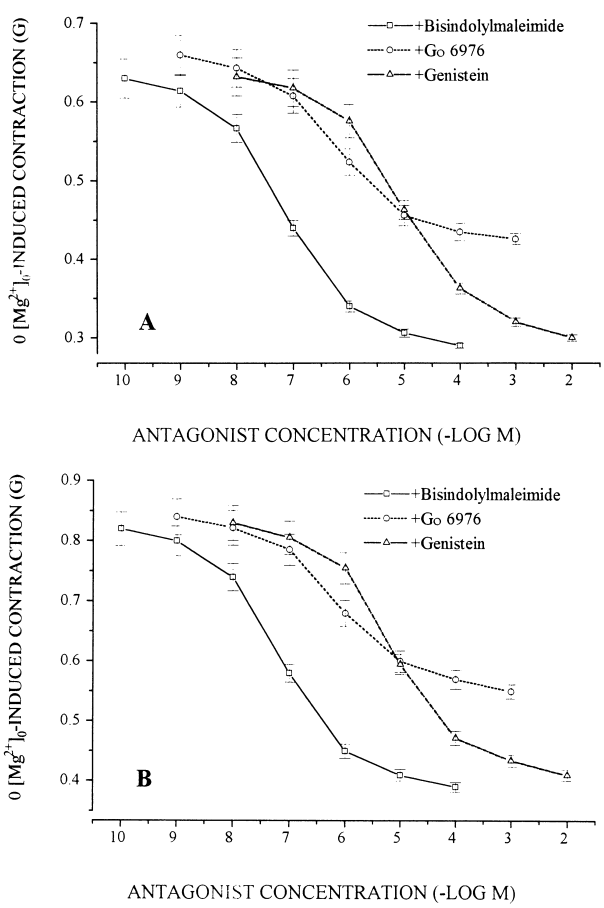

Fig. 4. Concentration-dependent inhibitory effects of Gö6976, bisindolylmaleimide and genistein on Mg^{2+}-free medium-induced contractions in isolated endothelium-intact (A) and -denuded (B) rat aortic rings. Pre-incubation time of these antagonists was 15 min. The data were obtained after 30 min of 0-mM $[Mg^{2+}]_0$ administration. Each point represents the mean ± S.E. expressed as percentage of 0-mM $[Mg^{2+}]_0$-induced maximal contraction. $N = 6$ each.

smooth muscle cells, using external standards containing 2.54 mM Ca^{2+}, and 0 mM Ca^{2+} plus 10 mM EGTA for maximum (R_{max}) and minimum (R_{min}) fluorescence ratios of the 340 and 380 nm images (Zhang et al., 1992a). $[Ca^{2+}]_i$ was calculated according to the following equation (Grynkiewicz et al., 1985):

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

A K_d of 224 nM was used for the fura-2/Ca^{2+} complex. B is the ratio of fluorescence intensity of fura-2 to Ca^{2+}:fura-2 complex excited at 380 nm.

2.3. Drugs

The following pharmacological agents were purchased from Sigma (St. Louis, MO): bisindolylmaleimide I, acetylcholine HCl, ethyleneglycol-bis (β-aminoethyl ether) *N,N′*-tetraacetic acid (EGTA), genistein and verapamil. Atropine sulfate was bought from MANN Res. Lab. (New York, NY). Fura-2-AM was purchased from Molecular Probes (Eugene, OR). Dimethyl sulfoxide (DMSO) and Gö6976 were purchased from CALBIOCHEM (La Jolla, CA). All other organic and inorganic chemicals were obtained from Fisher Scientific (Fair Lawn, NJ) and were of the highest purity.

2.4. Calculations and statistical analyses

The percentage contraction was expressed as the mean ± standard error of the mean (S.E.M.). Statistical evaluation of the results was carried out by analysis by Newman–Keuls Test and analysis of variance (ANOVA) using Scheffe's contrast test. The results were considered significant at a P-value of < 0.05.

3. Results

3.1. Extracellular Mg^{2+} deficiency-induced contraction and endothelium

Rapid removal of $[Mg^{2+}]_0$, or lowering $[Mg^{2+}]_0$, produced sustained, inverse-concentration- and time-dependent contractions in rat aortic ring segments (Figs. 1 and 2A and B) in the presence and absence of endothelium. The time course needed to achieve near-maximal contraction was about 30 min. There were significantly greater developed tensions in the endothelium-denuded rings, and in the endothelium-intact rings pre-treated with 150 μM L-NMMA, compared to that of the endothelium-intact rings (Figs. 1 and 2A, B; Fig. 3). There was no tension

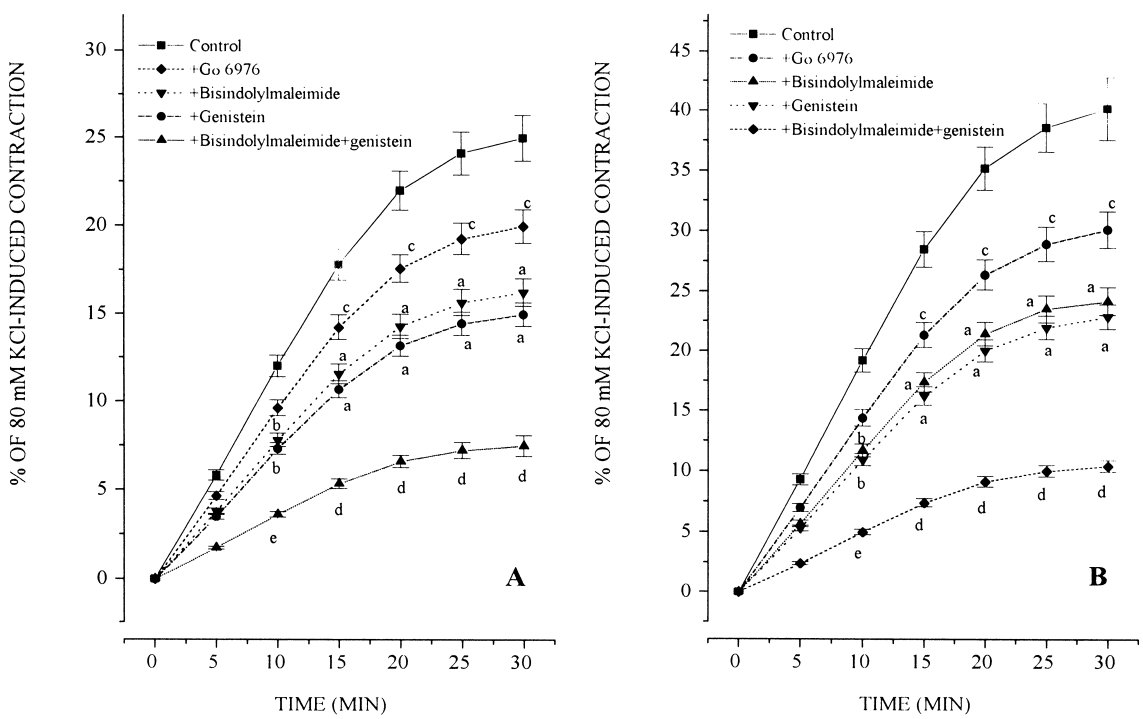

Fig. 5. Contractile effects to Mg^{2+}-free medium, on isolated endothelium-intact (A) and -denuded (B) rat aortic rings, obtained in the absence and presence of 10^{-6} M Gö6976, 5×10^{-7} M bisindolylmaleimide, 7.5×10^{-5} M genistein and 5×10^{-7} M bisindolylmaleimide I plus 7.5×10^{-5} M genistein. Pre-incubation time of these antagonists was 15 min. Each point represents the mean ± S.E. expressed as percentage of the tension developed by 80 mM KCl. (a) denotes $P < 0.01$, (b) denotes $P < 0.05$ compared to control, (c) denotes $P < 0.05$ compared to control and bisindolylmaleimide-treated group, (d) denotes $P < 0.01$ compared to control, bisindolylmaleimide-treated and genistein-treated groups, and (e) denotes $P < 0.05$ compared to control, bisindolylmaleimide-treated and genistein-treated groups. $N = 6$ each.

difference between endothelium-denuded rings and denuded rings pre-treated with 150 μM L-NMMA ($n = 6$, not shown, $P > 0.05$). Actual representative tracings for these responses are depicted in Fig. 1. Contractions produced in low $[Mg^{2+}]_0$ are composed of a very short, initial fast response (phasic component), followed by a longer, slower and sustained increase in tension (tonic component), which is exacerbated and amplified in endothelium-denuded aorta (Fig. 1).

3.2. Protein kinase C antagonists attenuate arterial smooth muscle contractions induced by low $[Mg^{2+}]_0$

As shown in Fig. 4A and B, pre-treatment of either intact or endothelium-denuded aortic rings with either Gö6976 [a protein kinase C_α and protein kinase $C_{\beta 1}$-selective antagonist (Husain and Abdel-Latif, 1998)] or bisindolylmaleimide I [a specific antagonist of protein kinase C (Deng et al., 1997)] inhibited, clearly, 0-mM $[Mg^{2+}]_0$-induced contractions, in a concentration-dependent manner. The concentrations producing approximately 50% of the maximal inhibitory effects (IC_{50} values) of Gö6976 and bisindolylmaleimide I were $\sim 9.56 \pm 0.38 \times 10^{-7}$ and $\sim 4.98 \pm 0.21 \times 10^{-7}$ M, respectively. The suppressant effect of bisindolylmaleimide I was clearly stronger than that of Gö6976 ($P < 0.05$). The effects of using IC_{50} levels of Gö6976 and bisindolylmaleimide on 0-mM $[Mg^{2+}]_0$-induced contractions of intact and denuded rat aorta are shown in Fig. 5A and B. After obtaining contractions of either endothelium-denuded or intact (not shown) rat aortic rings, which were induced by Mg^{2+}-free extracellular medium, the cumulative addition of either IC_{50} values of Gö6976 or IC_{50} values of bisindolylmaleimide I to the organ bath resulted in a reversal of the endothelium-independent contractile responses (Fig. 6A and B).

3.3. Effects of a tyrosine kinase inhibitor and protein kinase C antagonist on low $[Mg^{2+}]_0$-induced contractions

Fig. 4A and B indicate that pre-treatment of intact and denuded rat aortic rings with genistein resulted in a concentration-dependent inhibition of the arterial contractions induced by Mg^{2+}-free medium. The IC_{50} value of genistein for such contractions was $\sim 7.52 \pm 0.34 \times 10^{-5}$ M.

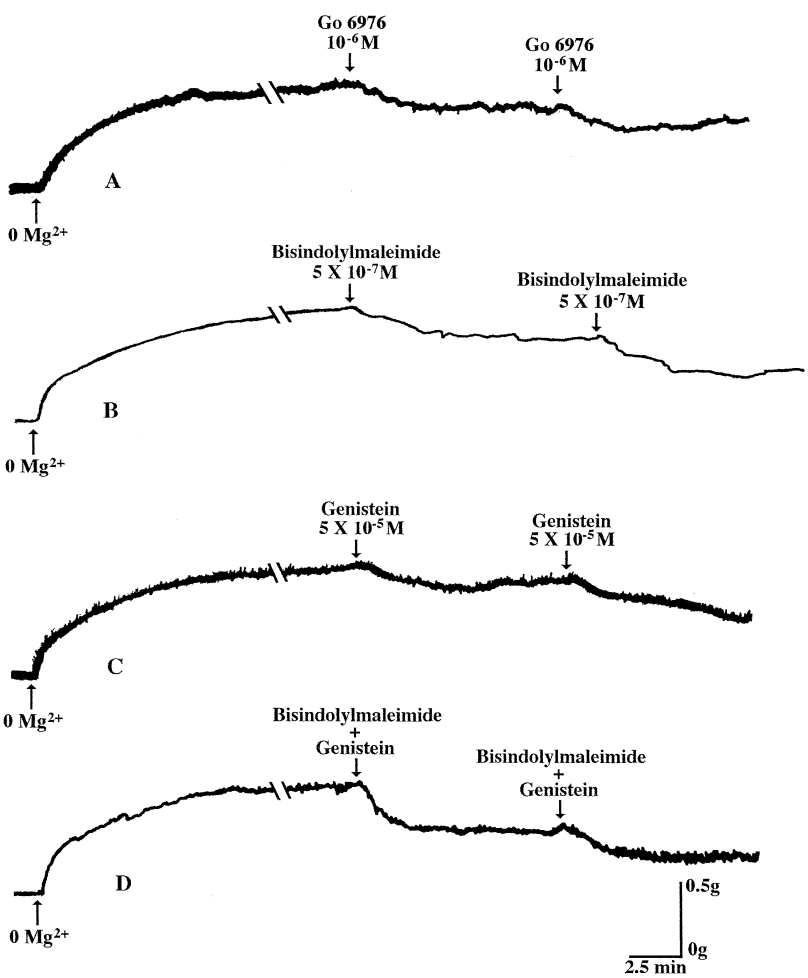

Fig. 6. Reversal of contractile responses of endothelium-denuded aorta rings to Mg^{2+}-free medium by addition of Gö6976 (A), bisindolylmaleimide I (B), genistein (C) and 5×10^{-7} M bisindolylmaleimide I plus 5×10^{-5} M genistein (D). Vertical bar: tension in g; horizontal bar: time in min.

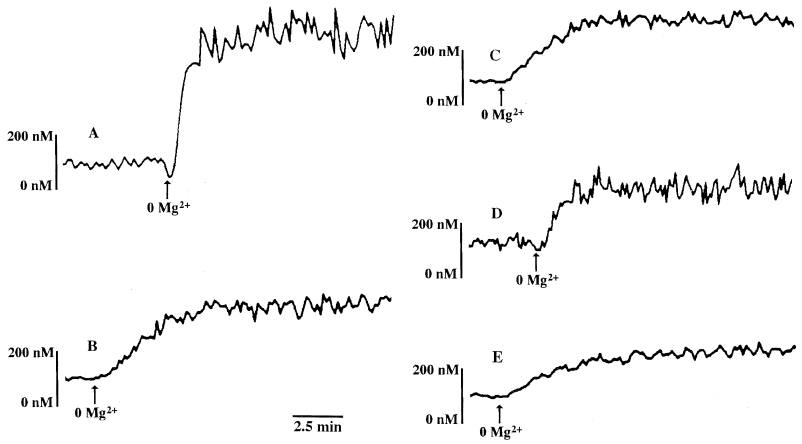

Fig. 7. Extracellular Mg^{2+}-free-induced elevation (A) of $[Ca^{2+}]_i$ in smooth muscle cells from rat aorta is modified by addition of 10^{-6} M Gö6976 (B), 5×10^{-7} M bisindolylmaleimide I (C) 7.5×10^{-5} M genistein (D), and 5×10^{-7} M bisindolylmaleimide I plus 7.5×10^{-5} M genistein (E). $[Ca^{2+}]_i$ was measured by fura-2 fluorescence as described under Section 2.

The IC_{50} levels of genistein-induced inhibition of 0-mM $[Mg^{2+}]_0$-induced contractions in intact and denuded rat aorta and a combination of 7.5×10^{-5} M genistein and 5×10^{-7} M bisindolylmaleimide I produced much more inhibition of $[Mg^{2+}]_0$-free-induced contractions, as shown in Fig. 5A and B. The cumulative employment of IC_{50} levels of genistein led to a prompt relaxation of the $[Mg^{2+}]_0$-free contractions (Fig. 6C). A much greater decrease of the $[Mg^{2+}]_0$-free contractions in the denuded aortic rings was noted by cumulative administration of IC_{50} levels of genistein plus IC_{50} levels of bisindolylmaleimide I (Fig. 6D).

3.4. Mg^{2+}-free medium and increments in $[Ca^{2+}]_i$ in single smooth muscle cells

Fig. 7 demonstrates that exposure of rat aortic smooth muscle cells to 0-mM $[Mg^{2+}]_0$ medium, containing 2.5 mM Ca^{2+}, produced a rapid elevation of $[Ca^{2+}]_i$ followed by a further rise to a plateau level, much higher than the basal level (approximately 600 nM from around 90 nM, Fig. 7A and Table 1). Surprisingly, pre-incubation of the cells with 10^{-6} M Gö6976 (Fig. 7B, Table 1), 5×10^{-7} M bisindolymaleimide (Fig. 7C, Table 1), or 5×10^{-5} genistein (Fig. 7D, Table 1) effectively prevents both the rapid increment in $[Ca^{2+}]_i$ and the additional rise of $[Ca^{2+}]_i$ to either the lower or higher steady-states, but does not alter the basal level of $[Ca^{2+}]_i$. The combination of bisindolymaleimide and genistein, on the $[Mg^{2+}]_0$-free induced increments in $[Ca^{2+}]_i$, is much stronger than that of either bisindolymaleimide or genistein alone (Fig. 7E, Table 1).

Table 1
Extracellular Mg^{2+}-free-induced elevation of $[Ca^{2+}]_i$ in primary cultured rat aortic smooth muscle cells is modified by pre-incubation with 10^{-6} M Gö6976, 5×10^{-7} M bisindolylmaleimide, 7.5×10^{-5} M genistein and 5×10^{-7} M bisindolylmaleimide plus 7.5×10^{-5} M genistein. Each point represents the mean ± S.E. of steady-states of the $[Ca^{2+}]_i$ values. The number of experiments is at least 12 each

Group	$[Ca^{2+}]_i$ (nM)
Control	105.8 ± 1.84
$[Mg^{2+}]_0$ alone	610.5 ± 5.69^{b}
$[Mg^{2+}]_0$ + Gö6976	$220.9 \pm 3.69^{a,c}$
$[Mg^{2+}]_0$ + bisindolylmaleimide I	$218.8 \pm 3.42^{a,c}$
$[Mg^{2+}]_0$ + genistein	$242.5 \pm 3.63^{a,c}$
$[Mg^{2+}]_0$ + bisindolylmaleimide and genistein	$158.6 \pm 2.26^{a,d}$

[a] $P < 0.01$ compared to control.
[b] $P < 0.001$ compared to control.
[c] $P < 0.01$ compared to $[Mg^{2+}]_0$ alone.
[d] $P < 0.01$ compared to all other values.

4. Discussion

One of the most interesting observations in this study is that removal of the endothelium causes stronger contractions in response to low $[Mg^{2+}]_0$ (i.e., 0.3 to 0.6 mM), levels found recently in the serum of patients with ischemic heart disease, hypertension and stroke using new specific Mg^{2+}-ion-selective electrodes (Altura and Altura, 1992; Altura et al., 1997a,b). This suggests, to us, that the endothelium is chronically producing a substance(s) which counters the effects of low $[Mg^{2+}]_0$. Pre-incubation of the intact aortic rings with a NO synthase antagonist, L-NMMA, in the present study, induces stronger contractions in response to low $[Mg^{2+}]_0$, indicating that the endothelium-released substance, countering the effects of low $[Mg^{2+}]_0$, is most likely NO. It should be pointed out here, that $[Mg^{2+}]_0$ and intact endothelial cells are known to be needed for action of a variety of vasorelaxants (Altura and Altura, 1987, 1995). It is, thus, tempting to speculate that $[Mg^{2+}]_0$ has possible dual roles in regulation of vascular tone; low $[Mg^{2+}]_0$ promoting elevation in basal tone and normal $[Mg^{2+}]_0$ promoting relaxation of basal tone, the net result being a homeostatic balance.

Activation of protein kinase C is an important mechanism by which vasoconstrictors act (Andrea and Walsh, 1992; Malarkey et al., 1996). An important observation presented, herein, is that, Gö6976 (a selective antagonist of protein kinase C_α as well as protein kinase $C_{\beta 1}$) and bisindolylmaleimide I (a specific protein kinase C antagonist), attenuated, markedly, the low $[Mg^{2+}]_0$-induced contractile responses of rat aortic segments, suggesting the probable involvement of protein kinase C activation in the arterial contractions induced by extracellular Mg^{2+} deficiency. The calculated IC_{50} values for Gö6976 and bisindolylmaleimide were $\sim 9.56 \pm 0.38 \times 10^{-7}$ and $\sim 4.98 \pm 0.21 \times 10^{-7}$ M, respectively. These values are only slightly higher than the reported K_i values for Gö6976 (about 2×10^{-8} M, Gschwendt et al., 1996) and bisindolylmaleimide ($1.6–2.0 \times 10^{-8}$ M, Toullec et al., 1991) for 50% inhibition of protein kinase C. Since the K_i values of these two antagonists were obtained at cellular levels and the IC_{50} values, herein, of these two antagonists were obtained at bioassay and tissue levels, the latter should be consistent with the former. It is interesting to point out that the inhibitory effects of Gö6976 on such contractions were smaller than that of bisindolylmaleimide I, but there are no differences between the actions of these two antagonists on $[Ca^{2+}]_i$ elevation in the cells, suggesting the probable involvement of protein kinase C activation (both Ca^{2+}-dependent and Ca^{2+}-independent isomers) in the arterial contractions and intracellular Ca^{2+} changes in the cells induced by 0 $[Mg^{2+}]_0$.

Recently, it has been shown that increments in $[Ca^{2+}]_i$ associated with certain drugs (e.g., ethanol), which promote contraction in vascular smooth muscle, are observed only after first depleting intracellular free Mg^{2+} (Altura et al., 1993); these alcohol-induced contractions are inhibited by the same protein kinase C antagonists used herein (Zheng et al., 1998; unpublished findings). In addition, it has been demonstrated that Mg^{2+} is a noncompetitive inhibitor of [^{3}H] IP_3 binding and IP_3-induced Ca^{2+}-release (Volpe et al., 1990). It is likely that decrements in $[Mg^{2+}]_0$ would alter the intracellular Mg^{2+} concentration in rat aortic muscle cells (Zhang et al., 1992c), and then the inhibitory effects of Mg^{2+} on IP_3 binding and IP_3-induced Ca^{2+} release would be, thus, attenuated or eliminated. In concert with this, IP_3-induced Ca^{2+} release from intracellular stores and protein kinase C activities in the cells would be elevated, and the smooth muscle cells would then be expected to undergo contraction.

The involvement of protein kinase C is reinforced by the present findings, in that in single rat aortic smooth muscle cells, pre-incubated with protein kinase C antagonists (Gö6976 and bisindolylmaleimide I), $[Mg^{2+}]_0$-free medium produced slow and smaller increases in $[Ca^{2+}]_i$, which suggests that both Ca^{2+} influx from the extracellular medium and Ca^{2+} release from intracellular stores were inhibited. Our results are consistent with several reports demonstrating: (1) inhibition of protein kinase C activity with staurosporine or chelerythrine can inhibit availability and long-opening of L-type Ca^{2+} channels in A7r5 cells (Obejero-Paz et al., 1998), (2) inhibition of protein kinase C activity blunts the relative increase in cytosolic free Ca^{2+} in rabbit afferent arterioles in response to angiotensin II (Salomonsson et al., 1997), and (3) inhibition of protein kinase C activity can inhibit, completely, the rise in $[Ca^{2+}]_i$ induced by alcohol in cerebral vascular smooth muscle cells (Zheng et al., 1998). It is, thus, tempting to speculate that inhibition of protein kinase C phosphorylation of Ca^{2+} channels, in aortic smooth muscle cell membranes, prevents the necessary rise in $[Ca^{2+}]_i$ produced by a $[Ca^{2+}]_0$-induced Ca^{2+} release, thus promoting relaxation of low $[Mg^{2+}]_0$-induced vascular contractions.

Protein tyrosine phosphorylation is an essential component in signal transduction pathways in smooth muscle cells (Malarkey et al., 1996). It has been pointed out, previously, that receptor-activated increases in $[Ca^{2+}]_i$ in cultured vascular smooth muscle cells may be coupled to receptor-activated increases in protein tyrosine phosphorylation (Semenchuk and Di Salvo, 1995). In the present study, the ability of genistein (an antagonist of protein tyrosine kinase) to impair extracellular Mg^{2+} deficiency-induced contractions [IC_{50} value herein for genistein was $\sim 7.52 \pm 0.34 \times 10^{-5}$ M, whereas the reported K_i value of genistein for protein tyrosine kinase is 1.2×10^{-5} M (O'Dell et al., 1991)], and to suppress both the rapid and stable increments in $[Ca^{2+}]_i$ in single rat aortic smooth muscle cells, could be used to implicate an involvement of tyrosine phosphorylation in vascular contractile responses of aortic muscle to low $[Mg^{2+}]_0$, which may be mediated, at least partially, by an elevation in $[Ca^{2+}]_i$ in rat aortic smooth muscle cells through activation of protein tyrosine kinase. This conclusion is well-supported by several lines of experimental data reported recently by other investigators: (1) genistein can suppress the erythropoietin-induced rise in $[Ca^{2+}]_i$ in vascular endothelial cells (Vogel et al., 1997); (2) platelet-derived growth factor BB elicits Ca^{2+} influx in vascular smooth muscle cells (VSMC) via a tyrosine kinase-dependent mechanism (Clunn et al., 1997); (3) genistein can inhibit the activity of L-type Ca^{2+} channels in VSMC form rat portal vein (Liu and Sperelakis, 1997); and (4) serotonin-evoked Ca^{2+} release from the sarcoplasmic reticulum in VSMC is blocked by genistein (Nelson et al., 1997). We are, thus, tempted to put forward the hypothesis that tyrosine kinase-induced phosphorylation of L-type Ca^{2+} channels in rat aortic smooth muscle cells is important in contraction induced by low $[Mg^{2+}]_0$.

Treatment of aortic smooth muscle with IC_{50} levels of bisindolylmaleimide I plus genistein blocks, almost completely ($> 90\%$), the low $[Mg^{2+}]_0$-induced contraction of endothelium-denuded rat aortic rings and elevation of $[Ca^{2+}]_i$ in aortic smooth muscle cells, supporting further the present conclusion that the change in vascular contractility induced by extracellular Mg^{2+} deficiency is medi-

ated by protein kinase C and tyrosine kinase signal transduction pathways.

Other recent findings have shown that within the pathophysiological range of extracellular Mg^{2+} concentrations (0.15–0.6 mM), fatty acid chain length and double bond content in lipid extracts of rat aortic smooth muscle are reduced progressively as $[Mg^{2+}]_0$ is lowered (Morrill et al., 1997). This finding, recently reported by our lab, supports previous findings, in intact aorta, that during Mg^{2+} deficiency, lipid peroxidation products, assayed as thiobarbituric acid-mediated materials, increased two-fold compared to control animals (Altura et al., 1990). The lipid peroxidation and lipid component changes in the vascular membranes, caused by extracellular Mg^{2+} deficiency, may be related, directly, to the opening of Ca^{2+} channels and the initiation of the cellular signal transduction pathways leading to the contractile responses of the vessels.

Based on all of the above and the new data presented herein, we tentatively propose that while it acts directly on vascular smooth muscle cell membranes, extracellular Mg^{2+} deficiency initiates contraction in rat aortic smooth muscle cells via Ca^{2+}-dependent activation of protein kinase C and protein tyrosine kinase, and probably, via as yet, unknown lipid peroxidative signals. The presence of viable endothelial cells seems to produce NO which modulates the adverse spasm-like actions of low $[Mg^{2+}]_0$.

Several clinical disease syndromes, including hypertension of unknown origin, preeclampsia–eclampsia of pregnancy, diabetes mellitus-induced hypertensive vascular diseases, essential hypertension, drug-induced hypertensive vascular diseases, several types of ischemic heart diseases and strokes are associated with hypomagnesemia (Sjögren et al., 1989; Altura and Altura, 1992, 1995; Altura et al., 1984, 1990, 1997a,b; Eisenberg, 1992; Rasmussen, 1993). Depending upon viability of the endothelial lining, $[Mg^{2+}]_0$ deficiency-induced peripheral vasoconstriction and vasospasm would be expected to, hemodynamically, lead to reduced organ blood flows, decreased tissue nutrition, decreased tissue oxygenation, and increased peripheral vascular resistance. The present studies, thus, could help to shed new light on the etiologies of diverse vascular disease states, and atherogenesis, and could be of considerable help in pin-pointing potential avenues for pharmacologic and therapeutic intervention, particularly in Mg-deficient states.

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