Low $[\text{Mg}^{2+}]_o$ induces contraction and $[\text{Ca}^{2+}]_i$ rises in cerebral arteries: roles of $\text{Ca}^{2+}$, PKC, and PI3

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Received 27 January 2000; accepted in final form 30 May 2000

Yang, Zhi-Wei, Jun Wang, Tao Zheng, Bella T. Altura, and Burton M. Altura. Low $[\text{Mg}^{2+}]_o$ induces contraction and $[\text{Ca}^{2+}]_i$ rises in cerebral arteries: roles of $\text{Ca}^{2+}$, PKC, and PI3. Am J Physiol Heart Circ Physiol 279:H2898–H2907, 2000.—Removal of extracellular $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+}]_o$) and pretreatment of canine basilar arterial rings with either an antagonist of voltage-gated $\text{Ca}^{2+}$ channels (verapamil), a selective antagonist of the sarcoplasmic reticulum $\text{Ca}^{2+}$ pump (thapsigargin [TSG]), caffeine plus a specific antagonist of ryanodine-sensitive $\text{Ca}^{2+}$ release (ryanodine), or a $\text{d-myo-inositol} 1,4,5$-trisphosphate $[\text{Ins}(1,4,5)\text{P}_3]$-mediated $\text{Ca}^{2+}$ release antagonist (heparin) markedly attenuates low extracellular $\text{Mg}^{2+}$ concentration ($[\text{Mg}^{2+}]_o$)-induced contractions. Low $[\text{Mg}^{2+}]_o$-induced contractions are significantly inhibited by pretreatment of the vessels with Go-6976 [a protein kinase C- (PKC-) and PKC-BI-selective antagonist], bisindolylmaleimide I (Bis, a specific antagonist of PKC), and wortmannin or LY-294002 [selective antagonists of phosphatidylinositol-3-kinases (PI3Ks)]. These antagonists were also found to relax arterial contractions induced by low $[\text{Mg}^{2+}]_o$ in a concentration-dependent manner. The absence of $[\text{Ca}^{2+}]_o$ and preincubation of the cells with verapamil, TSG, heparin, or caffeine plus ryanodine markedly attenuates the transient and sustained elevations in the intracellular $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+}]_i$) induced by low-$[\text{Mg}^{2+}]_o$ medium. Low $[\text{Mg}^{2+}]_o$-produced increases in $[\text{Ca}^{2+}]_i$ are also suppressed markedly in the presence of Go-6976, Bis, wortmannin, or LY-294002. The present study suggests that both $\text{Ca}^{2+}$ influx through voltage-gated $\text{Ca}^{2+}$ channels and $\text{Ca}^{2+}$ release from intracellular stores [both $\text{Ins}(1,4,5)\text{P}_3$ sensitive and ryanodine sensitive] play important roles in low-$[\text{Mg}^{2+}]_o$-induced contractions of isolated canine basilar arteries. Such contractions are clearly associated with activation of PKC isoforms and PI3Ks.

canine basilar arteries; extracellular magnesium concentration deficiency; calcium influx; intracellular calcium release; protein kinase C; phosphatidylinositol-3 kinase

Dietary deficiency of magnesium as well as abnormalities in $\text{Mg}^{2+}$ metabolism have been suggested to play important roles in hypertension, stroke, atherosclerosis, and diabetic vascular disease (for recent reviews, see Refs. 1, 3, 5, 10, 22, 34). During the past five or six years, using specific $\text{Mg}^{2+}$-selective electrodes that were pioneered in our laboratory, we have demonstrated that patients with severe untreated essential hypertension and stroke exhibit lowered levels of serum-ionized $\text{Mg}^{2+}$ (1, 3, 8, 9). Such low defined serum-ionized extracellular $\text{Mg}^{2+}$ concentration ($[\text{Mg}^{2+}]_o$) levels result in a rapid concentration-dependent rise in intracellular $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+}]_i$) in cultured cerebral arterial smooth muscle concomitant with contraction of these isolated primary vascular smooth muscle cells (8).

$\text{Ca}^{2+}$ is a major determinant of contractile force in all types of muscles. The initiation of contraction in vascular smooth muscle is believed to derive from a rise of free cytosolic $[\text{Ca}^{2+}]_i$. There are two ways to raise $[\text{Ca}^{2+}]_i$: 1) $\text{Ca}^{2+}$ can enter smooth muscle cells through $\text{Ca}^{2+}$ channels in the plasma membrane, such as voltage-gated $\text{Ca}^{2+}$ channels; and 2) $\text{Ca}^{2+}$ can be released from intracellular stores either by $\text{d-myo-inositol} 1,4,5$-trisphosphate $[\text{Ins}(1,4,5)\text{P}_3]$ or by $\text{Ca}^{2+}$-induced $\text{Ca}^{2+}$ release through the ryanodine receptor (23). Protein kinase C (PKC) and the thin filament-associated proteins have been considered to contribute in several ways to contractile regulation in vascular smooth muscle (16, 21), including a possible regulation of $[\text{Ca}^{2+}]_i$ and $\text{Ca}^{2+}$ channels. Recently phosphatidylinositol-3-kinases (PI3Ks), which phosphorylate phosphatidylinositol 4,5-bisphosphate ($\text{PIP}_2$) to phosphatidylinositol 3,4,5-trisphosphate ($\text{PIP}_3$), have received more attention because $\text{PIP}_3$ and PI3Ks have also been suggested to act as second messengers (31).

The myogenic tone of cerebral vessels is strongly dependent on the plasma concentration of $[\text{Mg}^{2+}]_o$ (4, 6, 7) and is believed to play an important role in the autoregulation of cerebral blood flow (7, 11). Recently, several clinical trials have demonstrated the therapeutic usefulness of administration of $\text{Mg}^{2+}$ in the treatment of stroke (26). However, the mechanism whereby low $[\text{Mg}^{2+}]_o$ alters cerebral arterial vascular tone may be complex, and whether the vascular action of $\text{Mg}^{2+}$ deficiency is associated with specific intracellular sig-
nal transduction pathways is less well defined. This prompted the present study to gain insight into the relationship between \([Mg^{2+}]_o\), deficiency-induced contractions, \(Ca^{2+}\) influx, intracellular \(Ca^{2+}\) release, and potential intracellular signaling pathways, such as PKC and PI3Ks.

**MATERIALS AND METHODS**

**General procedures.** Rings of canine basilar arteries were obtained from male mongrel dogs (18–22 kg) after administration of pentobarbital sodium anesthesia (40 mg/kg iv) and were placed in normal Krebs-Ringer bicarbonate solution at pH 7.4 containing (in mM) 118 NaCl, 4.7 KCl, 1.2 KH\(_2\)PO\(_4\), 1.2 MgSO\(_4\), 2.5 CaCl\(_2\), 10 dextrose, and 25 NaHCO\(_3\) (6). The rings were 3–4 mm in length. The segments were mounted on stainless steel pins under 2 g resting tension in isolated organ baths, attached to force transducers (Grass model FT 03) and connected to polygraphs (Grass model 7). The organ baths containing normal Krebs-Ringer bicarbonate solution were gassed continuously with 95% O\(_2\)-5% CO\(_2\) and warmed to 37°C. The segments were exposed, in the absence of any stimuli, to either low-Mg\(_2\) (0.6, 0.3, or 0.15 mM) or Mg\(_2\)-free (0.6 M) Krebs-Ringer bicarbonate solution containing 1.2 mM MgSO\(_4\) for 45 min, while it did relax the endothelium-intact segments (44, 45). When tissues were pretreated by various drugs, the drug was applied for at least 15 min before the concentration-response curves were obtained.

Ionization of magnesium in either low (0.15–0.6 mM) or 0 mM \([Mg^{2+}]_o\)-modified Krebs-Ringer bicarbonate solution was monitored using ion-selective electrodes (NOVA Biomedical, Waltham, MA) (3). For extracellular low-Mg\(_2\) or Mg\(_2\)-free experiments (after incubation in normal Krebs-Ringer bicarbonate solution containing 1.2 mM MgSO\(_4\) for 45 min), the rings were exposed, in the absence of any stimuli, to either low-Mg\(_2\) (0.6, 0.3, or 0.15 mM) or Mg\(_2\)-free Krebs-Ringer bicarbonate solution, and the bioassay data were then obtained. Responses to low-Mg\(_2\) (0.6, 0.3, or 0.15 mM) or Mg\(_2\)-free (0 mM) solutions and other drugs were expressed as either a percentage of the stable level of contraction induced by 80 mM KCl or grams of tension. All of the animal experimental procedures were approved by our institutional animal care and use committee.

Gö-6976, bisindolylmaleimide I (Bis), and LY-294002 were purchased from Calbiochem (La Jolla, CA). Phentolamine methanesulfonate was purchased from CIBA Pharmaceutical (Summit, NJ). Methysergide maleate was purchased from Sandoz Pharmaceuticals (Hanover, NJ). LY-294002 was purchased from Biomol Research Laboratories, (Plymouth Meeting, PA). All other organic and inorganic chemicals were obtained from Fisher Scientific (Fair Lawn, NJ) and were of the highest purity.

**Calculations and statistical analysis.** The contractile response \((g)\), percentage of maximal KCl-induced contraction, and [Ca\(^{2+}\)]_i were expressed as means \pm SE of the mean. Statistical evaluation of the results was carried out via analysis using the Newman-Keuls test and ANOVA using Scheffé’s contrast test. The results were considered significant at \(P < 0.05\).

**RESULTS**

\[[Mg^{2+}]_o\]-deficiency-induced contractions and extracellular \(Ca^{2+}\) influx. In medium in which the external \(Ca^{2+}\) concentration \(([Ca^{2+}]_o)\) is zero, removal of \([Mg^{2+}]_o\), from the medium induces a smaller transient \([Ca^{2+}]_i\) peak in the cells, which returns to the baseline level quickly without any plateau phase compared with the rapid rise and sustained plateau in \([Ca^{2+}]_o\) in the presence of normal \([Ca^{2+}]_o\) (Fig. 1A). In the presence of \([Ca^{2+}]_o\), preincubation with 5 \times 10^{-6} M verapamil (an antagonist of voltage-gated \(Ca^{2+}\) channels) for about 10 min almost diminishes completely the low \([Mg^{2+}]_o\)-induced \([Ca^{2+}]_i\) plateau and significantly reduces the transient \([Ca^{2+}]_i\) peak; calculated mean values for low \([Mg^{2+}]_o\)-induced \([Ca^{2+}]_i\) peaks in the absence of \([Ca^{2+}]_o\) and in the presence of verapamil are shown in Fig. 1B.

The low \([Mg^{2+}]_o\)-induced contractions of endothelium-denuded canine basilar arterial rings (Fig. 1C)

\[[Ca^{2+}]_i = K_4 \times B \times (R - R_{\text{min}})/(R_{\text{max}} - R)\]

where R is the fluorescence ratios of fura 2 obtained by dividing the 340-nm image by the 380-nm image, R_{\text{min}} is the minimum fluorescence ratios of fura 2 obtained in medium containing 0 mM Ca\(^{2+}\) plus 10 mM EGTA by dividing the 340-nm image by the 380-nm image, and R_{\text{max}} is the maximum fluorescence ratios of fura 2 obtained in medium containing 2.54 mM Ca\(^{2+}\) by dividing the 340-nm image by the 380-nm image. A dissociation constant \((K)\) of 224 mM was used for the fura 2-Ca\(^{2+}\) complex (24, 43, 44). B is the ratio of fluorescence intensity of fura 2 to the Ca\(^{2+}\)-fura-2 complex excited at 380 nm. Particular care was taken to minimize photobleaching of the dye. Experiments were carried out in total darkness, and exposure to excitation light was less than 2 s in all experiments.

**Experiments with removal of extracellular Ca\(^{2+}\).** For the extracellular Ca\(^{2+}\)-free experiments, the canine basilar arterial ring segments were equilibrated in Ca\(^{2+}\)-free normal Krebs-Ringer bicarbonate solution containing 0.2 mM EGTA for at least 90 min before initiation of the experiments.

**Drugs.** The following pharmacological agents were purchased from Sigma Chemical (St. Louis, MO); Bis HCl, ACh HCl, EGTA, heparin ammonium salt, propranolol HCl, ryanodine, and verapamil. Atropine sulfate was bought from MANN Research Laboratory (New York, NY). Cimetidine HCl and diphenhydramine HCl were received from Smith Kline and French Laboratories (Welwyn Garden City, Herts, UK). DMSO, thapsigargin (TSG), Gö-6976, and wortmannin were purchased from Calbiochem (La Jolla, CA). Phentolamine methanesulfonate was purchased from CIBA Pharmaceutical (Summit, NJ). Methysergide maleate was purchased from Sandoz Pharmaceuticals (Hanover, NJ). LY-294002 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). All other organic and inorganic chemicals were obtained from Fisher Scientific (Fair Lawn, NJ) and were of the highest purity.

**Calculations and statistical analysis.** The contractile response \((g)\), percentage of maximal KCl-induced contraction, and [Ca\(^{2+}\)]_i were expressed as means \pm SE of the mean. Statistical evaluation of the results was carried out via analysis using the Newman-Keuls test and ANOVA using Scheffé’s contrast test. The results were considered significant at \(P < 0.05\).
are significantly suppressed in both the absence of extracellular Ca²⁺ and the presence of 5 × 10⁻⁶ M verapamil. This is thus in close agreement with the low [Mg²⁺]₀-induced [Ca²⁺]ᵢ changes under the same conditions. Low [Mg²⁺]₀-induced mean tension values in the absence of [Ca²⁺]₀ and varying [Mg²⁺]₀ contractions and in the presence of verapamil are shown in Fig. 1D.

Collectively, these results may implicate a Ca²⁺ influx via mainly a voltage-dependent Ca²⁺-channel pathway in low [Mg²⁺]₀-induced contractions and concomitant [Ca²⁺]ᵢ changes in cerebral vascular muscle cells.

[Ins(1,4,5)P₃]ₐ deficiency-induced contractions and intracellular Ca²⁺ release. Because ryanodine is a “use-dependent” Ca²⁺ release-blocking agent (36), caffeine (10⁻² M) was used first in the smooth muscle cells from canine basilar arteries for an ~3-min period to open the ryanodine channels. Ryanodine (10⁻⁵ M) was then incubated in the tissue baths alone for ~5 min before the subsequent application of Mg²⁺-free medium (in the presence of ryanodine). Caffeine produces a transient [Ca²⁺]ᵢ peak (Fig. 2A). Addition of ryanodine slightly elevates the resting level of [Ca²⁺]ᵢ and markedly suppresses both the transient peak and sustained plateau of [Ca²⁺]ᵢ normally induced by medium containing no [Mg²⁺]₀ (Fig. 2A, compare with Fig. 1A). An Ins(1,4,5)P₃-mediated Ca²⁺-release antagonist, heparin, was next tested in our study. As shown in Fig. 2A, preincubation of the smooth muscle cells with 2 mg/ml heparin can be seen to dramatically reduce the increment in [Ca²⁺]ᵢ (both transient and stable phases). We
then examined the effect of a selective antagonist of the sarcoplasmic reticulum Ca\(^{2+}\) pump, viz. TSG, on low-[Mg\(^{2+}\)]\(_o\) medium-induced \([\text{Ca}^{2+}]_i\) increments. In the absence of external Ca\(^{2+}\), TSG (10\(^{-7}\) M) produces a transient \([\text{Ca}^{2+}]_i\) rise (Fig. 2A). A subsequent challenge with a medium containing 0 mM [Mg\(^{2+}\)]\(_o\) in the presence of TSG (under Ca\(^{2+}\)-free conditions) fails to induce the \([\text{Ca}^{2+}]_i\) peak and produces a very small, time-shortened \([\text{Ca}^{2+}]_i\) plateau (Fig. 2A); calculated mean values for different [Mg\(^{2+}\)]\(_o\) values are shown in Fig. 2B.

In denuded canine basilar arteries, in the presence of extracellular Ca\(^{2+}\), the application of 10\(^{-2}\) M caffeine produces a transient development and rise of contractile tension, which is followed by a rapid return to the resting level. Subsequent challenge with medium containing 0 mM [Mg\(^{2+}\)]\(_o\), after preincubation with 10\(^{-5}\) M ryanodine, produces smaller contractions of the arteries than those of the controls (Fig. 2C). Pretreatment of the arteries with 2.0 mg/ml heparin for \(\sim\)10 min markedly suppresses the contractions (both rapid and sustained components) induced by solutions with low [Mg\(^{2+}\)]\(_o\) (Fig. 2C). In the absence of extracellular Ca\(^{2+}\), TSG (10\(^{-7}\) M) produces a transient contraction and almost abolishes the 0 mM [Mg\(^{2+}\)]\(_o\) solution-induced
contractile tension in the vessels (Fig. 2C); mean values for the varying low [Mg\(^{2+}\)]\(_o\)-induced contractions in the presence of caffeine plus ryanodine, heparin, and TSG minus Ca\(^{2+}\) are shown in Fig. 2D.

These results implicate Ca\(^{2+}\) release [both Ins(1,4,5)P\(_3\) and ryanodine sensitive] in the initial action of low-[Mg\(^{2+}\)]\(_o\) medium on intracellular Ca\(^{2+}\) movement in the cerebral arterial smooth muscle cells.

**PKC antagonists attenuate [Mg\(^{2+}\)]\(_o\) deficiency-induced contractions.** As shown in Fig. 3, A and B, pretreatment of endothelium-denuded canine basilar arteries with Gö-6976 [a PKC-α and PKC-βI-selective antagonist (20)] or Bis [a specific antagonist of PKC (12)] significantly attenuates low-[Mg\(^{2+}\)]\(_o\) medium-induced contractions (both phasic and tonic components) in a concentration-dependent manner. The concentrations producing 50% of the maximal inhibitory effects (IC\(_{50}\) values) for Gö-6976 and Bis are 9.48 ± 0.41 × 10\(^{-7}\) M and 5.03 ± 0.19 × 10\(^{-7}\) M, respectively. Mean values for low [Mg\(^{2+}\)]\(_o\)-induced contractions in the presence of Bis and Gö-6976 are shown in Fig. 3C. After obtaining stable contractions of endothelium-denuded canine basilar arterial rings, which we induced using medium with no [Mg\(^{2+}\)]\(_o\), the cumulative addition of the IC\(_{50}\) amount of either Gö-6976 or Bis to the organ bath results in a reversal of the endothelium-independent contractile responses, in a concentration-dependent manner (data not shown).

**Effects of PKC antagonists on low [Mg\(^{2+}\)]\(_o\)-induced elevations in [Ca\(^{2+}\)].** Figure 4, A and B, illustrate that preincubation of primary cultured smooth muscle cells from canine basilar arteries with either Gö-6976 or Bis effectively prevents both the low-[Mg\(^{2+}\)]\(_o\)-medium-induced rapid increment in [Ca\(^{2+}\)]\(_i\) and the additional rise of [Ca\(^{2+}\)]. Lower steady states and a loss of the rapid peak increment in [Ca\(^{2+}\)]\(_i\) are then seen. Such inhibitory effects of these two antagonists display concentration-dependent effects. The IC\(_{50}\) values for Gö-6976 and Bis for such attenuation of the increases in [Ca\(^{2+}\)]\(_i\) are 7.68 ± 0.23 × 10\(^{-7}\) M and 3.54 ± 0.12 × 10\(^{-7}\) M, respectively, which is consistent with the reduced contractile responses induced by low-[Mg\(^{2+}\)]\(_o\) medium under the same conditions. Mean peak [Ca\(^{2+}\)]\(_i\) values obtained under different low-[Mg\(^{2+}\)]\(_o\) conditions in the absence and presence of the antagonists are shown in Fig. 4C.

**PI3K antagonists attenuate [Mg\(^{2+}\)]\(_o\) deficiency-induced contractions.** Figure 5, A and B, illustrates that the presence of wortmannin or LY-294002 [both are selective antagonists of PI3K (30, 39)] attenuates contractile responses (both phasic and tonic components) of endothelium-denuded canine basilar arteries to low-[Mg\(^{2+}\)]\(_o\) medium in a concentration-dependent manner. The calculated IC\(_{50}\) values for wortmannin and LY-294002 are 8.85 ± 0.36 × 10\(^{-7}\) M and 5.65 ± 0.23 × 10\(^{-6}\) M, respectively. Mean values for varying concentrations of low [Mg\(^{2+}\)]\(_o\)-induced contractions, in the presence of wortmannin or LY-294002, are shown in Fig. 5C. After achieving full contractile responses of the arterial rings to Mg\(^{2+}\)-free medium, cumulative administration of 5 × 10\(^{-7}\) M wortmannin or 10\(^{-6}\) M LY-294002 brings about significant relaxation of the endothelium-independent contractions (data not shown).
PI3K antagonists attenuate $[\text{Mg}^{2+}]_o$ deficiency-induced rises in $[\text{Ca}^{2+}]_i$. Figure 6A demonstrates that preincubation of the cells with $5 \times 10^{-7}$ M wortmannin or $10^{-5}$ M LY-294002 effectively inhibits both the low-$[\text{Mg}^{2+}]_o$ medium-induced transient $[\text{Ca}^{2+}]_i$ peak and the secondary plateau of $[\text{Ca}^{2+}]_i$ (to lower steady states) of basilar arterial smooth muscle cells. The inhibitory effects of these two antagonists show concentration-dependent effects (Fig. 6B). The calculated IC$_{50}$ values for wortmannin and LY-294002 are 7.26 ±
DISCUSSION

The main objectives of the present study were to explore whether the contractile effects of low-[Mg\textsuperscript{2+}]\textsubscript{o}, physiological salt solution on cerebral arteries may be in large measure mediated by Ca\textsuperscript{2+} influx, Ca\textsuperscript{2+} release, and activation of PKC and PI3Ks. We have recently found that low defined serum [Mg\textsuperscript{2+}]\textsubscript{o} levels result in a rapid concentration-dependent rise in [Ca\textsuperscript{2+}]\textsubscript{i} in cultured cerebral arterial smooth muscle concomitant with contraction of these isolated primary vascular smooth muscle cells (8). The low [Mg\textsuperscript{2+}]\textsubscript{o} values used herein (i.e., 0.3–0.6 mM) have been found recently in the serum of patients with stroke, hypertension, and ischemic heart diseases using new specific Mg\textsuperscript{2+}-selective electrodes (1, 3, 8).

The principal trigger for contraction of vascular smooth muscle is an elevation in [Ca\textsuperscript{2+}]\textsubscript{i}. In the present study, medium containing no [Mg\textsuperscript{2+}]\textsubscript{o} produced two phases of Ca\textsuperscript{2+} movement in smooth muscle cells from canine basilar arteries: a transient Ca\textsuperscript{2+} peak and a prolonged Ca\textsuperscript{2+} plateau (Fig. 1A). The transient [Ca\textsuperscript{2+}]\textsubscript{i} peak, induced by medium free of external Mg\textsuperscript{2+}, appears to be derived as a consequence of Ca\textsuperscript{2+} release from intracellular stores because it persists in the absence of extracellular Ca\textsuperscript{2+} and fails to occur after the store is emptied by TSG, a selective antagonist of the sarcoplasmic reticulum Ca\textsuperscript{2+} pump (Fig. 2A) (19).

In addition, we have noticed that both Ins(1,4,5)P\textsubscript{3} and ryanodine-sensitive Ca\textsuperscript{2+} stores are mobilized by low-[Mg\textsuperscript{2+}]\textsubscript{o} medium, because the Ca\textsuperscript{2+} release can be significantly suppressed by both heparin, an Ins(1,4,5)P\textsubscript{3}-sensitive Ca\textsuperscript{2+}-release antagonist (Fig. 2A) (19), and ryanodine, a specific antagonist of ryanodine-sensitive Ca\textsuperscript{2+} release (36). This finding, as far as we are aware, is the first direct and detailed evidence for low-[Mg\textsuperscript{2+}]\textsubscript{o}-induced intracellular Ca\textsuperscript{2+} release in cerebral vascular smooth muscle cells. The prolonged [Ca\textsuperscript{2+}]\textsubscript{i} plateau induced by low-[Mg\textsuperscript{2+}]\textsubscript{o} medium results clearly from Ca\textsuperscript{2+} influx, mainly through voltage-gated Ca\textsuperscript{2+} channels from the extracellular medium, because it disappears in the absence of extracellular Ca\textsuperscript{2+} and can almost be abolished by verapamil, an L-type voltage-gated Ca\textsuperscript{2+}-channel antagonist.

The marked attenuation of the contractile actions of low-[Mg\textsuperscript{2+}]\textsubscript{o} medium on canine basilar arterial smooth muscle in the absence of extracellular Ca\textsuperscript{2+} or that found with employment of verapamil, heparin, caffeine plus ryanodine, or TSG (in the absence of extracellular Ca\textsuperscript{2+}) (Fig. 1, C and D, and Fig. 2, C and D) is in agreement with and well supports the above-mentioned Ca\textsuperscript{2+} movements produced by low-[Mg\textsuperscript{2+}]\textsubscript{o} medium. Collectively, these findings indicate that influx of extracellular Ca\textsuperscript{2+} occurs mainly through voltage-gated Ca\textsuperscript{2+} channels, and that intracellular Ca\textsuperscript{2+} release from Ca\textsuperscript{2+} stores [both Ins(1,4,5)P\textsubscript{3} and ryanodine sensitive] is necessary for these low-[Mg\textsuperscript{2+}]\textsubscript{o} and [Mg\textsuperscript{2+}]\textsubscript{o}-free media-induced contractile responses. Our present findings are also well supported by previous investigations. It has been shown that essential hyper-
tension, stroke, and ischemic heart disease patients exhibit deficits in serum ionized Mg$^{2+}$ and demonstrate significant elevation in the serum ionized Ca$^{2+}$-to-serum-ionized Mg$^{2+}$ ratio, a sign of probable increased vascular tone and vasospasm (1, 5, 8). Exposure of primary cultured single canine cerebral vascular smooth muscle cells, rat aortic smooth muscle cells, and piglet single coronary arterial muscle cells to the low concentrations of serum-ionized Mg$^{2+}$ found in the hypertensive, stroke, and ischemic heart disease patients, e.g., 0.3–0.48 mM, resulted in rapid elevation in cytosolic free Ca$^{2+}$ (1, 5, 8, 9). Coincident with the rise in [Ca$^{2+}]_{i}$, many of the primary single cerebral, aortic, and coronary vascular cells went into spasm (5, 8, 9). Extracellular Mg$^{2+}$ has been shown previously by our group to inhibit Ca$^{2+}$ influx at the vascular smooth muscle membrane (6, 38, 41, 44). Previously, extracellular Mg$^{2+}$ has also been suggested to interfere with Ca$^{2+}$ release from intracellular bound sites in vascular smooth muscle, but this early speculation lacked experimental evidence to support this hypothesis (2, 33, 44). The results of the present study add considerable support to this previously advanced tenet.

Mg$^{2+}$ is known to be a noncompetitive antagonist of $[^{3}H]$(1,4,5)P$_3$ binding and (1,4,5)P$_3$-induced Ca$^{2+}$ release; saturating concentrations of (1,4,5)P$_3$ release less Ca$^{2+}$ from intracellular Ca$^{2+}$ stores at higher concentrations of free Mg$^{2+}$, and Mg$^{2+}$ controls (1,4,5)P$_3$-induced Ca$^{2+}$ release by affecting both the binding of (1,4,5)P$_3$ to its receptor sites and the release of Ca$^{2+}$ via Ins(1,4,5)P$_3$-gated Ca$^{2+}$ channels (40). It is more than likely that decrements in Mg$^{2+}$, would decrease the [Mg$^{2+}$]o in cerebral arterial smooth muscle cells (24, 43). The inhibitory effects of Mg$^{2+}$ on (1,4,5)P$_3$ binding and (1,4,5)P$_3$-induced Ca$^{2+}$ release would be thus attenuated or eliminated. In concert with this, (1,4,5)P$_3$-induced Ca$^{2+}$ release from intracellular storage sites in the cells would be elevated, and the smooth muscle cells would then be expected to undergo contraction.

The concept that PKC plays a pivotal role in tonic contraction of smooth muscle is relatively recent and comes from observations that phorbol esters, which are established activators of PKC, can produce slowly developing sustained contraction in a number of vascular tissues (16). Conventional PKCs, cPKCs (PKC-$\alpha$, PKC-$\beta$, PKC-$\delta$, and PKC-$\gamma$), require Ca$^{2+}$, phospholipids, and diacylglycerol (or phorbol esters). PKC is thought to phosphorylate smooth muscle myosin and myosin light-chain kinase in vitro (21), increase the Ca$^{2+}$ sensitivity of the contractile apparatus (14), and influence the sustained phase of agonist-induced contraction (16). An important observation presented herein is that Gö-6976 (a selective antagonist of PKC-$\alpha$ and PKC-$\beta$ isozymes) and Bis (a specific PKC antagonist) markedly attenuated the low [Mg$^{2+}$]o-induced contractile responses of canine basilar arterial segments, suggesting the probable involvement of PKC activation in such arterial contractions. This contention is supported by the IC$_{50}$ values found herein experimentally. The calculated IC$_{50}$ values for Gö-6976 and Bis were 9.48 ± 0.41 × 10$^{-7}$ M and 5.03 ± 0.19 × 10$^{-7}$ M, respectively. These values are only slightly higher than the reported inhibitory constant ($K_{i}$) values for Gö-6976 (2 × 10$^{-8}$ M (18)) and Bis (1.6–2.0 × 10$^{-8}$ M (37)) for 50% inhibition of PKC. Because 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.

The involvement of PKC in the low [Mg$^{2+}$]o-induced contraction pathway is reinforced by the present findings that in single smooth muscle cells from canine basilar arteries preincubated with PKC antagonists (Gö-6976 and Bis), medium that is [Mg$^{2+}$]o free produces slower and smaller increments in [Ca$^{2+}$]i, which suggests that both Ca$^{2+}$ influx from the extracellular medium and Ca$^{2+}$ release from intracellular stores were inhibited. Similarly, other investigators have demonstrated that: 1) inhibition of PKC activity with staurosporine or chelerythrine can inhibit availability and long opening of L-type Ca$^{2+}$ channels in A7r5 cells (29); 2) inhibition of PKC activity blunts the relative increase in cytosolic free Ca$^{2+}$ in rabbit afferent arterioles in response to ANG II (32); and 3) inhibition of PKC activity can inhibit, completely, the rise in [Ca$^{2+}$]i, induced by alcohol in cerebral vascular smooth muscle cells (45). It is thus tempting to speculate that inhibition of PKC phosphorylation of Ca$^{2+}$ channels in canine basilar arterial smooth muscle cell membranes prevents the necessary rise in [Ca$^{2+}$]i, produced by [Ca$^{2+}$]o influx and intracellular Ca$^{2+}$ release, thus promoting relaxation of low [Mg$^{2+}$]o-vasoconstrictive contractions.

The growing importance of PI3Ks in signal transduction has been pointed out over the past three years (13). A number of proteins have been shown to be associated with PI3Ks following stimulation of cells generally as a result of tyrosine-phosphorylated proteins associating via the PI3K p85 SH2 domains (15). We demonstrate herein for the first time that two potent antagonists of PI3Ks, wortmannin and LY-294002, significantly suppress low [Mg$^{2+}$]o-induced contractions in canine basilar arteries and the concomitant elevation of [Ca$^{2+}$]i, in canine basilar single arterial smooth muscle cells, indicating the likely involvement of products of PI3Ks [e.g., PI(3,4)P$_2$ and PI(3,4,5)P$_3$] in such vessel contractions. This contention gains further support from the experimentally derived IC$_{50}$ values. The calculated IC$_{50}$ values obtained herein for wortmannin and LY-294002 were 8.85 ± 0.36 × 10$^{-7}$ M and 5.65 ± 0.23 × 10$^{-6}$ M, respectively, which are consistent with the reported $K_{i}$ values of these two antagonists for PI3Ks (10$^{-8}$ M and 1.4 × 10$^{-6}$ M) (30, 39). Our present findings are consistent with and well supported by the previous studies for magnesium deficiency and some other vasoactive substances: 1) wortmannin attenuates the contraction of guinea pig gastric longitudinal smooth muscle induced by thrombin and epidermal growth factor (47); 2) the amplitude of contractions of the rat aorta induced by KCl, phenylephrine, and prostaglandin F$_{2\alpha}$ is de-
creased by wortmannin (35); and 3) addition of wortmannin or LY-294002 to HepG2 cells inhibits the release of intracellular Ca\(^{2+}\) induced by coactivation of phospholipase C\(\gamma\) and PI3K (31). We would, however, like to exercise a word of caution in that wortmannin, unlike LY-294002, has also been shown to be a potential inhibitor of myosin light-chain kinase. But it has recently been demonstrated that, in rat aortic smooth muscle, these PI3K inhibitors also attenuate low [Mg\(^{2+}\)]\(_o\)-induced vasoconstrictions (42). It is noteworthy that activation of PI3Ks leads to synthesis of its activities, at least one of which (e.g., PIP\(_3\)) has been pointed out to be a selective activator of PKC-\(\varepsilon\) (27). This may be another pathway by which low-[Mg\(^{2+}\)]\(_o\) could activate PKC in cerebral vascular smooth muscle cells.

From all of the above, our data suggest that although deficiency of extracellular Mg\(^{2+}\) acts directly on vascular smooth muscle cell membranes, it can initiate contraction in canine basilar arterial smooth muscle cells via Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels, intracellular Ca\(^{2+}\) release [both Ins(1,4,5)P\(_3\) and ryanodine sensitive], and activation of PKC and PI3Ks. The subsequent elevation in [Ca\(^{2+}\)]\(_i\) in the vascular smooth muscle cells appears to play a vital role in such contractile responses of the vessels when exposed to Mg\(^{2+}\)-deficient environments.

[Mg\(^{2+}\)]\(_o\)-deficiency-induced cerebral vasocostriction and vasospasm in situ would be expected to hemodynamic lead to reduced brain blood flows, decreased tissue nutrition, decreased tissue oxygenation, and increased cerebral vascular resistance and potentially a stroke. In this context, our laboratory has recently shown that a rapid reduction in cerebral spinal fluid and brain [Mg\(^{2+}\)] will result in vasospasms and rupture of cerebral microvessels in the intact living rat (4). There is a clear, documented, and growing shortfall in magnesium dietary intake, particularly among populations in Western world countries (see reviews, Ref. 5). Moreover, almost 100 stroke (both ischemic and hemorrhagic) patients have been reported recently to exhibit low levels of serum-ionized Mg\(^{2+}\) on admission to the emergency room (8). Since very early studies on the cerebral circulation, it has been suggested that cerebrovasospasm is a trigger in cerebral ischemia and stroke. However, the etiology of cerebrovasospasm remains elusive. The present studies could thus help shed some new light on the etiologies of cerebrovasospasm and diverse cerebral vascular disease states associated with low serum levels of Mg\(^{2+}\) and could be of considerable help in pinpointing potential avenues for pharmacological and therapeutic intervention, particularly in magnesium-deficient states.

This study was supported in part by National Institutes of Health Grant AA-08674 (to B. M. Altura).

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