Developmental changes in ryanodine- and IP$_3$-sensitive Ca$^{2+}$ pools in ovine basilar artery

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Nauli, S. M., J. M. Williams, S. E. Akopov, L. Zhang, and W. J. Pearce. Developmental changes in ryanodine- and IP$_3$-sensitive Ca$^{2+}$ pools in ovine basilar artery. Am J Physiol Cell Physiol 281: C1785–C1796, 2001.—To explore the hypothesis that cerebrovascular maturation alters ryanodine- and inositol 1,4,5-trisphosphate (IP$_3$)-sensitive Ca$^{2+}$ pool sizes, we measured total intracellular Ca$^{2+}$ with $^{45}$Ca and the fractions of intracellular Ca$^{2+}$ released by IP$_3$ and/or caffeine in furaptra-loaded permeabilized basilar arteries from nonpregnant adult and term fetal (139–141 days) sheep. Ca$^{2+}$ mass (nmol/mg dry weight) was similar in adult (1.60 ± 0.18) and fetal (1.71 ± 0.16) arteries in the pool sensitive to IP$_3$ alone but was significantly lower for adult (0.11 ± 0.01) than for fetal (1.22 ± 0.11) arteries in the pool sensitive to ryanodine alone. The pool sensitive to both ryanodine and IP$_3$ was also smaller in adult (0.14 ± 0.01) than in fetal (0.85 ± 0.08) arteries. Because the Ca$^{2+}$ fraction in the ryanodine-IP$_3$ pool was small in both adult (5 ± 1%) and fetal (7 ± 4%) arteries, the IP$_3$ and ryanodine pools appear to be separate in these arteries. However, the pool sensitive to neither IP$_3$ nor ryanodine was 10-fold smaller in adult (0.87 ± 0.10) than in fetal (8.78 ± 0.81) arteries, where it accounted for 72% of total intracellular membrane-bound Ca$^{2+}$. Thus, during basilar artery maturation, intracellular Ca$^{2+}$ mass plummets in noncontractile pools, decreases modestly in ryanodine-sensitive pools, and remains constant in IP$_3$-sensitive pools. In addition, age-related increases in IP$_3$ efficacy must involve factors other than IP$_3$ pool size alone.

The major goal of the present work was to test the hypothesis that maturation alters the relative sizes of the ryanodine- and IP$_3$-sensitive Ca$^{2+}$ pools in cerebral arteries. In previous studies, Staudeyman et al. (35) divided intracellular Ca$^{2+}$ stores into three distinct pools including those sensitive to 1) caffeine, 2) IP$_3$, and 3) both caffeine and IP$_3$. In light of recent evidence that other organelles may also contain significant intracellular Ca$^{2+}$ (10, 39), we have also examined the channel activation and Ca$^{2+}$ release may be influenced or even regulated by physiological and pathophysiological perturbations.

One of the most important physiological processes known to influence vascular contractility is maturation. Neonatal cerebral arteries exhibit similar active stresses, but because of their smaller wall thicknesses they produce less total contractile force than do corresponding adult arteries (8, 31). For multiple agonists, neonatal arteries exhibit greater receptor densities and agonist affinities, greater sensitivity (lower EC$_{50}$ values), and also altered patterns of Ca$^{2+}$ mobilization compared with corresponding adult arteries (2, 8, 23). In light of the central but variable role of ryanodine- and IP$_3$-induced Ca$^{2+}$ release in smooth muscle contractile responses, it is possible that developmental modifications of ryanodine- and IP$_3$-dependent pharmacomechanical coupling may play a role in age-related differences of vascular responses to receptor agonists. Consistent with this hypothesis are previous suggestions that IP$_3$-mediated Ca$^{2+}$ release is physiologically less important in immature than in mature smooth muscle (1). Such differences may result from a broad variety of maturational changes including smaller intracellular Ca$^{2+}$ stores, reduced sensitivity of IP$_3$- or ryanodine-sensitive receptors, and/or altered IP$_3$ or ryanodine receptor function in immature compared with mature cerebral arteries. One approach to choose among these possibilities would be to measure IP$_3$- and ryanodine-induced intracellular Ca$^{2+}$ release in mature and immature arteries. Such measurements have just recently become possible with the use of methods developed in permeabilized vascular preparations (see Fig. 1 of Ref. 12; Ref. 15).

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possible existence of a fourth pool resistant to both caffeine and IP$_3$ but releasable by Ca$^{2+}$ ionophore. Because our previous observations have indicated that the ovine basilar artery exhibits marked maturational changes in reactivity to multiple agonists that cannot be explained by differences in the receptor apparatus alone (22, 31) and also exhibits marked age-related changes in dependence on extracellular Ca$^{2+}$ for contraction (2), we chose this model for the present studies. Selection of the basilar artery for these studies was further justified by the well-established observations that arteries >150 μM in diameter, such as the basilar artery, are responsible for one-third or more of total adult cerebrovascular resistance (3) and may play an even more important regulatory role in neonates than in adults (31).

METHODS

General preparation. The Loma Linda University Animal Research Committee approved all procedures. Basilar arteries from adult sheep (18–24 mo old) and term (~140 days gestation) fetal sheep were placed in buffer with (in mM) 132 NaCl, 4.2 NaHCO$_3$, 5.9 KCl, 1.4 MgSO$_4$, 1.2 Na$_2$HPO$_4$, 1.6 CaCl$_2$, 11 dextrose, and 10 HEPES (pH 7.36). Segments 3–4 mm long were mounted on wires between a force transducer and a micrometer used to control passive stretch (1). All wires containing vanadate. When returned to normal PSS with vanadate these segments failed to relax, and the potassium-induced increase in cytosolic Ca$^{2+}$ persisted even after 10 min of incubation in normal PSS with vanadate. Thus 5 μM vanadate effectively inhibited Ca$^{2+}$ transport in our preparations.

To characterize ryandroine-induced release of intracellular Ca$^{2+}$, we exposed arterial segments, loaded with fura 2 and equilibrated with vanadate, to graded concentrations (0.1–30 μM) of ryandroine while continuously measuring contractile force and the Ca$^{2+}$ ratio. On completion of these measurements, the minimum fluorescence ratio was obtained by incubation of the preparations in nonfluorescent Ca$^{2+}$-ionophore Br-A-23187 (10 μM) for 15 min. As shown by Itoh et al. (16), this concentration of ionophore preferentially permeabilizes all intracellular organelles with minimal effects on sarcolemmal permeability.

Protocol 4: IP$_3$-induced Ca$^{2+}$ transients in permeabilized vascular preparations. Basilar artery segments were loaded with furaptra-AM (5 μM; premixed with 0.01% Pluronic F127) for 3.5 h at 37°C under protection from light. At this temperature furaptra accumulated mainly in the SR (12, 37). Intracellular organelles were loaded with furaptra-AM as follows. Permeabilization was necessary for sarcolemmal permeability.

The permeabilized segments were mounted in a fluorometer (in mM) 125 K-acetate, 5 EGTA, 4 ATP, 4 Mg-acetate, 1 dithiothreitol, 0.01 leupeptin, 1 NaN$_3$, and 10 HEPES at pH 6.9 (1). Intracellular Ca$^{2+}$ stores were loaded by increasing free Ca$^{2+}$ to 0.3 μM for 15 min, followed by a 5-min wash in Ca$^{2+}$-free buffer containing 0.05 mM EGTA. Intracellular Ca$^{2+}$ release was monitored by recording contractile responses to successive additions of 30 μM IP$_3$, with 15-min Ca$^{2+}$-free washouts between applications. Multiple lines of evidence indicate that 30 μM is an optimal IP$_3$ concentration for Ca$^{2+}$ release (1, 15). In control experiments, the effects of 30 μM IP$_3$ were evaluated in permeabilized preparations depleted of Ca$^{2+}$ by incubation with 10 μM A-23187 as previously described (16). At the end of each experiment, contractile responses to a maximally effective free Ca$^{2+}$ concentration (10 μM) were obtained.

Protocol 5: Relative Ca$^{2+}$ pool sizes based on IP$_3$ and ryandroine sensitivity in permeabilized vascular preparations. Intracellular organelles were loaded with furaptra-AM as described for protocol 4, after which the artery segments...
were maintained in 300 nM free Ca\(^{2+}\) and 10 \(\mu\)M GTP. The F\(_{346}\)-to-F\(_{374}\) ratio was recorded continuously except during administration of caffeine because millimolar concentrations of caffeine significantly increase the F\(_{346}\)-to-F\(_{374}\) ratio through a direct interaction with furaptra (26). Relative pool sizes were determined in two parallel experiments. In the first experiment, the decrease in the F\(_{346}\)-to-F\(_{374}\) ratio produced by 30 mM caffeine was taken as a measure of all ryanodine-sensitive Ca\(^{2+}\) pools. Caffeine was used because it produced a rapid release of Ca\(^{2+}\) whereas ryanodine-induced release was typically quite slow. Next, the arteries were exposed to 30 \(\mu\)M IP\(_3\) to indicate the size of the remaining IP\(_3\)-sensitive, ryanodine-resistant Ca\(^{2+}\) pool. In the second experiment, arteries were first exposed to 30 \(\mu\)M IP\(_3\), to release all IP\(_3\)-sensitive stores, and then to 30 mM caffeine, to release the IP\(_3\)-resistant but ryanodine-sensitive store. On conclusion of both experiments, the arteries were treated with 10 \(\mu\)M Br-A-23187 to determine the size of the pool resistant to both IP\(_3\) and ryanodine and establish the minimum fluorescence ratio.

**Protocol 6: Measurement of intracellular Ca\(^{2+}\) mass using \(^{45}\)Ca washout.** We measured total intracellular Ca\(^{2+}\) mass using \(^{45}\)Ca as described previously (50). Briefly, arteries were contracted for 2 min by exposure to a K\(^+\)-Krebs-bicarbonate buffer containing \(^{45}\)Ca at 1 \(\times\) 10\(^8\) cpm/ml with 3 \(\mu\)M histamine and 10 \(\mu\)M serotonin. The arteries were then relaxed for 18 min in Na\(^+\)-Krebs-bicarbonate buffer that also contained \(^{45}\)Ca at 1 \(\times\) 10\(^8\) cpm/ml. From each basilar artery, we prepared six adjacent artery segments, each of which was treated with a different number of contraction-relaxation cycles. After loading, the segments were washed in sequential wash vials (5 min each) containing ice-cold buffer with (in mM) 2 EGTA, 122.1 NaCl, 5.16 KCl, 2.5 MgSO\(_4\), 25.6 NaHCO\(_3\), and 11.08 dextrose continuously bubbled with 95% O\(_2\), 5% CO\(_2\). Arteries were then incubated with 10 \(\mu\)M Br-A-23187 to determine the size of the pool resistant to both IP\(_3\) and ryanodine and establish the minimum fluorescence ratio.

**Fig. 1.** Contractile responses to ryanodine-induced intracellular Ca\(^{2+}\) release in intact adult and fetal basilar artery segments. Artery segments with loaded Ca\(^{2+}\) stores were contracted with 1) 30 mM caffeine, 2) 30 \(\mu\)M ryanodine followed by 30 mM caffeine, or 3) 30 \(\mu\)M ryanodine alone. All contractions were obtained with extracellular Ca\(^{2+}\) buffered at either 1.6 mM or 10 mM. A: individual traces to illustrate response dynamics. B: comparison of the averaged contractile tensions of each response, normalized to the maximum contractile response to 122 mM K\(^+\). Note that pretreatment with ryanodine markedly reduced subsequent responsiveness to caffeine, suggesting that both agonists acted on the same Ca\(^{2+}\) pool. Vertical error bars indicate SE. *Significant differences \((P < 0.05)\) between corresponding adult \((n = 5)\) and fetal \((n = 7)\) basilar artery responses.
Experimental procedures.

Preparation of tissue.

Adult and fetal basilar arteries were obtained from ovine adult and fetal sheep that were used for other experiments. The arteries were isolated in 0.01 M HEPES-0.5% CO2 and then were weighed and counted. Cumulative washout curves were constructed, and the size and rate constant for the intracellular pool sizes were determined by fitting the data to

\[ y = A \cdot e^{(-k_1 \cdot t)} + B \cdot e^{(-k_2 \cdot t)} \]

where \( A \) is the mass of the extracellular Ca\(^{2+} \) pool, \( k_1 \) is the extracellular pool rate constant, \( t \) is wash time in minutes, \( B \) is the mass of all intracellular Ca\(^{2+} \) pools, and \( k_2 \) is the intracellular pool rate constant.

Values of pool size were plotted against their corresponding number of contraction cycles to indicate the rate of uptake and labeling of the intracellular pools. This relation was then fitted to determine both the steady-state magnitude of the intracellular Ca\(^{2+} \) mass and the number of contraction cycles required to label one-half of that mass using

\[ Y = (C \cdot N)/(D + N) \]

where \( Y \) is the measured intracellular Ca\(^{2+} \) mass, \( C \) is the maximum steady-state intracellular Ca\(^{2+} \) mass, \( N \) is the number of contraction cycles, and \( D \) is the number of contraction cycles required to reach half-maximal intracellular Ca\(^{2+} \) mass.

Data analysis and statistics. All values are means ± SE for the numbers of animals studied. Because we showed previously (31) that the maximum levels of potassium-induced contractile force per unit cross-sectional area (in 10\(^7 \) dyn/cm\(^2 \)) in ovine basilar arteries do not vary significantly between nonpregnant adults (3.63 ± 0.28, \( n = 37 \)) and term fetal lambs (3.66 ± 0.44, \( n = 18 \)), we normalized all contractile responses to the maximum contractile response to 122 mM potassium (K\(_{\text{max}} \)). This normalization corrected for age-related differences in smooth muscle mass and facilitated interage comparisons of contractile responses. All dose-response data were fitted to the logistic equation using nonlinear regression to calculate pD\(_2 \) values (−log ED\(_{50} \)). To calculate relative Ca\(^{2+} \) pool sizes, the results were combined from protocols 3, 4, and 5 and analyzed as a series of simultaneous equations using matrix algebra. The four pools defined were the pool releasable by both IP\(_3 \) and ryanodine, the pool releasable by IP\(_3 \) but resistant to ryanodine, and the pool releasable by both IP\(_3 \) and ryanodine, the pool releasable by ryanodine but resistant to IP\(_3 \), and the pool resistant to both IP\(_3 \) and ryanodine. The sum of all pools was assumed to equal 100% within each experiment. Because the extraction of total vascular Ca\(^{2+} \) was achieved using EGTA buffers of similar composition for determination of both minimum fluorescence ratios and 45Ca content, the total amounts of releasable Ca\(^{2+} \) measured in both the fluorescent measurement and 45Ca washout protocols were considered equal. Correspondingly, absolute pool sizes were calculated as the product of fractional pool size and the estimated total intracellular Ca\(^{2+} \) mass. All comparisons between means were performed using ANOVA with post hoc comparisons via Fisher’s protected least significant difference. All data sets were verified to be normally distributed before analysis by ANOVA. All comparisons with negative results had statistical powers of ≥0.8. Unless stated otherwise, \( n \) indicates the number of animals (not the number of segments) and statistical significance implies \( P < 0.05 \).

Materials. The IP\(_3 \) (d-myoinositol 1,4,5-trisphosphate), fura 2-AM, furaptra-AM, and Br-A-23187 used in these studies were obtained from Molecular Probes (Eugene, OR). The tissue solubilizer used (TS-2) was purchased from Research Products International (Mt. Prospect, IL). The 45Ca used was purchased from New England Nuclear (Boston, MA). All other chemicals and reagents were purchased from Sigma (St. Louis, MO) and were of the highest purity available.

RESULTS

This study used 207 basilar artery preparations obtained from 50 young adult sheep and 51 near-term fetuses. In arteries used for contractility measurements, unstressed baseline diameters averaged 0.62 ± 0.01 mm in adults and 0.53 ± 0.01 mm in fetuses. Maximum contractile tensions obtained in response to 122 mM potassium (K\(_{\text{max}} \)) averaged 5.58 ± 0.56 and 1.5%.
4.42 ± 0.26 g/cm of artery length in adult and fetal basilar arteries, respectively.

**Contractile effects of ryanodine- and caffeine-induced Ca\(^{2+}\) release in intact preparations.** The transient contractile responses to 30 mM caffeine were significantly less in adult than in fetal basilar arteries (Fig. 1). Responses to 30 μM ryanodine developed slowly, but their peak values were not significantly different from those to caffeine. Addition of caffeine immediately after ryanodine produced only small additional contractions. In artery segments first equilibrated 30 min in PSS buffered at 10 nM Ca\(^{2+}\), ryanodine-induced contractions were not significantly greater than zero.

**Contractile effects of IP\(_3\)-induced Ca\(^{2+}\) release in permeabilized preparations.** In permeabilized basilar arteries treated with 10 μM A-23187, administration of 30 μM IP\(_3\) failed to produce any changes in contractile force either in adult or fetal arterial preparations, indicating that A-23187 effectively emptied all IP\(_3\)-releasable Ca\(^{2+}\) stores. In preparations with Ca\(^{2+}\)-loaded stores, IP\(_3\) evoked contractions that were 20–40% of the maximum contractions evoked by exogenous Ca\(^{2+}\) (Fig. 2A). The IP\(_3\) evoked responses were, on average, threefold greater in adult than in fetal arteries. Repeated applications of 30 μM IP\(_3\) (without Ca\(^{2+}\) reloading between applications) progressively decreased the magnitudes of the contractile responses, less so in adult than in fetal arteries (Fig. 2B).

**Ryanodine-induced Ca\(^{2+}\) transients in intact vascular preparations.** Ryanodine evoked dose-dependent increases in cytosolic Ca\(^{2+}\). Because these increases occurred in the presence of 10 nM extracellular Ca\(^{2+}\), they indicate the release of intraorganellar Ca\(^{2+}\) (Fig. 3A). After treatment with Br-A-23187, Ca\(^{2+}\) concentrations attained relatively stable plateau values, indicating that 5 μM orthovanadate effectively blocked Ca\(^{2+}\) transport and that sarcolemmal permeability was unaffected by Br-A-23187. The ability of ryanodine to release Ca\(^{2+}\) was significantly less in adult than in fetal arteries (Fig. 3B). pD\(_2\) values for ryanodine were not significantly different in adult and fetal preparations.

![Graphs showing contractile responses](http://ajpcell.physiology.org/)

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**Fig. 3.** Ryanodine-induced transients in cytosolic Ca\(^{2+}\) in intact basilar arteries. In the presence of 5 μM vanadate and 10 nM extracellular Ca\(^{2+}\), ryanodine evoked a concentration-dependent release of Ca\(^{2+}\). All intraorganellar Ca\(^{2+}\) remaining after ryanodine was released by 10 μM Br-A-23187, after which Ca\(^{2+}\) attained a stable plateau (A). These plateau values decreased by only 0.58 ± 0.22 and 0.64 ± 0.18%/min in adult and fetal basilar arteries, respectively, indicating that 5 μM vanadate effectively blocked Ca\(^{2+}\) reuptake by sarcoplasmic reticulum Ca\(^{2+}\) pumps and Ca\(^{2+}\) extrusion by plasmalemmal Ca\(^{2+}\) pumps. pD\(_2\) values for the adult and fetal concentration-response relations did not differ significantly, but the Ca\(^{2+}\) fraction released by ryanodine was significantly smaller in adult than in fetal arteries (B). The vertical error bars indicate SE for 5 adults and 6 fetuses. F\(_{340}/F_{380}\), ratio of energies emitted at excitation wavelengths of 340 and 380 nm.
IP$_3$-induced Ca$^{2+}$ transients in permeabilized vascular preparations. To validate furaptra compartmentalization within the SR, we compared the dynamics of fluorescence generated during permeabilization with β-escin after furaptra loading at both 22 and 37°C (12). In both adult and fetal arteries loaded at 22°C, β-escin decreased fluorescence because of leakage of dye from the cytoplasm into the Ca$^{2+}$-free medium. In arteries loaded at 37°C, permeabilization induced little change in fluorescence, indicating that most dye was sequestered within intracellular organelles. These experiments also confirmed that very high concentrations of β-escin (≥1.5 mM) were required to destroy intracellular ultrastructure (40). Thus, for reasons articulated by Golovina and Blaustein (12), the intraorganellar Ca$^{2+}$ concentrations indicated in our furaptra preparations most probably reflected SR calcium, with small contributions possible from the nucleus or nuclear envelope (12, 37).

Baseline intraorganellar Ca$^{2+}$ concentrations were significantly greater in adult than fetal arteries, and exposure of these preparations to IP$_3$ evoked concentration-dependent declines in the F$_{346}$-to-F$_{374}$ ratio, indicating release of intraorganellar Ca$^{2+}$ (Fig. 4A). When normalized to total Ca$^{2+}$ released, IP$_3$-induced Ca$^{2+}$ release was significantly greater in adult compared with fetal arteries at all IP$_3$ concentrations (Fig. 4B). IP$_3$ sensitivity and total Ca$^{2+}$ release were not significantly different at ambient Ca$^{2+}$ concentrations of 150 or 300 nM in either age group. At 150 nM, the sizes of the IP$_3$-releasable pools were 63.0% and 13.3% of the ionophore-releasable Ca$^{2+}$ in adult and fetal arteries, respectively. At 300 nM Ca$^{2+}$, the corresponding values were 57.8% and 21.1%, respectively (Fig. 4C). In contrast, pD$_2$ values for IP$_3$ were not significantly different in adult and fetal preparations for either level of ambient Ca$^{2+}$ used.

![Fig. 4. IP$_3$-induced changes in intraorganellar furaptra fluorescence in β-escin-permeabilized adult and fetal basilar artery segments. Baseline organellar Ca$^{2+}$ concentrations averaged 140.7 ± 12.7 and 62.3 ± 9.3 μM in adult and fetal arteries, respectively (n = 7 each, P < 0.01). After permeabilization, administration of IP$_3$ in the presence of 10 μM GTP yielded concentration-dependent decreases in fluorescence. Ca$^{2+}$ remaining after IP$_3$ treatment was released with 10 μM Br-A23187 (A). 346/374, Ratio of furaptra fluorescence measured at wavelengths of 346 and 374 nm. When normalized to the total amount of Ca$^{2+}$ released, the magnitudes of IP$_3$-induced Ca$^{2+}$ release were significantly greater in adult than in fetal basilar arteries, regardless of ambient Ca$^{2+}$ concentration (B and C). pD$_2$ values for the adult and fetal release curves (range: 6.00–6.18) did not vary significantly with age or ambient Ca$^{2+}$ concentration (C). Vertical error bars indicate SE for n ≥ 5. *Significant age-related differences (P < 0.05).](http://ajpcell.physiology.org/10.220.33.3)
Ca\textsuperscript{2+} distribution based on IP\textsubscript{3} and ryanodine sensitivity in permeabilized vascular preparations. When arteries were exposed first to 30 \textmu M IP\textsubscript{3} (Fig. 5, left), the Ca\textsuperscript{2+} fraction released was significantly greater in adult (63.8\%) than fetal (19.4\%) arteries, but the fraction released by subsequent 30 mM caffeine was significantly less in adult (4.0\%) than fetal (8.5\%) arteries. The remaining Ca\textsuperscript{2+} released by 10 \textmu M Br-A-23187 was then significantly less in adult (32.2\%) than in fetal (72.1\%) arteries. When arteries were exposed first to caffeine (Fig. 5, right) the Ca\textsuperscript{2+} released was significantly smaller in adult (8.8\%) than in fetal (15.0\%) arteries and subsequent release by IP\textsubscript{3} was significantly greater in adult (59.4\%) than in fetal (13.2\%) arteries. Br-A-23187 then released significantly less Ca\textsuperscript{2+} in adult (31.8\%) than in fetal (71.9\%) arteries.

The Ca\textsuperscript{2+} release results were used to define a series of linear equations with four unknowns, one for each Ca\textsuperscript{2+} pool. These equations assumed only that Br-A-23187 released all membrane-bound intracellular Ca\textsuperscript{2+}, that pool sizes were consistent within a given age group, and that agonist affinities were uniform across all receptors of a given type. The estimated sizes of the pool sensitive to both ryanodine and IP\textsubscript{3} were quite small (range: 5–7\% of total releasable Ca\textsuperscript{2+}), as were the sizes of the pool sensitive to ryanodine only (range: 4–10\% of total releasable Ca\textsuperscript{2+}) (Fig. 6). The pool sensitive to IP\textsubscript{3} only varied dramatically with age and was fourfold larger in adult than in fetal arteries, whereas the pool resistant to both ryanodine and IP\textsubscript{3} was twofold smaller in adult than in fetal arteries.

Total Ca\textsuperscript{2+} mass and pool size based on 45Ca uptake and washout. Repeated cycles of contraction and relaxation rapidly labeled all intracellular Ca\textsuperscript{2+} pools (Fig. 7A). Half-maximal labeling was achieved with 0.9 and 1.2 cycles in adult and fetal arteries, respectively (P =
not significant; Fig. 7B). Adult basilar arteries contained significantly less Ca$^{2+}$ mass on a dry weight basis (2.7 nmol/mg) than did fetal basilar arteries (12.2 nmol/mg). In contrast, the rate constant of washout of $^{45}$Ca was similar in adult and fetal arteries and averaged $0.016 \pm 0.001$ and $0.013 \pm 0.002$ s$^{-1}$, respectively.

To estimate the Ca$^{2+}$ mass in each pool, the relative pool size (Fig. 6) was multiplied by the intracellular Ca$^{2+}$ mass (Fig. 7B). The resulting absolute size of the pool sensitive to IP$_3$ only was similar in the adult (1.6 nmol/mg) and fetus (1.7 nmol/mg) (Fig. 8). All other pools, however, were significantly smaller in adult than fetal arteries. The pool sensitive to ryanodine only averaged 0.11 and 1.22 nmol/mg in adult and fetal arteries, respectively. Corresponding values for the pool sensitive to both IP$_3$ and ryanodine were 0.14 and 0.85 nmol/mg. The most dramatic age-related difference in pool size was obtained for the pool resistant to both IP$_3$ and ryanodine; here, the masses averaged 0.87 and 8.78 nmol/mg for adult and fetal basilar arteries, respectively.

**DISCUSSION**

The distribution of Ca$^{2+}$ within vascular smooth muscle is highly heterogeneous and dynamic and varies with artery type, size, and location (20, 29). Studies of this distribution have identified multiple Ca$^{2+}$ pools, the most important being those sensitive to IP$_3$ or ryanodine. The relative size and independence of these pools remain uncertain, however, as some studies suggest that IP$_3$ and ryanodine release a common Ca$^{2+}$ pool (30) whereas others suggest that IP$_3$ and ryanodine release functionally and spatially distinct Ca$^{2+}$ stores (18) (43). In addition, the impact of vascular maturation on these stores is uncertain, although it is clear that Ca$^{2+}$ distribution and handling are considerably different in the contractile and synthetic smooth muscle phenotypes (21, 44) and that contractility changes considerably during postnatal maturation (31). In relation to these issues, the present results offer two main findings. First, there are at least four functionally independent calcium stores in fetal and adult basilar arteries. Second, the relative sizes of each of these stores change significantly with postnatal development.

Maturation and cerebrovascular intracellular Ca$^{2+}$ mass. An essential step for measurement of absolute Ca$^{2+}$ pool size was determination of total intracellular Ca$^{2+}$ mass. The nonlinear tracer kinetic approach we used enabled estimates of mass in adult arteries (2.7 nmol/mg dry wt) consistent with published values (1–3 nmol/mg dry wt) (32) and also provided the first known estimates of fetal basilar Ca$^{2+}$ content. From contractility measurements, Nakanishi et al. (27) concluded that intracellular Ca$^{2+}$ content must be much smaller in adult than fetal mesenteric arteries. Brunette (5) also reported that accelerated skeletal development in late fetal and early postnatal life is supported by increased active transport of Ca$^{2+}$ across the placenta, which more than doubles tissue Ca$^{2+}$ during late gestation to values markedly greater than in adults. Against this background, our observation that basilar Ca$^{2+}$ content averaged more than fourfold less in adult than in fetal arteries appears reasonable and consistent with published evidence.

IP$_3$-sensitive Ca$^{2+}$ pool. Relative to total releasable Ca$^{2+}$, the IP$_3$-sensitive pool accounted for a much larger Ca$^{2+}$ fraction in adult (64%) than in fetal (19%)
arteries even though mass within the IP₃-sensitive pool was equivalent in adult and fetal arteries. Because pD₂ values for IP₃-induced Ca²⁺ release agreed with published values (15) but did not vary with age, the IP₃ receptors involved were probably of the same type and phosphorylation state in both age groups (41). Given that IP₃ receptor density changes little during ovine cerebrovascular maturation (49), the ability of IP₃ to release Ca²⁺ appears unaffected by maturation in ovine basilar arteries. Thus age-related changes in IP₃-induced Ca²⁺ release cannot explain the observed age-related differences in the coupling between IP₃ concentration and contractile response (Fig. 2). Because IP₃-induced Ca²⁺ release was similar at 150 and 300 nM Ca²⁺ (Fig. 4), differences in sensitivity to ambient Ca²⁺ concentration within the low physiological range also cannot explain age-related differences in the contractile efficacy of IP₃ (15, 19).

If sensitivity to IP₃ and the mass of Ca²⁺ released by IP₃ are similar in adult and fetal basilar arteries, then differences in coupling between IP₃ and contraction must involve some component downstream from Ca²⁺ release. This downstream component cannot be myofilament Ca²⁺ sensitivity, because this is significantly less in adult than in fetal arteries, particularly during agonist-stimulated contractions (1). Alternatively, Ca²⁺ release geometry may be more favorable for myofilament activation in adult than in fetal basilar arteries. Consistent with this possibility, smooth muscle cells have very different dimensions and volumes in adult and fetal ovine cerebral arteries (7). In addition, if IP₃-induced Ca²⁺ release is similar but depletion of the IP₃-sensitive pool is slower in adult than in fetal arteries (Fig. 2), the fetal SR must be less able to reserquester released Ca²⁺. Correspondingly, Ca²⁺ transport protein (sarcoplasmic or endoplasmic reticular Ca²⁺-ATPase, SERCA) abundance in cardiac SR is greater in adult than in fetal sheep (25).

_Ryanodine-sensitive Ca²⁺ pool._ Ryanodine produced concentration-dependent increases in cytosolic Ca²⁺ with similar pD₂ values in both age groups (Fig. 3), suggesting that the receptor involved was probably the same in both adult and fetal arteries. Because ryanodine released Ca²⁺ slowly (Fig. 1) and can hyperpolarize the plasmalemma through activation of Ca²⁺-sensitive potassium channels (17), we also used caffeine to release Ca²⁺ from the ryanodine-sensitive pool. Caffeine and ryanodine released Ca²⁺ from a common pool (Fig. 1; Ref. 20), although they activate the ryanodine receptor differently (48). Nonspecific effects of caffeine...
on adenosine receptors (4), phosphodiesterase (42), ion channels (13), or IP₃ receptors (24) should have been negligible at the caffeine concentration we used, particularly because caffeine was thoroughly washed out before subsequent treatments. Consistent with this view, the pool size estimates obtained with ryanodine (Fig. 3) and caffeine (Fig. 5) experiments were quite similar.

The ryanodine-sensitive pool contained a smaller fraction of total Ca²⁺ and less mass in adult compared with fetal basilar arteries (Figs. 6 and 8). Correspondingly, magnitudes and rates of ryanodine- and caffeine-induced contractions were significantly less in adult than fetal basilar arteries (Fig. 1), although some of this difference might be accounted for by reduced myofilament Ca²⁺ sensitivity in adult arteries (1). Ryanodine-induced contractions required extracellular Ca²⁺, as also recently reported for ovine middle cerebral artery (22). Thus ryanodine-induced calcium release stimulated Ca²⁺ entry either directly or through store-operated Ca²⁺ entry, as recently reported in renal smooth muscle (9). Whereas the small ryanodine-sensitive pool in adult arteries might easily be depleted and thus be likely to stimulate capacitative Ca²⁺ entry, this is less likely in fetal arteries, where the ryanodine-sensitive pool was markedly larger and thus harder to deplete. Age-related differences in ryanodine-sensitive pool size further suggest that the capacity for Ca²⁺-induced Ca²⁺ release should be less in adult than fetal basilar arteries. Undoubtedly, these age-related differences in ryanodine-sensitive Ca²⁺ release are species dependent, as ryanodine receptors are completely non-functional at birth in rat cerebral arteries (11) and are absent in proliferating cells of the rat aorta (44).

Overlap between IP₃ and ryanodine-sensitive Ca²⁺ pools. Despite reported overlap between ryanodine- and IP₃-sensitive pools (19, 30), other evidence suggests that these pools are functionally distinct (18, 43). More IP₃ receptors are found in central than in peripheral locations (47). Only ryanodine-sensitive Ca²⁺ pools near the sarcolemma mediate Ca²⁺-induced Ca²⁺ release (19), Ca²⁺ spark formation (17), and superficial buffer barrier function (19). Consistent with this functional specialization, caffeine failed to deplete the IP₃-sensitive pool and IP₃ did not deplete the caffeine-sensitive pool (Fig. 5) in ovine basilar arteries. Furthermore, the pool sensitive to both IP₃ and ryanodine averaged only 5–7% of total releasable Ca²⁺ regardless of age (Fig. 6). These data suggest functional separation between the IP₃- and ryanodine-sensitive Ca²⁺ pools in the ovine basilar artery. Consistent with the smaller ryanodine-induced contractions in adult arteries (Fig. 1), the pool sensitive to both IP₃ and ryanodine was smaller in adult (0.14 nmol/mg) than in fetal (0.85 nmol/mg) basilar arteries. This latter difference reflects a trend for less mass in adult than fetal artery Ca²⁺ pools.

IP₃ and ryanodine-resistant Ca²⁺ pool. Ca²⁺ mass in the pools insensitive to IP₃ and ryanodine was 10-fold smaller in adult than in fetal arteries (Fig. 8) but accounted for a major fraction of membrane-bound Ca²⁺ in both age groups (Fig. 6). The extra fetal Ca²⁺ was probably not within the mitochondria (39), golgi (46), or nucleus (10) and instead was most probably located within the endoplasmic reticulum (ER), which contains most second messenger Ca²⁺ in noncontractile cells (33). Consistent with this possibility, ER volume is generally smaller in mature than in immature arteries (27), which, in turn, reflects less synthetic activity and more smooth muscle cells with a contractile phenotype in adult compared with fetal arteries (28). Alternatively, this large fetal Ca²⁺ store may simply be an SR fraction without functional IP₃ or ryanodine receptors. In either case, vascular maturation and differentiation appear to be associated with loss of approximately two-thirds of all fetal vascular Ca²⁺, most of which is lost from a membrane-bound Ca²⁺ pool insensitive to either IP₃ or ryanodine. A major consequence of this large fetal noncontractile pool was that it dramatically reduced the fraction of cell Ca²⁺ in the IP₃-sensitive pool, even though the mass of IP₃-releasable Ca²⁺ was the same in fetal and adult arteries.

Functional implications. Similar to values reported for other preparations (12, 37), organellar Ca²⁺ concentrations averaged 141 and 62 μM in adult and fetal basilar arteries, respectively. These concentrations, combined with the observation that total organellar Ca²⁺ mass was much smaller in adult than fetal arteries, predicts that total organellar volume must be dramatically smaller in adult than fetal basilar arteries. The large fetal organellar volume, which possibly includes ER or SR without functional IP₃ or ryanodine receptors, appears to attenuate the ability of IP₃-released Ca²⁺ to induce contraction, even though the Ca²⁺ mass released is similar to that in the adult. Restriction of diffusion or compartmentalization (20, 36) may limit Ca²⁺ activation of contractile proteins less in mature than in immature arteries. Conversely, ryanodine-evoked Ca²⁺ release appears less effectively coupled to contraction in adult than in fetal arteries, perhaps because of a larger ryanodine-sensitive pool in fetal arteries. The larger fetal ryanodine-sensitive pool further suggests that Ca²⁺-induced Ca²⁺ release and Ca²⁺ spark formation may be enhanced in fetal arteries, but this has not been found in other preparations (11), suggesting that an immature vascular morphology, which includes a large organellar volume, may also influence the distribution and function of Ca²⁺ released from ryanodine-sensitive pools. More importantly, the present data indicate that in the ovine basilar artery Ca²⁺ is distributed among four functionally independent stores, with little overlap between the IP₃- and ryanodine-releasable pools. With maturation, intracellular Ca²⁺ mass falls dramatically in noncontractile pools, decreases modestly in ryanodine-sensitive pools, and remains relatively constant in IP₃-sensitive pools, clearly indicating that postnatal development has a dramatic impact on both the function and distribution of Ca²⁺ in ovine basilar arteries.
REFERENCES


