Myogenic contractility is more dependent on myofilament calcium sensitization in term fetal than adult ovine cerebral arteries

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Myogenic contractility is more dependent on myofilament calcium sensitization in term fetal than adult ovine cerebral arteries. Am J Physiol Heart Circ Physiol 293: H548–H556, 2007. First published March 23, 2007; doi:10.1152/ajpheart.00134.2007.—Regulation of cytosolic calcium and myofilament calcium sensitivity varies considerably with postnatal age in cerebral arteries. Because these mechanisms also govern myogenic tone, the present study used graded stretch to examine the hypothesis that myogenic tone is less dependent on calcium influx and more dependent on myofilament calcium sensitization in term fetal compared with adult cerebral arteries. Term fetal and adult posterior communicating cerebral arteries exhibited similar myogenic responses, with peak tensions averaging 24 and 26% of maximum contractile force produced in any given tissue in response to an isotonic Krebs buffer containing 122 mM K+ (Kmax) at optimum stretch ratios (working diameter/unstressed diameter) of 2.19 and 2.23, respectively. Graded stretch increased cytosolic Ca2+ concentration at stretch ratios >2.0 in adult arteries, but increased Ca2+ concentration only at stretch ratios >2.3 in fetal arteries. In permeabilized arteries, myogenic tone peaked at a stretch ratio of 2.1 in both fetal and adult arteries. The fetal %Kmax values at peak myogenic tone were not significantly different at either pCa 7.0 (23%) or pCa 5.5 (25%) but were significantly less at pCa 8.0 (8.4 ± 2.3%). Conversely, adult %Kmax values at peak myogenic tone were significantly less at both pCa 8.0 (10.4 ± 1.8%) and pCa 7.0 (16%) than at pCa 5.5 (27%). The maximal extents of stretch-induced increases in myosin light chain phosphorylation in intact fetal (20%) and adult (17%) arteries were similar. The data demonstrate that the cerebrovascular myogenic response is highly conserved during postnatal maturation but is mediated differently in fetal and adult cerebral arteries.

β-escin; fura 2; length-tension relations; myosin light chain phosphorylation

The transition from late fetal to postnatal life involves numerous changes in vascular morphology and function that significantly alter contractility in brain arteries (2, 15, 23, 58). For excitable tissues, many of these functional differences are a consequence of the relatively small total body mass of calcium present in most mammalian fetuses at term (60). This reduced calcium mass has important effects on the sizes of intracellular calcium pools and the signaling pathways used for cell activation (49). Specifically, contractile tone is more dependent on calcium influx in immature than in mature arteries (1). Conversely, immature arteries exhibit an enhanced ability to increase myofilament calcium sensitivity in response to G protein-dependent contractile agonists (2, 3). In contrast to these well-established effects of postnatal development, however, the influence of maturation on myogenic reactivity have not been well studied, even though it is clear that myogenic tone is critically important for cardiovascular homeostasis, regardless of age (12). Because cerebral autoregulation is highly dependent on myogenic reactivity (33), appears to be regulated quite differently in mature and immature cerebral arteries (28), and is highly vulnerable to pathophysiological insults (61), a close examination of the mechanisms mediating myogenic reactivity in immature cerebral arteries is due.

In adult cerebral arteries, myogenic contractions depend on both an increase in cytosolic calcium concentration and stretch-induced increases in myofilament calcium sensitivity (35, 53). These increases in calcium sensitivity, in turn, can be partitioned between effects of stretch on the relations between cytosolic calcium concentration and the extent of myosin light chain (MLC) phosphorylation, and the parallel effects of stretch on the relations between MLC phosphorylation and contractile force. As originally proposed by Murphy and Walker (48), the relation between cytosolic calcium concentration and the extent of MLC phosphorylation can be considered to be the result of the family of mechanisms that together govern thick-filament regulation. Alternatively, relations between the extent of MLC phosphorylation and contractile force reflect the combined influence of those mechanisms that determine thin-filament reactivity. These mechanisms include the influences of thin-filament regulatory proteins, such as caldesmon, heat shock protein (HSP) 27, and HSP20 (8, 17, 42, 43), as well as integrin-linked interactions of the actin cytoskeleton with the extracellular matrix (40). The relative importance of these general classes of mechanisms during myogenic responses remains unstudied in cerebral arteries of any age. Similarly, the ability of stretch to activate or modulate any of these mechanisms remains completely unstudied, particularly in immature cerebral arteries.

Given the importance of myogenic tone for cerebrovascular regulation, and evidence that the calcium-dependent contractile mechanisms governing myogenic tone are quite different in mature and immature cerebral arteries, the present study explores the hypothesis that myogenic tone is less dependent on calcium influx and more dependent on myofilament calcium sensitization in fetal compared with adult cerebral arteries. To test this hypothesis, we examined myogenic responses in posterior communicating cerebral arteries taken from term fetal and nonpregnant adult sheep. Owing to the possible contribution of the vascular endothelium to myogenic responses in...
cerebral arteries (19), the endothelium was gently removed in all arteries studied. The protocols involved application of graded stretch, normalized as a ratio of working diameter divided by unstressed diameter, followed by recordings of the corresponding changes in cytosolic calcium via fura 2 photometry. These protocols also included rapid freezing of artery segments at varying durations of graded stretch to enable determination of the corresponding extent of MLC phosphorylation, as measured using Western blots of membranes transferred from urea gels. Parallel experiments in intact and β-escin-permeabilized arteries helped identify the relative importance of changes in cytosolic calcium and myofilament calcium sensitization in myogenic responses to artery stretch. Together, these approaches provided a unique view of the effects of postnatal maturation on the roles of cytosolic calcium transients and myofilament calcium sensitivity in the myogenic reactivity of ovine cerebral arteries.

MATERIALS AND METHODS

**General preparation.** All procedures and protocols strictly followed all federal rules and regulations governing the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of Loma Linda University. Posterior communicating arteries were harvested from young nonpregnant adult sheep (18–24 mo old) and near-term (~140 days of gestation) fetuses euthanized with an overdose of pentobarbital sodium (60 mg/kg iv). We chose to study the posterior communicating artery because this artery plays a critical regulatory role in shunting Circle of Willis blood flow between the anterior and posterior cerebral territories, and its length permits the study of up to eight artery segments per animal. Following their removal from the surface of the brain, the arteries were placed in a calcium-replete sodium Krebs buffer solution that was continuously bubbled with 95% O2–5% CO2 and contained the following (in mM): 122 NaCl, 25.6 NaHCO3, 5.17 KCl, 2.49 MgSO4, 1.60 CaCl2, 2.56 dextrose, 0.0270 EGTA, and 0.114 ascorbic acid. Extracellular and loose connective tissue was gently removed. The arteries were then cut into 4-mm segments for both fetal and adult arteries and then mounted on wires suspended between a force transducer that was attached to a micrometer used to adjust resting length and determine the passive diameter of each individual tissue segment. The tissue diameter recordings were utilized to obtain the optimal stretch ratios necessary to obtain maximal isometric force in each segment, as previously described (16). To minimize endothelium-mediated effects, we removed the vascular endothelium by gently rotating each arterial segment around a mounting wire several times to scrape the entire luminal surface. All buffers also included 100 μM Nω-nitro-L-arginine methyl ester and 100 μM nitro-L-arginine to ensure complete inhibition of endothelial nitric oxide synthase and nitric oxide release.

**Determination of active myogenic tone in intact arteries.** Tissue segments were equilibrated for 30 min in calcium-replete Na+ Krebs buffer bubbled with 95% O2–5% CO2 at 37°C (normal ovine core temperature). Unstressed artery diameter (slack length) was determined as before, and artery stretch was calculated as a stretch ratio (D/Do). For each segment studied, the Do was initially set at 1.3, after which each segment was permeabilized with HEPES buffer at either pCa 8, 7, or 5.5 and allowed to equilibrate. Thereafter, artery stretch was calculated as a stretch ratio (D/Do), which was the working diameter divided by the unstressed diameter. As shown previously (16, 50), this approach normalized the increments of stretch applied to the resting unstressed artery diameter and thereby minimized the effects of differences in initial artery diameter. For each segment studied, the D/Do was initially set at 1.3, after which the artery was allowed to equilibrate until a stable total tension (recorded continuously via computer) was obtained (usually in ~10 min). The artery segment was then stretched to a D/Do of 1.5 and allowed to reequilibrate. This cycle was repeated in D/Do increments of 0.2 until a final D/Do of 2.5 was obtained. Then the arteries were frozen in liquid nitrogen, after which they were incubated in a calcium-free Na+ Krebs buffer containing 1 mM EGTA. As shown previously (58), freezing the arteries before incubation in EGTA accelerates the rate of calcium chelation and produces optimally reproducible estimates of passive tension. Following this treatment, the levels of tension observed at each D/Do ratio were again recorded to define the passive length-tension relation in each segment. Active myogenic tone was calculated as the difference between the initial total tension and the subsequent passive tension measured at each D/Do ratio.

**Stretch-induced changes in global cytosolic calcium.** To enable measurement of global cytosolic calcium, arterial segments were mounted in a Jasco fluorescent photometer (Jasco CAF-110), and slack length was determined after which the segments were stretched to a D/Do ratio of 1.3, then were loaded with 5 μM fura 2-AM in the presence of 0.01% pluronic acid for 3 h at 25°C. The loading buffer also included the following (in mM): 122.1 NaCl, 25 HEPES, 5.16 KCl, 2.4 MgSO4, 50 μM ETPA, 11.1 dextrose, and 1.60 CaCl2. Following loading, the segments were washed in the same buffer without fura 2-AM and then gently warmed to 38°C (normal ovine core temperature) and bubbled with 95% O2–5% CO2. After 30 min of equilibration to allow desaturation of fura 2-AM, the initial 340- to 380-nm ratio (340/380 ratio) was recorded, after which each segment was contracted with 120 mM potassium and then washed with normal buffer. Following this wash and equilibration, each segment was stretched to one and only one D/Do ratio between 1.5 and 2.5 (in increments of 0.2). During and following this stretch, changes in the 340/380 ratio were recorded along with contractile force. Once responses were stabilized, the segments were washed with fresh buffer and then exposed to buffer containing 2 mM EGTA without calcium in the presence of 10 μM ionomycin to determine minimum tissue signal intensity recorded during exposure to near-zero concentrations of free calcium (Fmin) values. After stable 340-to-380 ratio values were obtained, calcium was added at a final concentration of 24 μM to obtain maximum tissue signal intensity recorded during exposure to saturating concentrations of free calcium (Fmax). Autofluorescence was then determined via manganese quench (71). Intracellular calcium values were then determined using the Grynkiewicz equation (25).

**Determination of active myogenic tone in permeabilized arteries.** Tissue segments were equilibrated for 30 min in calcium-replete Na+ Krebs buffer bubbled with 95% O2–5% CO2 at 25°C. Unstressed artery diameter (slack length) was determined as before, and artery stretch was calculated as a stretch ratio (D/Do). For each segment studied, the D/Do was initially set at 1.3, after which each segment was permeabilized with HEPES buffer (in mM): 110 K(CH3COO), 6 Mg(CH3COO)2, 5 EGTA, 5 ATP, 5 DTT, 20 HEPES, 0.0100 calmodulin, 0.01 leupeptin containing 150 μM β-escin at pH 6.0. After equilibration, each tissue segment was washed with HEPES buffer at pH 8.0 and allowed to equilibrate. The segments were then incubated with HEPES buffer at either pH 8, 7, or 5.5 and allowed to equilibrate. The concentration of pH 8 represented a sub-baseline concentration that should eliminate most, if not all, calcium-dependent responses. The pH 7 concentration represented the approximate average basal concentration of calcium in uncontracted term fetal and adult ovine cerebral arteries (1). Conversely, the concentration of pH 5.5 represented a maximal concentration previously shown to yield maximal contractile responses in both term fetal and adult ovine cerebral arteries (2). The artery segment was then stretched to a D/Do of 1.5, and the total tension (recorded continuously via computer) was obtained (usually in ~10 min). The artery segment was stretched to D/Do 1.7 and allowed to reequilibrate. This cycle was repeated in D/Do increments of 0.2 until a final D/Do of 2.5 was obtained. Then the arteries were frozen in liquid nitrogen, after which they were incubated in a calcium-free Na+ Krebs buffer containing 1 mM EGTA. Following this treatment, the levels of tension observed at each D/Do ratio were again recorded to define the passive length-
tension relation in each segment. Active myogenic tone was calculated as the difference between the initial total tension and the subsequent passive tension measured at each \( \Delta D/D_0 \) ratio.

**Determination of MLC phosphorylation in intact arteries.** Multiple adjacent tissue segments from both fetal and adult posterior communicating arteries were mounted in 5-ml baths and were allowed to equilibrate for 30 min in calcium-replete Na\(^+\)–Krebs buffer solution constantly bubbled with 95% O\(_2\)–5% CO\(_2\) at 38°C (normal ovine core temperature). Unstressed artery diameter (slack length) was defined as the artery diameter observed at a passive tension of 0.03 g, and this measurement was made in each artery segment. Thereafter, artery stretch was calculated as a stretch ratio \( (D/D_0) \) (at slack length, \( D/D_0 = 1 \)). After equilibration, each tissue segment was quickly stretched to a desired stretch ratio and frozen at exactly 10 s after stretch. The freeze time of 10 s was used because preliminary experiments indicated that the time to peak phosphorylation following quick stretch in intact arteries did not vary significantly with either age or \( D/D_0 \) (between 1.5 and 2.3) and averaged 9.8 ± 5.1 s (N = 56). Each frozen segment’s dry weight was measured and extracted in buffer (pH 8.6) containing 8 M urea, 10% glycerol, 0.04% bromophenol blue, and (in mM) 20 Tris, 1.5 M Tris at pH 8.6 in 40% glycerol, and 10% ammonium persulfate. The stacking gel consisted of 30%/1.6% acrylamide, 1.0 M Tris at pH 9.0, and 0.1 M glycine tank buffer was used. Identical amounts of total protein were loaded for each sample on 10% acrylamide native gels and run for 2.5 h at 200 V. Proteins were transferred onto nitrocellulose membranes at constant current (50 mA) for 3 h. The nitrocellulose membranes were then blocked with Tris-buffered saline (pH 7.5) and run for 2.5 h at 200 V. Proteins were transferred onto nitrocellulose membranes at constant current (50 mA) for 3 h. The nitrocellulose membranes were then blocked with Tris-buffered saline (pH 7.5) containing 5% milk for 1 h. After blocking, the membranes were placed in Tris-buffered saline buffer with 5% milk and 0.1% Tween 20 with primary mouse monoclonal anti-MLC\(_{20}\) (20-kDa MLC; clone MY-21) at a titer of 1:300 for 3 h and then visualized with horseradish peroxidase-conjugated goat anti-mouse secondary antibody at a titer of 1:1,000 for 1 h. Membranes were scanned to determine the levels of phosphorylated and nonphosphorylated MLC blots. Percent MLC phosphorylation was then plotted against time to identify the effect of stretch on myosin phosphorylation at each pCa for each group.

**Calculation, data analysis, and statistics.** Myogenic tone was calculated as the difference between total contractile tone and passive contractile tone, which was determined following freezing in liquid nitrogen and subsequent incubation in 1 mM EGTA. Reported values of active myogenic tone are expressed relative to the maximum contractile capacity of each artery, as defined by the response to calcium-replete Krebs buffer containing 120 mM K\(^+\). Measurements of cytosolic calcium were initially obtained as fura 2 340/380 ratios, which were subsequently corrected for \( F_{max}, F_{min}\), and autofluorescence and then reported as values of the Grynkiewicz ratio (25). These ratio values were not converted to cytosolic calcium concentrations due to uncertainties regarding the actual value of the fura 2 \( K_d \) values in our preparations. Instead, calcium concentration estimates were normalized relative to the maximal change in calcium produced in response to Krebs buffer containing 120 mM K\(^+\) as obtained in each individual artery preparation. Myosin phosphorylation ratios were reported as percent total phosphorylation, as determined by the ratio between the mass of the phosphorylated band divided by the sum of the phosphorylated and unphosphorylated bands. All statistical comparisons were performed using ANOVA routines with age as one factor and either stretch ratio or time as the other factor. Homogeneity of variance was verified for all ANOVA procedures using Cochrans’ analysis. All sample distributions were verified for normalcy via analysis of kurtosis and skewness values. When multiple measurements were taken from a single artery segment (see Figs. 1, 3, and 5), comparisons between groups were performed using a repeated-measures ANOVA. When multiple segments were taken from a single animal and used in the same protocol, the results were averaged and counted as an average value for the number of animals studied.

**RESULTS**

Effects of graded stretch on myogenic tone in intact cerebral arteries. Fetal and adult cerebral arteries exhibited similar myogenic responses with peak tensions averaging 23.9 ± 6.8 and 26.0 ± 4.8% of maximum contractile force produced in any given tissue response to an isotonic Krebs buffer containing 122 mM K\(^+\) (\(K_{\text{max}}\)) values at \( D/D_0 \) values of 2.19 ± 0.04 and 2.23 ± 0.02, respectively (Fig. 1). None of these values varied significantly with age.

Effects of graded stretch on cytosolic calcium concentration in cerebral arteries. When equilibrated at a stretch ratio of 1.3, values of the Grynkiewicz ratio averaged 0.221 ± 0.019 and 0.214 ± 0.027 in fetal (\(N = 33\)) and adult (\(N = 38\)) arteries, respectively. Graded stretch produced no significant increases in these ratio values at \( D/D_0 \) values <2.0 in either fetal or adult cerebral arteries (Fig. 2). There was, however, an increase in values of the Grynkiewicz ratio at \( D/D_0 \) ratios >2.0 for adult cerebral arteries. No increases in ratio values were observed in fetal arteries until \( D/D_0 \) ratios >2.3 were applied. Thus significantly more stretch was required to elicit increases in fetal compared with adult arteries.

Effects of graded stretch on myogenic tone in permeabilized cerebral arteries. Both fetal and adult cerebral arteries exhibited stretch-induced myogenic tone under constant calcium...
The stretch ratios that produced half-maximal (EC50) MLC

...given tissue in response to an isotonic Krebs buffer containing 122 mM K3.0%) or pCa 5.5 (24.8...

...tone were not significantly different at either pCa 7.0 (22.7...

...values at any stretch ratio. Kmax, maximum contractile force produced in any given tissue in response to an isotonic Krebs buffer containing 122 mM K+.

As shown in Fig. 4, both fetal and adult cerebral arteries exhibited similar patterns of stretch-induced MLC phosphorylation in intact cerebral arteries. However, there was no significant difference in the maximal extent of MLC phosphorylation above baseline between fetal (19.9 ± 4.9%) and adult (17.1 ± 2.7%) cerebral arteries. There were also no significant differences in basal MLC phosphorylation between fetal (18.0 ± 2.3%) and adult (15.9 ± 2.8%) arteries. The stretch ratios that produced half-maximal (EC50) MLC phosphorylation were also highly similar in fetal (1.68 ± 0.10) and adult (1.64 ± 0.18) cerebral arteries, respectively.

Effects of graded stretch on the extent of MLC phosphorylation in permeabilized cerebral arteries. When the arteries were held at a pCa of 8.0, basal values of %MLC phosphorylation were not significantly different in fetal (21.0 ± 2.1%) and adult (22.0 ± 1.9%) arteries (Fig. 5). Rapid stretch from a D/D0 of 1.3 to a D/D0 of 1.9 had no time-dependent effects on phosphorylation at pCa 8.0 in either fetal or adult arteries over a time course of 3 min. In arteries held at a pCa of 7.0, baseline phosphorylation was significantly elevated in fetal (40.5 ± 1.4%) but not adult (23.0 ± 1.9%) arteries. Rapid stretch at pCa 7.0 from a D/D0 of 1.3 to a D/D0 of 1.9 produced no significant increase in the extent of MLC phosphorylation in either fetal or adult arteries over a time course of 2 min. After 3 min of stretch, the averaged fetal value of phosphorylation (44.8 ± 0.5%) at pCa 7.0 was slightly but significantly elevated over baseline; no such difference was observed in the adult arteries. In arteries held at pCa 5.5, baseline values of phosphorylation were not significantly different but that the responses at pCa 8.0 were significantly less than observed at pCa 7.0 in either fetal (42.0 ± 1.4%) or adult (26.0 ± 1.2%) arteries. Rapid stretch from a D/D0 of 1.3 to a D/D0 of 1.9 at pCa 5.5 significantly enhanced %MLC phosphorylation above basal levels to 37.0 ± 5.5% and 46.1 ± 1.5% in adult and fetal arteries, respectively.

**DISCUSSION**

...that determine vascular reactivity change progressively throughout fetal development and early postnatal life, particularly in the cerebral circulation (58). Immature cerebral arteries exhibit greater dependence on calcium influx, and less dependence on intracellular calcium release, than is typical of functionally mature adult cerebral arteries (1, 49). In parallel, immature cerebral arteries also demonstrate a greater ability to enhance myofilament calcium sensitivity when stimulated by G protein receptor agonists (2). Together, these maturational changes have a major impact on

**REFERENCES**

...cerebral arteries plotted as a Grynkiewicz ratio (Rg) for fetal and adult cerebral arteries. Shown here are values of change above baseline in the Grynkiewicz ratio (ΔRg)

Fig. 1. Myogenic length-tension relations in intact fetal and adult cerebral arteries. Segments of fetal and adult posterior communicating arteries were stretched from a stretch ratio [working diameter/unstressed diameter (D/D0)] of 1.3 to a ratio of 2.7 before (total tension) and after (passive tension) freezing in liquid N2 and incubation in 1 mM EGTA. Myogenic tone was calculated as the difference between total and passive tensions at each stretch ratio, then expressed as a percentage of the corresponding response to 120 mM K+ in each segment. Error bars indicate SEs for arteries from 6 adults and 7 fetuses, respectively. There were no significant differences between fetal and adult values at any stretch ratio. Kmax, maximum contractile force produced in any given tissue in response to an isotonic Krebs buffer containing 122 mM K+.

Fig. 2. Relations between stretch and cytosolic calcium in fetal and adult cerebral arteries. Increases in fetal artery cytosolic calcium were observed only at a stretch ratio 2.0. At stretch ratios between 2.0 and 2.3, stretch increased cytosolic calcium, but only in adult arteries. Increases in fetal artery cytosolic calcium were observed only at a stretch ratio of 2.5. **Significantly greater than baseline (average ΔRg values between stretch ratios of 1.3 and 1.7).** Error bars indicate SE of the mean for N = 5 in both experimental groups.
many aspects of overall cerebrovascular regulation, including endothelial function (72), responses to hypoxia (56), and the role of the perivascular adrenergic innervation (57). Aside from this broad variety of work, however, relatively little is known about how postnatal maturation influences myogenic reactivity in the cerebral circulation. Although evidence of the myogenic response in fetal arteries was first reported many years ago (63), only recently has this response been reported in fetal cerebral arteries (19, 20). To examine the mechanistic basis for this response, the present study explored the main hypothesis that postnatal maturation modulates the relative importance of each of the main mechanisms governing the myogenic response, with emphasis on changes in the relations among myogenic stretch, cytosolic calcium concentration, extent of MLC phosphorylation, and contractile force (48). Our first objective was to compare the relations between artery stretch and myogenic tone as a function of postnatal age. As shown in Fig. 1, both fetal and adult cerebral arteries exhibited robust myogenic contractions whose magnitudes (fetal: 23.9 ± 6.8%; adult: 26.0 ± 4.8% \( K_{\text{max}} \)) were similar and agreed well with previously published results in both cerebral (19, 35, 54) and noncerebral (7, 55, 64, 65, 66) arteries. In contrast to most previous measurements of myogenic tone in either cerebral or fetal arteries, our measurements were not based on diameter responses to changes in artery pressure. Instead, our stretch stimulus was normalized relative to passive diameter to obtain stretch ratios that enabled direct estimates of optimal diameter and stretch, as previously described (16). These estimates of optimal stretch were very similar in fetal (D/\( D_0 \) = 2.19 ± 0.04) and adult (D/\( D_0 \) = 2.23 ± 0.02) arteries, indicating that the mechanisms determining the myogenic response are fully developed in fetal cerebral arteries. This result was unexpected, given that regulation of contractility differs greatly between fetal and adult cerebral arteries (2, 3). In turn, this result suggests that the mechanisms mediating the myogenic response may be differ-
stretch, however, significantly increased myogenic tone in both age groups (Fig. 1). At stretch ratios >1.9, however, only adult arteries exhibited a significant rise in calcium, whose peak correlated closely with maximum myogenic tone. In contrast, stretch significantly increased calcium in fetal arteries only at a much greater stretch ratio of 2.5, and this may have been attributable to forced dilatation (53). Together, these results demonstrate that cytosolic calcium is more sensitive to graded stretch in adult than in fetal cerebral arteries. From a more general perspective, these results also reinforce the view that low levels of myogenic tone do not require large steady-state increases in cytosolic calcium. Given that highly localized calcium transients can play a major role in regulation of smooth muscle contraction (36, 74), it remains possible that stretch-induced calcium influx might also involve localized calcium transients, as has been implied in recent reviews of myogenic reactivity (12, 30). If so, such transients would constitute a calcium-mediated response to stretch that would be very difficult to observe using fura 2, which is subject to extensive spatial averaging (67).

To evaluate the possibility that stretch-induced myogenic contractions in our preparations were being mediated by highly regional changes in calcium, we examined the effects of graded stretch on myogenic tone in β-escin permeabilized cerebral arteries. As shown by others, β-escin permeabilization enables equilibration of the cytosol with the extracellular medium (59), eliminates gradients in calcium throughout the cell, and simultaneously stabilizes calcium at any desired concentration. Under these conditions, any changes in localized calcium should be buffered by regional EGTA, which is present at high concentrations throughout the cell. In arteries held at a low-calcium concentration of 10 nM (pCa 8), graded stretch still produced small but significant myogenic contractions in both fetal and adult arteries (Fig. 3). The peak magnitudes of these contractions were similar in fetal (8.4 ± 2.3% Kmax) and adult (10.4 ± 1.8% Kmax) arteries. However, when ambient calcium was held at 100 nM (pCa 7.0), myogenic responses to stretch were significantly greater in fetal (22.7 ± 3.0% Kmax) than adult (15.7 ± 4.2% Kmax) arteries. These results suggest that stretch enhanced myofilament calcium sensitivity, as previously suggested (41), and the magnitude of this effect was greater in fetal than adult cerebral arteries. These observations are consistent with previous reports that agonist-induced myofilament calcium sensitization is upregulated in fetal compared with adult cerebral arteries (2). In addition, the stretch ratios at which peak myogenic tone was observed were similar in the permeabilized and intact preparations in both age groups. When the arteries were incubated and held at high-calcium concentrations of 3 μM (pCa 5.5), age-related differences in myogenic tone disappeared; the relation between stretch and peak tone was significantly enhanced in adult (26.6 ± 3.0% Kmax) but not fetal (24.8 ± 5.0% Kmax) cerebral arteries. Again, the stretch ratios at which peak myogenic tone was observed were similar in the permeabilized and intact preparations in both age groups. Together, these results suggest that 100 nM calcium is sufficient to obtain a maximal response in fetal, but not adult, arteries. This helps explain why significant myogenic tone was observed (Fig. 1) in the absence of stretch-induced increases in cytosolic calcium in fetal arteries at stretch ratios <1.9 (Fig. 2). The development of myogenic tone

ent in fetal and adult arteries, despite the fact that the overall response was independent of age.

The isometric preparations used in this study offered several advantages that simplified comparisons among arteries of different size and also facilitated direct measurements of wall calcium by minimizing movement of the artery wall. This enabled repeated measurements of cytosolic calcium during graded stretch in both fetal and adult cerebral arteries (Fig. 2). Consistent with reports of similar experiments in other preparations (22, 29, 53, 62, 68, 69), gradual stretch did not produce a significant increase in calcium at any stretch ratio (D/D0) <1.9 in either fetal or adult arteries. The same range of gradual

Fig. 5. Relations between stretch and MLC phosphorylation in permeabilized fetal and adult cerebral arteries. Both fetal and adult cerebral arterial segments were equilibrated at a D/D0 of 1.3 at pCa values of 8.0, 7.0, or 5.5, then frozen at 0, 1, 2, or 3 min after stretch to a D/D0 of 1.9. Percent MLC phosphorylation was determined as described in Fig. 4 legend. Rapid stretch significantly increased MLC phosphorylation in both fetal and adult arteries. *Significantly greater than baseline (time 0) at each pCa. The phosphorylation time course was significantly different at pCa 5.5 than that at either pCa 7.0 or 8.0 in adult arteries. For fetal arteries, the phosphorylation time courses at both pCa 5.5 and 7.0 were significantly different than that at pCa 8.0. Error bars indicate SE of the mean for 64 fetal and 64 adult arteries taken from 16 fetuses and 16 adults. Individual N values for each pCa in each age group are indicated in the legends.
in the absence of major changes in global calcium is consistent with previously reported observations (22, 29, 53, 62).

In light of the age-related differences observed in stretch-induced changes in global calcium (Fig. 2), we explored the possibility that the effects of gradual stretch on MLC phosphorylation might also vary with postnatal age. When artery segments were rapidly frozen after 10 s of stretch, MLC phosphorylation increased significantly, as previously reported (6), but the response was closely similar in fetal and adult cerebral arteries (Fig. 4). Because significant increases in MLC phosphorylation occurred in both fetal and adult arteries at a stretch ratio of 1.9 that was not associated with significant increases in calcium (Fig. 2), the data suggest a possible contribution from calcium-independent kinases in the response to stretch. Recent studies have identified several candidates capable of phosphorylating serine 19 of MLC (5, 14, 34, 37, 51, 52, 70, 73). Another possibility is that stretch inhibits phosphatases that reduce the level of MLC phosphorylation, and, here again, several possible mechanisms for this effect have been reported. These include calcium-independent roles for arachidonate (24), zipper-interacting protein kinase (34), zipper-interacting protein kinase-like kinase (38), integrin-linked kinase (13, 31, 47), and protein kinase C (27).

To test the possibility that stretch-induced changes in MLC phosphorylation are mediated by highly regional changes in calcium, we also examined the effects of stretch on MLC phosphorylation in permeabilized arteries. When adult arteries were held at low-calcium concentrations of 10 or 100 nM, stretch induced no changes in phosphorylation over either very short or long durations (Fig. 5). In adult arteries held at 3 μM calcium, however, stretch stimulated a gradual increase in MLC phosphorylation that reached a peak in ~2 min. This response suggests that calcium is required for stretch-induced increases in MLC phosphorylation but that some mechanism other than changes in cytosolic calcium are also involved in the development of myogenic tone at calcium concentrations at or below 100 nM (Fig. 3). One possibility is that stretch inhibits MLC phosphatase activity (13, 24, 27, 34, 38, 39, 47), which would be evident only if a significant fraction of MLC were phosphorylated. Such a system would be calcium dependent but would not require calcium transients to exhibit stretch-induced changes in MLC phosphorylation. This arrangement could also involve possible stretch-induced changes in MLC kinase activity, as reported in other preparations (46). Clearly, in adult cerebral arteries, calcium is required for stretch to stimulate increased MLC phosphorylation and maximum myogenic contraction (Figs. 1 and 3), even though calcium transients are not.

In contrast to adult cerebral arteries, fetal cerebral arteries did not exhibit stretch-induced increases in MLC phosphorylation when cytosolic calcium concentrations were held constant (Fig. 5). At very low calcium concentrations of 10 nM, baseline MLC phosphorylation was similar to that observed in the adult. Increasing the calcium concentration to only 100 nM elevated MLC phosphorylation to near maximal levels: at both 100 nM and 3 μM calcium, the extent of MLC phosphorylation was near maximal and did not respond to stretch. Consistent with the results shown in Fig. 3 and previous findings that myofilament calcium sensitization is markedly upregulated in the fetus (2, 3), the data suggest that 100 nM calcium are sufficient to support near maximal MLC phosphorylation in fetal cerebral arteries at a stretch ratio of 1.3 or higher. In addition, these data also support the view that the effects of stretch-induced increases in calcium are near maximal at low levels of stretch and that calcium increases observed at high levels of stretch may reflect forced dilatation and not a physiological response (53). As for the adult arteries, this response could involve parallel effects of stretch on both calcium-dependent MLC phosphorylation, as well as coupling between stretch and inhibition of MLC phosphatase activity. However, some additional mechanism must also be involved. As shown in Fig. 3, stretch clearly enhanced myofilament calcium sensitivity in fetal cerebral arteries. For this response to occur in the absence of stretch-induced changes in MLC phosphorylation, stretch must also enhance thin-filament reactivity. Correspondingly, a broad variety of recent studies has suggested that such mechanisms may play a key role in myogenic reactivity (4, 9, 10, 17, 18, 21, 44). For example, rates of actin polymerization can be increased by stretch, which in turn can elevate the number of active binding sites for myosin cross bridges, resulting in greater force development (4, 10, 11, 21, 44). In addition, stretch can activate protein kinase C and rho kinase (35, 66), leading to increased phosphorylation and inhibition of actin regulatory proteins such as caldesmon, HSP20, and HSP27 (8, 17, 18, 42). Aside from the mechanisms involved, the data strongly suggest that the relative importance of these individual mechanisms in determining the overall myogenic response is markedly different in fetal and adult cerebral arteries.

Overall, the present data support the view that the myogenic response is highly conserved in cerebral arteries, regardless of postnatal age. Aside from this consistency, the relations between stretch and cytosolic calcium, and between stretch and myofilament calcium sensitization, appear to be regulated very differently in fetal and adult cerebral arteries. These age-dependent differences appear to be a natural consequence of corresponding differences in the ability to store and release intracellular calcium and are consistent with the general view that myofilament calcium sensitivity is markedly upregulated in fetal compared with adult arteries. A key feature of the present data, however, is the suggestion that stretch-induced modulation of thin-filament reactivity may be more important for the myogenic response in fetal compared with adult cerebral arteries. This suggestion predicts that it may be valuable to further explore the role of thin-filament regulatory proteins in the myogenic response, particularly in relation to the postnatal development of cerebrovascular reactivity.

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