Maturation Enhances the Sensitivity of Ovine Cerebral Arteries to the ATP-Sensitive Potassium Channel Activator Lemakalim

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ABSTRACT. A wide variety of previous studies have demonstrated that arterial reactivity and contractility change dramatically during maturation. In light of recent findings that binding sites for glibenclamide, a ligand for ATP-sensitive potassium (K_{ATP}) channels, become more abundant with age in many tissues, the present studies examine the hypothesis that maturational changes in vascular reactivity involve changes in arterial electrophysiologic characteristics. To test this hypothesis, we determined the dose-response relation to lemakalim, a selective activator of K_{ATP} channels, in isolated endothelium-denuded segments of the second (2B, internal diameter $\approx 200 \mu\text{m}$) and fourth (4B, internal diameter $\approx 125 \mu\text{m}$) branches of middle cerebral arteries taken from newborn (3–7 d old) and adult sheep. At $100 \mu\text{M}$, lemakalim completely relaxed serotonin-induced tone in all arteries. However, $-\log \text{ED}_{50}$ values were 29 to 43 times greater in adult (2B, 7.15 ± 0.38 ; 4B, 6.61 ± 0.42) than in newborn (2B, 5.52 ± 0.25 ; 4B, 5.15 ± 0.24) segments. Correspondingly, Hill values were significantly smaller in adults (2B, 0.47 ± 0.17 ; 4B, 0.71 ± 0.30) than in newborns (2B, 1.40 ± 0.35 ; 4B, 3.30 ± 0.92). These findings demonstrate that K_{ATP} channels are less sensitive to activation in newborn than in adult cerebral arteries. Given the important influence of K_{ATP} channels on vascular tone, and their possible role in many cardiovascular responses, the present data suggest that maturational increases in the activity of K_{ATP} channels contribute significantly to age-related changes in cerebrovascular contractility. (*Pediatr Res* 35: 729–732, 1994)

Abbreviations

2B, second branch of the middle cerebral artery
4B, fourth branch of the middle cerebral artery
 K_{ATP} channels, ATP-sensitive potassium channels
 pD_2 , negative log of the concentration of a drug that produces half its maximal response

The transition from newborn to adult produces major changes in the contractile characteristics of most arteries. Newborn cerebral arteries, for example, generally have thinner walls, produce less total force, and have different sensitivities to receptor agonists than their adult counterparts (1). Aside from speculated differences in receptor densities or contractile protein content, the

mechanistic bases for these age-related differences in vascular reactivity remain unknown.

One possible explanation of such differences is that vascular electrophysiologic characteristics change with age. As shown in many studies, vascular contractility is tightly coupled to membrane potential, which in turn is dominated by membrane permeability to potassium such that changes in outward potassium current dramatically affect contractile responses to all receptor agonists (2). In adult arteries, one of the main channels carrying outward potassium current is the K_{ATP} channel (3). Activation of these channels by either decreases in ATP (4) or increases in ADP (5) hyperpolarizes the membrane, reduces voltage-sensitive calcium channel activity, and reduces the cytosolic calcium concentration available to support contraction (6). As shown by studies of $^{42}\text{K}^+$ efflux (7) and direct electrophysiologic measurements (8), these channels can be selectively activated by cromakalim, a benzopyran, and blocked by glibenclamide, a sulfonylurea.

Ligand-binding studies using derivatives of both glibenclamide and cromakalim indicate that K_{ATP} channels are ubiquitously but heterogeneously distributed among all types of electrically excitable tissues (9). From the few studies that have examined such binding in neonatal tissues, it seems that the density of these channels is very low at birth and increases with maturation (10). Functional studies of these channels in neonatal tissues, thus far limited to heart and lung tissues (11), also suggest the presence of these channels, but comparisons with corresponding adult tissues have yet to be made. Thus, the present studies are the first to examine K_{ATP} channel function, as reflected by the dose-response relation for lemakalim, the maximally active stereoisomer of cromakalim, in corresponding newborn and adult arteries. Owing to the high incidence of cerebrovascular complications in neonates (12), resistance arteries from the cerebral circulation were examined.

MATERIALS AND METHODS

All procedures and protocols were approved by the Animal Research Committee of Loma Linda University and followed all guidelines put forth in the NIH *Guide for the Care and Use of Laboratory Animals*. We obtained segments of the second and fourth branches of middle cerebral arteries from newborn lambs (5–7 d old) and nonpregnant adult sheep (18–24 mo of age), removed external adipose and connective tissue, removed the endothelium by passing a small-diameter wire through the lumen of the vessel a number of times, then cut the arteries into ring segments 1 mm long. We selected segments with an internal diameter of approximately $200 \mu\text{m}$ from 2B and segments with an internal diameter of approximately $130 \mu\text{m}$ (4B) and mounted them on $24\text{-}\mu\text{m}$ diameter tungsten wires between a low-compliance transducer (BG-10, Kulite Semiconductors, Ridgefield, NJ) and a micrometer used to vary baseline stretch. We obtained

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micrometer readings, and thereby diameter measurements, for each arterial segment under unstressed conditions (at ≈ 7 mg tension). Inside vessel diameter was calculated as twice the distance between the two wires divided by π . The artery segments were then stretched to optimal lengths of 2.2 (newborn 2B), 2.7 (newborn 4B), 2.9 (adult 2B), and 3.3 (adult 4B) times unstressed diameter, as previously described (13).

Before protocol initiation, we equilibrated the arteries at 38.5°C for at least 30 min in a bath continuously bubbled with 95% O₂–5% CO₂ and superfused with bicarbonate Krebs solution containing (in mM): 122 NaCl, 5.56 dextrose, 25.6 NaHCO₃, 5.17 KCl, 2.49 MgSO₄, 1.60 CaCl₂, 0.114 ascorbic acid, and 0.027 disodium EDTA. During all experiments the contractile tensions were continuously digitized, normalized, and recorded by an on-line computer. After initial equilibration, we determined endothelial integrity by exposing arteries contracted with 1 μ M serotonin to either 1 μ M acetylcholine or 10 μ M ADP. We discarded arteries that relaxed more than 10% in response to either of these agents.

Dose-response protocol. Preliminary cumulative dose-response experiments with lemakalim indicated that prolonged exposure leads to tachyphylaxis and loss of the dose-response relation. Thus, in these dose-response experiments we repeatedly contracted the arteries, then exposed them to a single concentration of lemakalim after each contraction. After equilibration of the artery segments at baseline tension, we first contracted them with an isotonic Krebs solution containing 120 mM potassium and then returned them to resting conditions. Approximately 15 min later, we contracted the arteries with 1 μ M serotonin. When the contraction stabilized, we added 0.01 nM lemakalim and recorded the ensuing response. Thereafter, the segments were rested and washed in normal sodium Krebs solution for 15 min, then contracted again with 1 μ M serotonin. Lemakalim was again added, but this time at 0.10 nM. Using this wash-contraction-relaxation cycle, we obtained relaxation responses to lemakalim over the concentration range of 10⁻¹¹ to 10⁻³ M. After this series of measurements, the arteries were again washed in normal Krebs solution for 30 min, and then contracted again with 120 mM potassium Krebs solution to verify that the segments had not fatigued. Next, the segments were washed again in normal sodium Krebs solution, then exposed for 30 min to 30 μ M glibenclamide. After glibenclamide incubation, the arteries were contracted with 1 μ M serotonin and then exposed to 1 μ M lemakalim.

Statistics and calculations. We calculated and expressed relaxant responses as the percent relaxation of the maximum initial tone attained just before the addition of lemakalim. The maximum percent relaxation, pD₂ (–log ED₅₀), and Hill coefficient (related to slope) for each dose-response relation were determined by fitting the dose-response data with the logistic equation using computerized nonlinear regression. For all variables, we verified homogeneity of variance using Bartlett's test. For maximum percent relaxation, pD₂, and Hill coefficient, we used two-way analyses of variance with maturational age (newborn or adult) and artery size (second or fourth branch) as the factors. We evaluated the effects of repeated contraction with serotonin and potassium using a repeated measures analysis of variance. Individual post-hoc differences between groups were analyzed using Duncan's multiple range test. Unless otherwise indicated, statistical significance implies $p < 0.05$.

RESULTS

All values are given as the mean $\pm$ SEM. Eight newborns and eight adults provided a total of 64 artery segments. Newborn segment diameters averaged 199 $\pm$ 4 and 125 $\pm$ 4 μ m in the 2B and 4B segments, respectively. Corresponding adult values were 193 $\pm$ 4 and 123 $\pm$ 5 μ m. Artery diameters did not vary significantly between newborns and adults in either branch order.

To verify that repeated contractions and relaxations did not

impair contractility, we compared the magnitudes of the first and last contractions to potassium in each artery group. The first and last contractions did not vary significantly in any group and agreed within an overall average difference of <8%. The average responses to potassium in the newborn were 460 $\pm$ 73 mg and 424 $\pm$ 59 mg in 2B and 4B segments, respectively. Corresponding adult values were significantly greater and averaged 681 $\pm$ 95 mg and 634 $\pm$ 111 mg. Similarly, we analyzed the magnitudes of the repeated responses to serotonin and found that these responses did not vary significantly over time in any group. Average newborn responses to serotonin were 347 $\pm$ 62 mg and 337 $\pm$ 32 mg in 2B and 4B segments, respectively. Corresponding adult values were not significantly different and averaged 413 $\pm$ 86 mg and 405 $\pm$ 31 mg.

Maximal concentrations of lemakalim completely relaxed serotonin-induced tone in all groups (Fig. 1). The percent relaxation observed at 100 μ M lemakalim in the newborn averaged 97 $\pm$ 1% and 98 $\pm$ 1% in the 2B and 4B segments, respectively. Corresponding adult values were 96 $\pm$ 3% and 92 $\pm$ 4%. In contrast, the pD₂ values for lemakalim changed dramatically with maturation (Fig. 1). Newborn pD₂ values averaged 5.52 $\pm$ 0.25 (2B) and 5.15 $\pm$ 0.24 (4B). Corresponding adult pD₂ values were significantly greater and averaged 7.15 $\pm$ 0.38 and 6.61 $\pm$ 0.42. The pD₂ values did not vary significantly between 2B and 4B segments in either age group.

Coincident with the age-related changes in pD₂ were marked changes in the Hill coefficient. As calculated in the present study, the Hill coefficients quantify the slope of the relation between input (drug concentration) and output (relaxation response) and may be taken as a simple index of dose-response "gain." Newborn Hill coefficients averaged 1.40 $\pm$ 0.35 (2B) and 3.30 $\pm$ 0.92 (4B). Corresponding adult Hill values were significantly smaller and averaged 0.47 $\pm$ 0.17 and 0.71 $\pm$ 0.30. Hill values did not vary significantly between 2B and 4B segments in either age group.

In all groups, 30 μ M glibenclamide blocked the relaxation responses induced by 1 μ M lemakalim. In the presence of 30 μ M glibenclamide, lemakalim produced relaxations averaging only 2.2 $\pm$ 2.7% and 0.6 $\pm$ 2.9% in the newborn 2B and 4B segments, respectively. Corresponding adult values were 4.1 $\pm$ 4.3% and 9.8 $\pm$ 2.2%.

DISCUSSION

K_{ATP} channels have now been demonstrated in most excitable tissues, including the cerebral arteries of many laboratory animal species (14). In these studies, activation of K_{ATP} channels has consistently produced hyperpolarization and vasodilation. The cellular mechanisms mediating these responses do not seem to involve either cyclic nucleotides (15) or pertussis toxin-sensitive G proteins (16). Functional studies further suggest that K_{ATP} channels may help govern resting membrane potential and basal tone in pulmonary arteries of the adult (17) but not the newborn (11). Other studies of adult animals indicate that K_{ATP} channels may also play a role in the acute vasodilation associated with reactive hyperemia (18), septic shock (19), hypoxia (20), and blood flow autoregulation (21). Evidence that these channels may be involved in long-term adaptations to conditions such as diabetes (22) has also recently been obtained.

The broad range of physiologic responses in which K_{ATP} channels may participate has motivated recent efforts to find endogenous activators of these channels. Several candidates have thus far been identified in adult tissues, the most important of which seems to be endothelium-derived hyperpolarizing factor, which activates K_{ATP} channels in some vascular beds (23) and other types of potassium channels in others (24). The vasodilator, calcitonin gene-related peptide, also seems to activate K_{ATP} channels in some (2) but not all (25) arteries. Responses to a growing variety of other compounds have also been suggested to involve activation of K_{ATP} channels including arachidonic acid (26),

Fig. 1. Effects of maturation on lemakalim dose-response relations. Dose-response data for lemakalim, expressed as percent relaxation of initial contractile tone induced by $1 \mu\text{M}$ serotonin, is shown in the upper panels. Maturation was associated with a substantial left-shift in the lemakalim dose-response relation for both second branch (upper left panel) and fourth branch (upper right panel) segments taken from newborn and adult sheep. These data were fitted with the logistic equation to determine values for pD_2 ($-\log \text{ED}_{50}$), Hill coefficient, and maximum percent relaxation. For all artery groups, the maximum percent relaxation was close to 100%. Average values of pD_2 and the Hill coefficient are shown in the lower panels. For all panels, error bars indicate SEM for the numbers of animals indicated in parentheses and asterisks indicate statistically significant differences at the $p < 0.05$ level. Maturation was associated with a marked elevation in the pD_2 and a depression of the Hill coefficient in both artery sizes.

epoxyeicosatrienoic acids (27), adenosine (28), endothelin (29), and superoxide (30).

Although K_{ATP} channels are ubiquitously distributed and may play a role in a broad variety of responses, the sensitivity of these channels to cromakalim in adult tissues seems to be quite uniform. Across eight recent studies, which together determined the dose-response relation for cromakalim in adult arteries from five different vascular beds in four different species (7, 14, 31–36), pD_2 values ($-\log \text{ED}_{50}$) were remarkably uniform and averaged 6.73 ± 0.13 . This value agrees well with the adult pD_2 values of 6.61 ± 0.42 (2B) and 7.15 ± 0.38 (4B) obtained in the present study. Also consistent with previous findings is our observation that maximal concentrations of lemakalim produced complete relaxation in all artery types.

In contrast to results obtained in adult arteries, our determinations of the lemakalim pD_2 values in newborn arteries were markedly lower and averaged 5.52 ± 0.25 (2B) and 5.15 ± 0.24 (4B) (Fig. 1). Because receptor sensitivity is determined by both receptor number and affinity, the low newborn pD_2 values we obtained may be explained by a reduced density of K_{ATP} channels given previous findings that glibenclamide binding sites are more sparse in newborn than adult tissues (10). Consistent with this interpretation are the significant age-related decreases in Hill coefficient we observed. Although the Hill coefficient data cannot rule out age-related increases in the affinity of these channels for lemakalim, the Hill data are more compatible with maturational decreases in affinity. Full appreciation of the exact reasons why pD_2 values for lemakalim were depressed in the newborn arteries must await future parallel determinations of K_{ATP} channel density and affinity for lemakalim in both newborns and adults.

From a functional perspective, the present data suggest that K_{ATP} channels are less sensitive to physiologic activation in newborn than in adult cerebral arteries. If so, these findings suggest that basic electrophysiologic differences may underlie many age-related differences in cardiovascular responses. Be-

cause the present data were obtained from arteries less than $200 \mu\text{m}$ in diameter, our findings further suggest that these age-related differences in electrophysiologic characteristics reside in the small resistance arteries where most vascular regulation occurs. If K_{ATP} channels are indeed less abundant in newborn than adult arteries, then other potassium channels, such as the large-conductance calcium-activated channel (37), may regulate the outward current normally carried by K_{ATP} channels in adult arteries and therefore may also function differently with age. Clearly, the finding that K_{ATP} channels are less sensitive to activation in newborn than in adult arteries has many functional implications. Fortunately, a growing diversity of pharmacologic tools is simplifying the study of these channels (38) and should in the near future help elucidate the diverse and important roles these channels play in functional cardiovascular maturation.

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