Role of Ca\(^{2+}\) channels in NE-induced increase in [Ca\(^{2+}\)]\(_i\) and tension in fetal and adult cerebral arteries

WEN LONG, YU ZHAO, LUBO ZHANG, AND LAWRENCE D. LONGO

Center for Perinatal Biology, Departments of Physiology, Pharmacology, and Obstetrics and Gynecology, Loma Linda University, School of Medicine, Loma Linda, California 92350

Long, Wen, Yu Zhao, Lubo Zhang, and Lawrence D. Longo. Role of Ca\(^{2+}\) channels in NE-induced increase in [Ca\(^{2+}\)]\(_i\) and tension in fetal and adult cerebral arteries. Am. J. Physiol. 277 (Regulatory Integrative Comp. Physiol. 46): R286–R294, 1999.—In vascular smooth muscle, elevation of agonist-induced intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) occurs via both Ca\(^{2+}\) release from intracellular stores and Ca\(^{2+}\) influx across the plasma membrane. In the cerebral vasculature of the fetus and adult the relative roles of these mechanisms have not been defined. To test the hypothesis that plasma membrane L-type and receptor-operated Ca\(^{2+}\) channels play a key role in NE-induced vasoconstriction via alterations in plasma membrane Ca\(^{2+}\) flux and that this may change with developmental age, we performed the following study. In main branch middle cerebral arteries (MCA) from near-term fetal (~140 days) and nonpregnant adult sheep, we quantified NE-induced responses of vascular tension and [Ca\(^{2+}\)]\(_i\) (by use of fura 2) under standard conditions in response to several Ca\(^{2+}\) channel blockers and in response to zero extracellular Ca\(^{2+}\). In fetal and adult MCA, maximal NE-induced tensions (g) were 0.91 ± 0.12 (n = 10) and 1.61 ± 0.13 (n = 12), respectively. The pD\(_2\) values for NE-induced tension were both 6.0 ± 0.1, whereas the fetal and adult maximum responses (\%K\(_{\text{max}}\)) were 107 ± 16 and 119 ± 7, respectively. The fetal and adult pD\(_2\) values for NE-induced increase of [Ca\(^{2+}\)]\(_i\) were 6.2 ± 0.1 and 6.4 ± 0.1, respectively, whereas maximum [Ca\(^{2+}\)]\(_i\) responses were 81 ± 9 and 103 ± 15% of K\(_{\text{max}}\), respectively. After 10\(^{-5}\) M NE-induced contraction, nifedipine resulted in dose-dependent decrease in vessel tone and [Ca\(^{2+}\)]\(_i\), with pC\(_{50}\) values for fetal and adult tensions of 7.3 ± 0.1 and 6.6 ± 0.1, respectively (P < 0.01; n = 4), whereas pC\(_{50}\) for [Ca\(^{2+}\)]\(_i\) responses were 7.2 ± 0.1 and 6.9 ± 0.1, respectively. The pC\(_{50}\) values for tension for diltiazem and verapamil were somewhat lower but showed a similar relationship. The receptor-operated Ca\(^{2+}\) channel blocker 2-nitro-4-carboxyphenyl-N,N-diaphenyl carbamate showed little effect on NE-induced vessel contractility or [Ca\(^{2+}\)]\(_i\). In the absence of extracellular Ca\(^{2+}\) for 2 min, 10\(^{-5}\) M NE resulted in markedly attenuated responses of adult MCA tension and [Ca\(^{2+}\)]\(_i\) to 39 ± 7 and 73 ± 9% of control values (n = 4). For fetal MCA, exposure to extracellular Ca\(^{2+}\) concentration resulted in essentially no contractile responses (n = 4). Similar blunting of NE-induced tension and [Ca\(^{2+}\)]\(_i\) was seen in response to 10\(^{-3}\) M lanthanum ion. These findings provide evidence to suggest that especially in fetal, but also in adult, ovine MCA, Ca\(^{2+}\) flux via L-type calcium channels plays a key role in NE-induced contraction. In contrast, Ca\(^{2+}\) flux via receptor-operated Ca\(^{2+}\) channels is of less importance. This developmental difference in the role of cerebrovascular plasma membrane Ca\(^{2+}\) channels may be an important association with increased Ca\(^{2+}\) sensitivity of the fetal vessels.

IN CEREBROVASCULAR SMOOTH muscle, agonist-induced elevation of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) occurs through G protein-coupled mechanisms involving Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) (24). Stimulation of \(\alpha_1\)-adrenergic receptors (\(\alpha_1\)-AR) by norepinephrine (NE) results in production of inositol 1,4,5-trisphosphate [Ins(1,4,5)P\(_3\)], which stimulates SR Ca\(^{2+}\) release via Ins(1,4,5)P\(_3\)-receptors (6, 27, 28). In most smooth muscle, NE also induces Ca\(^{2+}\) influx through both voltage-gated and receptor-operated channels (19, 29), and electrophysiological studies have demonstrated the importance of membrane potential in the regulation of cerebral vascular tone (21, 29). Nonetheless, the extent to which L-type voltage-gated and/or receptor-operated Ca\(^{2+}\) channels are important in NE-induced contraction of cerebral arteries is not established. Neither is the role of these Ca\(^{2+}\) channels in cerebral arteries of the fetus established, nor how their function changes with development.

We and others have demonstrated that adrenergic-mediated cerebrovascular reactivity changes dramatically with development from fetus to newborn to adult (25, 27, 31, 41). Changes in membrane \(\alpha_1\)-AR density (27), Ins(1,4,5)P\(_3\) synthesis (27), and Ins(1,4,5)P\(_3\) receptor density (43) appear to be involved in developmental differences in NE-induced contractility. However, changes in these factors alone cannot explain fully the changes in vascular contractility (26, 27). Thus differences in related intracellular signaling mechanisms in addition to membrane receptor binding events appear to play an important role in the genesis of age-related differences in cerebrovascular tone.

The present studies test the hypotheses that Ca\(^{2+}\) flux via L-type plasma membrane Ca\(^{2+}\) channels and/or receptor-operated Ca\(^{2+}\) channels plays a key role in NE-induced contraction of cerebral arteries and that the role of these channels is different in the fetus from the adult.

METHODS

Experimental animals and tissues. For these studies, we used middle cerebral arteries (MCA) from normoxic control near-term fetuses (~140 days) and nonpregnant adult sheep (~2 yr) obtained from Nebeker Ranch (Lancaster, CA) that had been maintained near sea level (~300 m). The ewes were anesthetized and killed with 100 mg/kg intravenous pentobarbital sodium, following which we obtained isolated cerebral artery segments. We have shown that this method of death

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
that no significant effect on vessel reactivity compared with use of other anesthetic agents (31). All studies were performed in isolated main branch MCA (adult ~450 μm; fetus ~350 μm) cleared of adipose and connective tissue, as previously described (27, 28). To avoid the complication of endothelial-mediated effects, we removed the endothelium by carefully inserting a small wire three times (27, 28). To confirm endothelium removal, we contracted the vessel with 10^{-3} M 5-hydroxytryptamine and at the plateau added 10^{-6} M ADP. Vessels that relaxed >20% after this treatment were rejected for further study. Cerebral arteries were used immediately for simultaneous measurements of [Ca^{2+}]i and tension (36). Unless otherwise noted, all chemical compounds were purchased from Sigma (St. Louis, MO). Contractility measurements. We cut the MCA into rings of 2 mm in length, mounted them on two tungsten wires (0.13 mm diameter; A-M Systems, Carlsborg, WA), attaching one wire to an isometric force transducer (Kent Scientific, Litchfield, CT) and the other to a post attached to a micrometer used to vary resting tension in a 5-ml tissue bath mounted on a jar: CAF-110 intracellular Ca^{2+} analyzer (Jasco, Easton, MD). We equilibrated the arteries at 38°C for 30 min in a bicarbonate Krebs solution (pH 7.4) containing (in mM) 115.2 NaCl, 22.14 NaHCO_3, 7.88 d-glucose, 4.7 KCl, 1.18 KH_2PO_4, 1.16 MgSO_4, 1.80 CaCl_2, 0.114 ascorbic acid, and 0.027 Na_2 EDTA continuously bubbled with 95% O_2-5% CO_2. We obtained micrometer and therefore inside diameter measurements for each arterial segment under unstressed conditions (~0.1 g tension) and similarly at optimum resting tension. To establish optimal resting tension for the studies, we performed K^+-induced contractility experiments with resting tension ranging from 0.2 to 0.5 g (n = 4 each). In both fetal and adult arteries, optimal results for both tension and fluorescence ratio were obtained at 0.3 g, the value chosen for the present studies. The vessel inside diameter measurements, in combination with measurements of vessel wall thickness, length, and potassium-induced force, enabled calculation of force per unit cross-sectional area, as previously described (30, 31).

Intracellular calcium measurement in smooth muscle. MCA rings were equilibrated under 0.3 g tension at 25°C for 40 min before loading with the acetoxymethyl ester of fura 2 (fura 2/AM; Molecular Probes, Eugene, OR), a fluorescent Ca^{2+} indicator (15). To sequester the dye in the cytosol, loading of the smooth muscle with the fura 2 was performed by incubating it for 4 h at 25°C in Krebs buffer containing 5 μM fura 2, 0.17% DMSO, 0.02% Cremophore EL, and 0.1% BSA. After loading, the vessel ring was washed with Krebs buffer five times in 30 min to allow for complete hydrolysis of the fura 2 ester groups by endogenous esterase. Fura 2 fluorescence and force were measured simultaneously at 38°C. Vessels were illuminated alternatively (125 Hz) at excitation wavelengths of 340 and 380 nm by means of two monochromators in the light path of a 75 W xenon lamp. Tissue fluorescence emission was measured at 510 nm by a photomultiplier. The fluorescence intensity at each excitation wavelength (F_{340} and F_{380}) and the ratio of these fluorescence values (R_{340/380}) were recorded with a time constant of 250 ms and stored with the force signal.

When fura 2 is present only as free acid there is an inverse change in fluorescence at 340 and 380 nm excitation. At the end of the study we determined the maximum R_{340/380} (R_{max}) by stimulation with 10^{-4} M NE and 10^{-5} M ionomycin in a phosphate-free, bicarbonate-free 120 mM K^+, 5 mM Ca^{2+} Krebs buffer. The minimum R_{340/380} (R_{min}) was elicited by chelating Ca^{2+} with 50 mM EGTA. R_{max} and R_{min} were used to normalize changes in fluorescence ratios obtained in different preparations. Because of uncertainty about the dissociation constant for [Ca^{2+}], under intracellular conditions, we used R_{340/380} as an indicator of relative changes in [Ca^{2+}], rather than calculating actual [Ca^{2+}]. Although some investigators may prefer the transformation of fluorescence to [Ca^{2+}], in tissues such as cerebral arteries the presentation of the ratio is less ambiguous.

We first contracted the arterial segment by adding 120 mM isosonic potassium chloride to the Krebs buffer. After peak tension was reached, we washed the artery with normal sodium Krebs solution and allowed it to return to baseline tension for 15 min. Subsequent contractions were then induced with NE at half log doses (10^{-8}–10^{-4} M). In earlier studies in ovine cerebral arteries, we have shown this to produce maximal stable contractions (31). During all contractility experiments, we continuously digitized, normalized, and recorded contractile tensions and the fluorescence ratio (R_{340/380}) using an online computer. For all vessels we evaluated the ratio for tension and fluorescence ratio by measuring the maximum peak height, and expressing it as percent K_{max} (a measure of "efficacy") and calculated pD_2 (the negative logarithm of the EC_{50}, or half-maximal concentration, for NE and an index of tissue "sensitivity" or "potency").

Role of Ca^{2+} channel blockers. To determine the role of L-type Ca^{2+} channels in MCA arteries (n = 4 each group), we quantified NE-induced tension and [Ca^{2+}]i after blockade by each of three groups of agents as follows: dihydropryridines (e.g., nifedipine, 10^{-9}–10^{-5} M), phenylalkylamines (e.g., verapamil, 10^{-9}–10^{-5} M), and benzothiazepines (e.g., diltiazem, 10^{-9}–10^{-5} M). For each of these agents we first quantified the [Ca^{2+}]i and tension in response to 10^{-5} M NE. After a 40-min washout and reequilibration, we repeated the experiment after administration of 10^{-5} M NE to increase [Ca^{2+}]i, and Emax. Then, at the plateau of the response, we added increasing concentrations of the Ca^{2+} channel blocker to examine the inhibition of the [Ca^{2+}]i, signal and tension. For each compound we determined threshold concentration, EC_{50} or pEC_{50} (negative logarithm of the half-maximal inhibitory concentration), and maximum response. We also quantified these parameters after the addition of the Ca^{2+} channel antagonist lanthanum ion (10^{-3} M LaCl_3) for 3 min (34, 42).

Because LaCl_3 forms an La_2(CO_3)_{3-} precipitate in Krebs buffer, those studies of La^{3+} effects were conducted in HEPES-buffered salt solution containing (in mM) 120 NaCl, 6.0 KCl, 1.8 CaCl_2, 1.2 MgCl_2, 11.4 d-glucose, and 5.0 HEPES. pH was adjusted to 7.4 by titration with NaOH, and this solution was gassed with 100% O_2. To examine the role of receptor-operated or nonselective cation channels, we used the blocker 2-nitro-4 carboxyphenyl-N,N-diphenyl carbamate (NCDC) (34).

Role of extracellular Ca^{2+}. To determine the role of extracellular Ca^{2+} in traversing calcium channels, we measured [Ca^{2+}]i and tension after NE stimulation at the value of half-maximal contraction (pD_2) after vessels had been exposed to calcium-free (~10^{-6} M of Ca^{2+} with EGTA 5 × 10^{-4} M to chelate Ca^{2+}) Krebs buffer for 2 min (n = 4 each). Again, we evaluated the contractile response for tension and fluorescence ratio by measuring the maximum peak height and expressing it as percent K_{max}. We quantified the duration of contractile response by measuring the peak width (s) at 50% of peak height, arbitrarily defining this at t_{50}.

Statistical analysis. All values were calculated as means ± SE. In all cases, n refers to the number of vessel segments (which corresponds to the number of animals) studied. Because of the nature of these studies, several statistical tests were used to test for significant differences. For testing differences between two groups, we used a simple unpaired Student’s t-test. For multiple comparisons, one- and two-way analysis of variance (ANOVA) was performed.
ANOVA (vessel, age) coupled with Duncan's multiple range test was used. Where appropriate, we used ANOVA with repeated measures. The correlation between tension and fluorescence ratio was determined by linear regression (8). A P value of <0.05 was considered significant.

RESULTS

Contractile and [Ca$^{2+}$], responses to potassium and norepinephrine. Figure 1A shows the NE-induced contractile tension (g) changes of main branch MCA in response to increasing NE concentration under control conditions for fetus and adult. Fetal and adult maximal K$^+$-induced tensions were 0.79 ± 0.06 (n = 10) and 1.43 ± 0.08 g (n = 12), respectively. The corresponding maximal NE-induced tensions (g) were 0.91 ± 0.12 and 1.61 ± 0.13, respectively (P < 0.01), whereas the corresponding stress values (10^6 dynes/cm²) were 0.36 ± 0.05 and 0.41 ± 0.05, respectively. The corresponding pD$_2$ values were 6.0 ± 0.1 for each. Figure 1B shows the NE-induced tension as percent of maximal response. The NE maximum values as %$K_{max}$ for fetus and adult MCA were 107 ± 16 and 119 ± 7, respectively (Fig. 1, inset). The Hill coefficients of the fetal and adult NE dose-response curves were 1.03 ± 0.12 and 1.02 ± 0.14, respectively.

Figure 1C shows the fura 2 fluorescence ratios (R$_{340/380}$), a measure of free Ca$^{2+}$ concentration in response to NE, for fetal and adult MCA. For adult MCA, NE produced dose-dependent increases of [Ca$^{2+}$]i with a pD$_2$ value of 6.4 ± 0.1 and %$K_{max}$ of 103 ± 15 (Fig. 1, inset). The NE-induced pD$_2$ value for the fluorescence ratio for fetal MCA was 6.2 ± 0.1, whereas the %$K_{max}$ was 81 ± 9. Neither the pD$_2$ values nor the maximal K$^+$-induced tensions (%$K_{max}$) were significantly different between fetus and adult. The relation of NE-induced tension (%$K_{max}$) to fluorescence ratio (%$K_{max}$) for fetal and adult MCA were plotted, the slopes being 1.66 ± 0.1 (r$^2$ = 0.90) and 1.40 ± 0.1 (r$^2$ = 0.78), respectively (P < 0.05).

Role of “L-type” Ca$^{2+}$ channels. To determine the role of L-type Ca$^{2+}$ channels in fetal and adult MCA contractile responses, we quantified NE-induced tensions and [Ca$^{2+}$]i after administration of three L-type Ca$^{2+}$ channel blockers. As shown in Fig. 2A, in fetal and adult MCA (n = 4 each), nifedipine administered in half-log doses from 10$^{-9}$ to 10$^{-5}$ M produced dose-dependent inhibition of contraction to 10$^{-5}$ M NE, with pIC$_{50}$ values of 7.3 ± 0.1 and 6.6 ± 0.1, respectively (P < 0.01). As shown in Fig. 2B, in fetal and adult MCA, the pIC$_{50}$ values for the inhibition of NE-induced [Ca$^{2+}$]i response to nifedipine were 7.2 ± 0.1 and 6.9 ± 0.1, respectively (P < 0.05) (see Table 1).

In response to verapamil, NE-induced vascular tension in fetal and adult MCA (n = 4 each) was also inhibited, with pIC$_{50}$ values of 6.7 ± 0.1 and 6.1 ± 0.1, respectively (P < 0.01) (Table 1). The pIC$_{50}$ values for verapamil-induced inhibition of the NE-induced [Ca$^{2+}$]i in fetal and adult MCA were each 5.9 ± 0.1 (Table 1). Diltiazem also inhibited NE-induced vascular tension in fetal and adult MCA (n = 4 each), with pIC$_{50}$ values of 6.2 ± 0.1 and 5.2 ± 0.1, respectively (P < 0.01). For diltiazem, the pIC$_{50}$ values for [Ca$^{2+}$]i in fetal and adult MCA were 5.8 ± 0.1 and 5.4 ± 0.1, respectively (P < 0.05) (Table 1).

Role of receptor-operated Ca$^{2+}$ channels. To evaluate the role of receptor-operated Ca$^{2+}$ channels in fetal and
Role of extracellular \([\text{Ca}^{2+}]\). To determine the role of extracellular \([\text{Ca}^{2+}]\) in NE-induced contraction, we exposed both fetal and adult MCA to zero extracellular \([\text{Ca}^{2+}]\) for 2 min before contraction with \(10^{-5}\) M NE. As shown in Fig. 3, A and B, in response to \(10^{-5}\) M NE in the presence of 1.8 mM extracellular \([\text{Ca}^{2+}]\) (\([\text{Ca}^{2+}]_o\)), adult MCA showed a typical rise in vascular tension with a sustained plateau phase and increase in \([\text{Ca}^{2+}]_i\). In the absence of \([\text{Ca}^{2+}]_o\), however (\(n = 4\)), tension (Fig. 3C) showed an initial rise to only 39 ± 7% of that peak tension in the presence of \([\text{Ca}^{2+}]\). This was followed by a rapid decline to baseline values, rather than maintaining a plateau. The \(t_1/2\) for the contraction was 60 ± 12 s. The peak fluorescence ratio under these conditions (Fig. 3D) was 73 ± 8% of the peak ratio in the presence of \([\text{Ca}^{2+}]\) (Fig. 3B); however, the \(t_1/2\) was 23 ± 8.0 s. As predicted and as seen in Fig. 3D, after the change to \([\text{Ca}^{2+}]_o\)-free buffer, baseline fluorescence ratio decreased significantly (−0.10 ± 0.03) over the 2-min period. In contrast, baseline tension remained relatively constant. When the adult artery was exposed to zero \([\text{Ca}^{2+}]_o\) for 10 rather than 2 min (\(n = 3\)), there was no detectable NE-induced response of either tension or \([\text{Ca}^{2+}]_i\).

Figure 4, A and B, shows the \(10^{-5}\) M NE-induced increase in tension and \([\text{Ca}^{2+}]_i\) in fetal MCA in the presence of 1.8 mM \([\text{Ca}^{2+}]_o\). Tension and \([\text{Ca}^{2+}]_i\) plateaued in a manner similar to that of the adult. However, in contrast to the adult, in the absence of extracellular \([\text{Ca}^{2+}]\) (\(n = 4\); Fig. 4C), after NE stimulation both the initial rise in tension was much less (−6 ± 2% that of control) and the plateau was markedly attenuated. \([\text{Ca}^{2+}]_i\) (Fig. 4D) showed a similar attenuated response (12 ± 3% of control). Again, after the change to \([\text{Ca}^{2+}]_o\)-free buffer, the baseline fluorescence ratio decreased significantly (−0.12 ± 0.02).

After exposure to \([\text{Ca}^{2+}]_o\)-free buffer, the vessel segments were allowed to reequilibrate in \([\text{Ca}^{2+}]_o\) Krebs buffer for 40 min and then were replaced in \([\text{Ca}^{2+}]_o\)-free buffer for 2 min and NE exposure was repeated. Under these conditions, the contractile and fluorescence ratio responses to the second agonist exposure were somewhat less than that of the first. For fetal and adult MCA, NE-induced tensions (%K\(_{\text{max}}\)) were 5 ± 1 and 22 ± 8, respectively. Fluorescence values (%K\(_{\text{max}}\)) were 8 ± 1 and 14 ± 4, respectively. For adult MCA, the \(t_1/2\) for these responses were only half of that of the first NE exposure, e.g., 26 ± 3 and 13 ± 1 for tension and fluorescence ratio, respectively. For fetal MCA, the \(t_1/2\) values were 29 ± 7 and 20 ± 2, respectively.

To explore further the role of extracellular \([\text{Ca}^{2+}]\) channels in contraction of fetal and adult cerebral arteries, before giving \(10^{-5}\) M NE we administered lanthanum ion (\(10^{-3}\) M LaCl\(_3\)) for 2 min to irreversibly block the several types of plasmalemmal \([\text{Ca}^{2+}]_i\) channels (\(n = 4\) each). As shown in Fig. 5, A and B, in adult MCA after La\(^{3+}\) pretreatment, the contractile and \([\text{Ca}^{2+}]_i\) responses to NE stimulation were significantly attenuated (43 ± 7% for each peak response). For fetal MCA in

---

**Table 1. Negative logarithms of pIC\(_{50}\) values of \([\text{Ca}^{2+}]_i\) channel blockers**

<table>
<thead>
<tr>
<th></th>
<th>Tension</th>
<th>[Ca(^{2+})]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fetus</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>7.3 ± 0.1†</td>
<td>6.6 ± 0.1</td>
</tr>
<tr>
<td>Verapamil</td>
<td>6.7 ± 0.1†</td>
<td>6.1 ± 0.1</td>
</tr>
<tr>
<td>Diltiazam</td>
<td>6.2 ± 0.1†</td>
<td>5.2 ± 0.1</td>
</tr>
<tr>
<td>NCDC</td>
<td>4.3 ± 0.1*</td>
<td>4.8 ± 0.1</td>
</tr>
</tbody>
</table>

Values are means ± SE. pIC\(_{50}\), 50% inhibitory concentration; NCDC, 2-nitro-4-carboxyphenyl-N,N-diphenyl carbamate. *P < 0.05, †P < 0.01, different from adult.
the presence of La³⁺ (Fig. 5, C and D), there was essentially no NE-induced response of either tension or [Ca²⁺].

**DISCUSSION**

The present studies are the first to present simultaneous measurements of [Ca²⁺] and tensions of cerebral arteries of fetus and adult and their responses to NE. As such they offer several important observations. First is the significant role of extracellular Ca²⁺ in NE-induced contractility in fetal and adult MCA, with the extracellular Ca²⁺ dependence for the fetus being greater than that for the adult. For NE-induced tension and fluorescence ratios in fetal and adult MCA, neither the pD₂ values nor the maximum tension and fluorescence ratios expressed as %Kₘₚ were significantly different from one another (Fig. 1). Nonetheless, in terms of %Kₘₚ, tension as a function of %Kₘₚ fluorescence ratio (i.e., [Ca²⁺]), the fetal MCA showed significantly increased sensitivity compared with that of the adult. Second, fetal MCA showed a dramatically greater sensitivity to voltage-gated Ca²⁺ channel blockade compared with that of the adult (Fig. 2 and Table 1). This was especially evident for nifedipine (Fig. 2), but was also true for verapamil and diltiazem (Table 1). By comparison, both fetal and adult MCA were relatively insensitive to NCDC blockade of receptor-operated channels. In agreement with this, MCA of both fetus and adult showed great dependence on extracellular Ca²⁺ for contraction, with that for the fetus being greater than that of the adult (Figs. 3–5). In the absence of extracellular Ca²⁺, the fetal MCA peak contractile responses and fluorescence ratios were more markedly attenuated and the plateaus were not sustained in comparison with those of the adult. Determination of the [Ca²⁺] and tension in the presence of Ca²⁺ channel blockers and zero [Ca²⁺]₀ demonstrates the important role of Ca²⁺ channels and other non-Ins(1,4,5)P₃ mechanisms in NE-induced contraction in both fetal and adult cerebral arteries.

These differences between fetal and adult calcium handling mechanisms fit with previous studies from our group and others on developmental differences in cerebral artery contractility. Fetal arteries develop less tone but have greater aminergic activity than those of adult (31), newborn MCA require more transmembrane calcium uptake than the adult (44), fetal arteries show greater calcium sensitivity (1, 3), and fetal arteries rely
less on Ins(1,4,5)P_3-mediated contractile mechanisms (27, 43).

Vascular tension and [Ca^{2+}]_{i}, is a key determinant of vascular contractility. Ca^{2+} flux across the plasma membrane from the extracellular space occurs via a resting Ca^{2+} leak and the several types of Ca^{2+} channels: the voltage dependent or L-type, which is the most common, receptor-operated channels, stretch-activated channels, and others.

Both fetal and adult MCA exhibited a highly reproducible K^+- and NE-induced dose-dependent increase in tension and [Ca^{2+}]_{i}. In a previous study, we reported the pD_2 for ovine fetal and adult MCA as 6.3 ± 0.1 and 6.2 ± 0.1, respectively (27). Also, in a study of NE responsiveness of second- and fourth-order MCA of newborn lambs vs. adult sheep, Elliott and Pearce (13) demonstrated markedly increased tissue sensitivity (e.g., pD_2) in fourth-order MCA of younger animals, but not the second-order branches. The pD_2 values for fourth-order newborn and adult MCA were 6.2 ± 0.2 and 5.2 ± 0.2, respectively, whereas in second-order branches the values were 5.9 ± 0.2 and 5.5 ± 0.2, respectively (13). These values compare to the present values of 6.0 ± 0.1 in each vessel. The previous studies used larger vessel segments (~5 mm length) compared with the present study (2 mm), as well as somewhat different vessel bath ionic concentrations. In addition, Akopov and coworkers (1) reported increased Ca^{2+} sensitivity of common carotid and basilar arteries in 9-day-old rabbits compared with adults. These changes appeared to be associated with a G protein-dependent mechanism (1). The present results of greater NE sensitivity of fetal, compared with adult, MCA are in agreement with these studies. Of interest, and in contrast to findings of studies of some workers (7, 40), Elliott and Pearce (13) showed no change in NE sensitivity as a function of cerebral artery diameter in a given age group. This was also the case in the baboon (16).

Because of the prolonged duration of this study (3 h after loading with fura 2), to examine the possibility of desensitization to NE and to determine the reproducibility of the NE-induced contraction results over time, we repeated the NE dose response three times (with 5 min for dose response followed by 40 min washout and reequilibration in Krebs buffer) over a total period of ~180 min (n = 5). At 10^{-6} M (approximately EC_{50})...
NE-induced tension and fluorescence ratio each decreased (~20% by the second and third contractions).

Some may argue that the [Ca\textsuperscript{2+}] data should be presented in absolute values rather than as the fluorescence ratio. As noted above, because of uncertainty about K_D for [Ca\textsuperscript{2+}], use of the ratio is less ambiguous. Nonetheless, regardless of the method used, the conclusions would be identical.

Plasma membrane calcium channels and contractility. In several tissues, calcium handling has been shown to vary with developmental age. For instance, neonatal myocardium is much more dependent on transmembrane Ca\textsuperscript{2+} flux and less dependent on Ca\textsuperscript{2+} release from intracellular storage sites than adult myocardium (11, 20). This agrees with the present study. In addition, several lines of evidence indicate that the volume of sarcoplasmic reticulum (i.e., Ca\textsuperscript{2+} storage site) in vascular smooth muscle is less in immature tissues (38) as well as in the smaller arteries (4, 37).

In addition to releasing compartmentalized Ca\textsuperscript{2+} from intracellular stores, vascular smooth muscle can increase [Ca\textsuperscript{2+}] by evoking Ca\textsuperscript{2+} influx from the extracellular space via several types of Ca\textsuperscript{2+} channels: voltage-dependent L-type, receptor operated, stretch activated, and/or others (17, 19, 29). Other investigators (1, 4, 21, 35) have noted that cerebral arteries are more dependent on Ca\textsuperscript{2+} flux through voltage-gated plasma membrane channels than arteries from other vascular beds. This is especially true for smaller cerebral arteries (2). Results of the present study also agree with findings that cerebral arteries of the newborn sheep are more dependent on extracellular Ca\textsuperscript{2+} than those of the adult (44). In rat tail artery (18) and rat skeletal muscle (32) membrane electrical properties change with age. This suggests that the voltage-current relations for voltage-sensitive Ca\textsuperscript{2+} channels may also differ in fetal vs. adult cerebral arteries, and this needs to be explored.

L-type calcium channel blockers. Vascular L-type Ca\textsuperscript{2+} channels can be blocked by several classes of compounds: dihydropyridines, phenylalkylamines, and benzothiazepines (10, 14, 33). As demonstrated in the present study, the dihydropyridine nifedipine was much more effective in blocking fetal MCA (pIC\textsubscript{50} for tension = 7.3 \pm 0.1), compared with that of the adult (pIC\textsubscript{50} = 6.6 \pm 0.1). Nonetheless, both the phenylalkamine verapamil (fetal and adult pIC\textsubscript{50} = 6.7 \pm 0.1 and 6.1 \pm 0.1, respectively) and the benzothiazepine diltiazem (fetal and adult pIC\textsubscript{50} = 6.2 \pm 0.1 and 5.2 \pm 0.1).
respectively, see Table 1) were markedly effective in this regard. These findings further support the idea that fetal cerebral arteries are more dependent on extracellular Ca\(^{2+}\) than are those of the adult. In addition, the data on inhibition of contractility suggest that in the presence of the Ca\(^{2+}\) channel blockers the Ca\(^{2+}\) sensitivity of fetal MCA is greater than that of the adult, e.g., greater inhibition of tone for a given [Ca\(^{2+}\)], (see Fig. 2).

We caution that the age-related differences in sensitivity of cerebral artery to blockers of L-type voltage-operated Ca\(^{2+}\) channels, may not necessarily reflect differences in the relative roles of these channels in transmembrane Ca\(^{2+}\) flux or the development of tension. A number of related processes may relate to the latter. In addition, the density of these channels and their molecular structure may differ considerably between fetus and adult. Thus these several factors may contribute to an apparent developmental change in sensitivity to Ca\(^{2+}\) channel blockers. We currently are exploring these possibilities.

Receptor-operated and other calcium channel blockers. Several studies have demonstrated a class of Ca\(^{2+}\)-permeable receptor-activated, nonselective cation channels in vascular smooth muscle, which are insensitive to the classical Ca\(^{2+}\) channel blockers (5, 23, 34). To date, a pharmacological classification of these channels has not been possible because of the lack of specific and potent pharmacological blockers. Perhaps surprisingly, in the present studies in both adult and fetal MCA, except at high concentrations, the receptor-operated Ca\(^{2+}\) channel blocker NCDC (34) displayed little effect on NE-induced contractility and thus presumably on transmembrane Ca\(^{2+}\) flux.

As noted above, the blockade of essentially all plasma membrane Ca\(^{2+}\) channels by lanthanum ions, with resultant diminution of NE-induced increases in tension and [Ca\(^{2+}\)]\(_i\) (Fig. 5), confirms the key role of plasmalemml calcium channels in cerebral artery contractile responses. This is compatible with findings in human uterine artery (22), rabbit renal artery (42), rat aorta (12), and dog coronary artery (39). In addition, it further emphasizes the dependence of cerebral arteries on extracellular Ca\(^{2+}\) because of small intracellular stores (9), this being even more true for the fetus than the adult.

Perspectives

NE-induced vascular tension (or force or stress) is an index of \(\alpha\)-adrenergic receptor-mediated smooth muscle activation. In vitro this represents the integrated output of several complex signal transduction cascades that converge on [Ca\(^{2+}\)]\(_i\) and myofilaments. Previously, we demonstrated major differences between fetal and adult cerebral artery \(\alpha_1\)-AR density and NE-induced Ins(1,4,5)P\(_3\) responses (27), Ins(1,4,5)P\(_3\)-Receptor density (43), and other factors (31). However, none of these differences fully account for the developmental differences in NE-induced contractility.

The present studies are the first to quantify simultaneously [Ca\(^{2+}\)]\(_i\) and tension in fetal and adult cerebral arteries and to demonstrate in these vessels that plasma membrane L-type (but not receptor operated) Ca\(^{2+}\) channels play a key role in the NE-induced contractile response. Fetal cerebral arteries demonstrate almost total dependence on extracellular Ca\(^{2+}\). This dependence on extracellular Ca\(^{2+}\) is associated with fetal cerebral arteries also having increased Ca\(^{2+}\) sensitivity compared with those of the adult. For both fetus and adult, plasma membrane Ca\(^{2+}\) channels are probably an important regulating element of cerebral artery contraction in vivo as well as in vitro. Nonetheless, the role of changes with development in the many other elements of the signal transduction cascade that modulate NE-induced Ca\(^{2+}\) flux and contraction needs to be explored. These include the influence of \(\alpha_3\)-AR subtypes, intracellular sarcoplasmatic reticulum Ca\(^{2+}\) stores, protein kinase C and protein kinase A, enzyme phosphorylation, the several K\(^+\) channels, and so forth. These topics are the subject of current studies in the elucidation of the mechanisms of adrenergic-mediated cerebral vascular contraction and their change with development.

We thank Brenda Kreutzer for preparing the manuscript. This work was supported by National Institutes of Health Grants HD/HL-03807 and PO1-HD-31226 to L. D. Longo.

Address for reprint requests and other correspondence: L. D. Longo, Center for Perinatal Biology, Loma Linda University, School of Medicine, Loma Linda, CA 92350 (E-mail: llongo@som.llu.edu).

Received 14 August 1998; accepted in final form 31 March 1999.

REFERENCES

CEREBRAL ARTERY PLASMAMEMAL Ca2+ CHANNELS


