Decreased Expression of Voltage- and Ca^{2+}-Activated K^{+} Channels in Coronary Smooth Muscle During Aging

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Abstract—Aging is the main risk factor for coronary artery disease. One characteristic of aging coronary arteries is their enhanced contractile responses to endothelial vasoconstricting factors, which increase the risk of coronary vasospasm in older people. Because large-conductance voltage- and Ca^{2+}-activated K^{+} channels (MaxiK) are key regulators of vascular tone, we explored the possibility that this class of channels is diminished with increasing age. Using site-directed antibodies recognizing the pore-forming α subunit and electrophysiological methods, we demonstrate that the number of MaxiK channels is dramatically diminished in aged coronary arteries from old F344 rats. Channel density was reduced from 52±9 channels/pF (3 months old) to 18±5 channels/pF (25 to 30 months old), which represents a 65% reduction in the older population. Pixel intensity of Western blots was also diminished by ≈50%. Moreover, the age-related decrease in the channel protein expression was also evident in humans, which showed ≈80% reduction in 61- to 70-year-old subjects compared with 3- to 18-year-old youngsters and ≈45% reduction compared with 19- to 56-year-old adults. In agreement with a reduction of MaxiK channel numbers in aging coronary arteries, old coronary arteries from F344 rats contract less effectively (≈70% reduction) than young coronary arteries when exposed to the MaxiK channel blocker iberiotoxin. The contraction studies indicate that under physiological conditions, MaxiK channels are tonically active, serving as a hyperpolarizing force that opposes contraction. Thus, reduced expression of MaxiK channels in aged coronary arteries would lead to a decreased vasodilating capacity and increased risk of coronary spasm and myocardial ischemia in older people. (Circ Res. 2001;88:210-216.)

Key Words: vascular smooth muscle ■ coronary arteries ■ K^{+} channels ■ Ca^{2+}-activated K^{+} channels ■ aging

Aging is a main risk factor for coronary heart disease, which remains the leading cause of death in developed countries.¹ In human coronary arteries, spontaneous contractile activity seems to be more frequent in older subjects,²,³ increasing the risk of vasospasm. In addition, there is evidence that aging induces increased responses of rat coronary and mesenteric arteries to endothelial constricting factors and K⁺.⁴-⁶ The increase in K⁺-induced contractions in aging animals suggests a change in K⁺ channel function or expression as age progresses. Coronary arteries possess several types of K⁺ conductances; the large-conductance, voltage-dependent, and Ca^{2+}-activated K⁺ channel (MaxiK, BK) is particularly abundant and plays a key role in regulating arterial tone.⁷-¹⁰

MaxiK channels regulate membrane potential and intracellular Ca^{2+} ([Ca^{2+}]) in various smooth muscles¹¹,¹² (compare with Archer et al¹³ and Cornfield et al¹⁴). Under basal conditions, MaxiK channels are tonically active and act as a hyperpolarizing force that reduces the activity of voltage-dependent Ca^{2+} channels and Ca^{2+} influx and, thus, oppose contraction.¹² In fact, MaxiK blockade causes smooth muscle contraction.¹⁰,¹¹ Given the crucial role of MaxiK in setting the point of vascular contractility, we examined the possibility that these channels are diminished in number in aging coronary arteries. A decrease in MaxiK channel expression would provide a mechanism to explain coronary increased excitability as age progresses.

In the present study, we show that expression of MaxiK channel protein in aging coronary smooth muscle is substantially decreased not only in rats but also in humans. Functional (pharmacomechanical and electrophysiological) experiments were confirmed with immunochemistry using a polyclonal antibody raised against an intracellular epitope of the pore-forming α subunit (slo) of MaxiK channels.⁷

Materials and Methods

F344 male rats (young, 3 months old; old, 25 to 30 months old) were killed according to a protocol approved by UCLA. Human coronary arteries from explanted hearts were used. The protocol received institutional review committee approval. Demographic data for human subjects are presented in the Table.

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Isometric Contraction

Arterial rings (inner diameter 0.15 to 0.3 mm, 3 mm long) were equilibrated ~60 minutes in Krebs solution. Before experimentation, rings were progressively stretched to an optimal tension (maximal increase in tension in response to KCl). Optimal wall tension was ~120 mg for young and ~200 mg for old coronary arteries. KCl (80 mmol/L)-induced contraction was similar in 33 of 40 old rings (68±12%, n=33 rings) compared with young coronary rings (66±10%, n=45 rings). Consistent with previous studies, the mean value of all old samples gave significant differences (123±19% in old, n=40 versus 66±10% in young, n=45). Thus, data were normalized to the response of each ring to 80 mmol/L KCl. Equivalent results were obtained with or without normalization.

Cell Isolation

Single coronary myocytes were used within 6 to 8 hours after isolation. Vessels (~1-mm pieces) were incubated for 15 minutes at 37°C with Ca2+-free Hanks’ solution containing 30 U/mL papain, 2 mg/mL BSA, and 1 mmol/L dithiothreitol followed by 10 minutes with 2.1 U/mL collagenase F and 0.2 U/mL collagenase H.

Electrophysiology

Whole-cell currents were measured using patch electrodes (3 to 5 MΩ) filled with (in mmol/L) potassium methanesulfonate (Mes) 140, CaCl2 0.1, MgCl2 2, HEPES 10, glucose 10, and EGTA 0.146, pH 7.4, pCa 7, with/without 150 μg/mL nystatin. The bath solution was (mmol/L) KMes 5, NaMes 135, CaCl2 0.1, MgCl2 2, HEPES 10, and glucose 10, pH 7.4. Data acquisition and analysis were performed using pCLAMP (Axon Instruments).

Nonstationary variance analysis was performed using 1 mmol/L 4-aminoypyridine and/or maintaining the cells at a depolarized holding potential (HP) to avoid a small Kv current component (<7%). To attain maximal open probability (Po), experiments were performed in the presence of the MaxiK channel opener NS1619 (50 to 100 μmol/L). NS1619 eliminated the need to use a high concentration of [Ca2+], or extremely high potentials (≥200 mV) that would otherwise damage the cells. Data were fit using the following equation:

\[ \sigma^2_i(t) = I_{n_0}i(t) - \frac{1}{N} \]

where \( \sigma^2_i(t) \) is the variance as a function of time, \( I_{n_0} \) is the mean current as a function of time, \( i \) is the unitary current, and \( N \) is the total number of functional channels. Limiting Po was calculated from \( Po = I_{n_0}i(t)/N \); it was identical in young and old coronary arteries (0.64±0.06 versus 0.65±0.04).

Tissue Lysates, Membrane Preparations, and Immunohistochemistry

Rat coronary and pulmonary arteries were homogenized in ~50 μL ice-cold hypotonic buffer containing 20 mmol/L HEPES-KOH and 1 mmol/L EDTA, pH 7.4, and supplemented with 0.1 mmol/L phenylmethylsulfonyl fluoride, 1 μmol/L pepstatin A, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 10 mmol/L CHAPS and centrifuged at 1000g. The solubilized protein was directly used for immunoblotting. Human membrane preparations, immunoblotting, and immunohistochemistry were as described previously.
supports the idea that changes in current density are likely larger in young coronary myocytes. This result strongly equivalent bulk Ca$^{2+}$.

Under the same experimental conditions and with capacitance in single coronary myocytes from old and young shows typical whole-cell MaxiK currents normalized to cell

subunit with specific antibodies (see Figure 4). Figure 2A functional MaxiK channels using electrophysiology (Figure

To test the hypothesis that during aging MaxiK channels are reduced in numbers, we directly quantified the number of MaxiK channels are decreased during aging in

aging rats (Figure 1B) versus young rats (Figure 1A). This dramatic difference is plotted in Figure 1C where the maxi-
difference in contraction force were calculated using $\frac{|F_i - F_0|}{F_0} \times 100$, where $F_0$ is the final force and $F_i$ is the initial force. Dose-response curves were well-fitted to a Hill function (continuous line): $\frac{\text{max}}{1 + \left(\frac{E_{C50}}{|IbTx|}\right)^n}$, where $N$ is the Hill coefficient. Mean values were as follows: young (●), EC$_{50}$=9±1 nmol/L, N=1.3±0.15, maximum contraction=100±4% (n=13 rats, 13 to 25 rings for each point); old (○), EC$_{50}$=15±6 nmol/L, N=1.5, maximum contraction=19±3% (n=11 rats, 15 to 22 rings for each point). Maximum contraction was significantly reduced by ~70% in old animals. Unless otherwise stated, in this and subsequent figures, values are mean±SE. Student’s t test was used. $P<0.05$ was considered statistically significant.

Number of functional MaxiK channels decreases in aging coronary myocytes. A, Records illustrate whole-cell current density (100 nmol/L IbTx-sensitive component) from young and old rats. Pulses: −100 to 100 mV every 10 mV. Currents were normalized to the capacity C (in pF) of each cell.Inset, Mean MaxiK current density-voltage relationships: young (●), n=10; old (○), n=7. B and C, Examples of variance versus mean current plots. Whole-cell perforated patches. Data were well-fitted (continuous line) to Equation 1 with young (B), N=1146 channels, i=10 pA (expected i=22 pA); old (C), N=411 channels, i=9.5 pA (expected i=28 pA). The reduced single-channel current can be explained by internal blockade by Mg$^{2+}$.

Insets, Mean current and variance during the pulse. Panel B inset, Limiting Po=0.77, C=16.5 pF, pulse=100 mV, and HP=−30 mV. Panel C inset, Limiting Po=0.8, C=16 pF, pulse=150 mV, and HP=−60 mV. Cell capacity was practically the same in young and old cells: young, 16±1 pF (n=9); old, 20±4 pF (n=10). D, Mean number of active channels/pF. Old coronary arteries have lower functional channel density than young coronary arteries. *Statistically different in this and subsequent figures.

due to differences in channel expression rather than changes in bulk [Ca$^{2+}$] levels in young versus old coronary myocytes. The inset in Figure 2A shows the mean current density versus voltage plot, demonstrating that, at each potential, old coronary myocytes have a lower current density than young coronary myocytes.

The number (N) of functional MaxiK channels in single myocytes was directly assessed using the nonstationary variance analysis. In this method, a limiting Po value larger than 0.5 has to be reached to adequately fit the variance versus mean current curve and obtain N (see Materials and Methods). Hypothetical changes in [Ca$^{2+}$], may change the pulse-voltage values necessary to reach limiting Po but will not modify the shape of the variance versus mean current plot and thus will not change the fitted N values. Therefore, N values obtained with this method are independent of [Ca$^{2+}$], and therefore possible changes in [Ca$^{2+}$] levels in young versus old would not affect the results. To estimate the number of functional channels, cells were repetitively pulsed to 100 to 150 mV for 50 ms, and variance versus mean current curves were constructed. Figure 2B shows an example of the nonstationary noise analysis in a young cell. The inset shows superimposed traces of the corresponding mean current and variance. In this cell, we estimated a total of 1146 active channels. In comparison, a cell from an old coronary
artery had ≈400 channels (Figure 2C). The number of functional channels obtained from the nonstationary variance analysis was normalized to the capacity of each cell (Figure 2D). It is clear that myocytes from old coronary arteries possess a lower number of functional channels (18±6 channels/pF, n=10 cells; 5 rats) than those from young arteries (52±9 channels/pF, n=9 cells; 4 rats), with a 65% reduction in the older population.

MaxiK Channel Antibodies Reveal Loss of Protein in Aging Coronary Arteries From F344 Rats and Humans

Western blot analysis was consistent with the functional measurements. Labeling was performed with an affinity-purified polyclonal antibody targeted to the carboxyl terminus of the MaxiK pore-forming α subunit.7 Figure 3A demonstrates that the amount of MaxiK protein with a molecular mass of ≈120 kDa is less in old than in young coronary arteries. Normalized optical density (OD) was 0.74±0.08 (n=7) for young coronary arteries, whereas for old coronary arteries it was 0.4±0.05 (n=7). Interestingly, this age-dependent diminution was not observed in cell homogenates from pulmonary arteries (young, 0.8±0.1 versus old, 0.76±0.08; n=4). As an additional control, we used the same blots to determine the relative amount of protein kinase G (PKG)-I in old and young coronary arteries. A polyclonal antibody against the mammalian PKG-Iα and PKG-Iβ isoforms recognized a doublet in coronary preparations (≈75 to 80 kDa); whereas, a single band was labeled in the pulmonary artery. These results suggest that rat coronary arteries express both PKG-Iα and β isoforms.22 Quantification by densitometry showed that although there is a small tendency of PKG to diminish in old vessels (coronary and pulmonary), the differences were not significant. The blots in Figures 3A and 3B also show the specificity of the anti-MaxiK and anti-PKG antibodies when the antigenic peptides were preadsorbed to the antibodies.

In agreement with the Western blot and functional studies, immunocytochemistry of freshly isolated cells and of coronary rings demonstrated that aged coronary arteries expressed a reduced amount of MaxiK α-subunit protein. Figure 4A shows examples of isolated cells used for patch-clamp recordings labeled with anti-MaxiK antibody, whereas Figure 4B illustrates images of the MaxiK channel signals obtained...
in coronary rings. Quantification of the pixel intensity in the smooth muscle layer was performed in paired experiments and demonstrated that the level of expression of MaxiK channels in aging coronary arteries is ∼50% of the channels expressed in young animals. Pixel intensity was 40±6 (n=4) in young coronary arteries versus 21±5 (n=4) in old coronary arteries.

Moreover, when we examined human coronary samples from explanted hearts, there was a correlation between age and expression levels of MaxiK channels as examined by Western blotting. Figure 5 shows that the density of MaxiK channel protein (normalized to PKG) is diminished with advancing age. Normalized OD was 0.55±0.1 (n=7) for the young (3 to 18 years old); it decreased to 0.3±0.04 (n=16) in the adult population (19 to 56 years old), and was further suppressed to 0.1±0.02 (n=10) for older subjects (61 to 70 years old). Antibodies were as in Figure 3.

Discussion

Aging is known to affect cellular excitability, producing impaired cell function. Major age-related diseases are neuro-

degenerative, cardiovascular, and immunological. In the aging brain, the Ca^{2+} hypothesis postulates that aging induces sustained changes in cellular mechanisms of [Ca^{2+}], regulation. Cellular mechanisms that can alter both excitability and Ca^{2+} homeostasis are voltage-dependent ion channels. Consistent with this view, in hippocampal neurons, aging produces an increase in the number of L-type Ca^{2+} channels. In addition to a massive increase in [Ca^{2+}], that may lead to cell death, modification of Ca^{2+} entry through this class of channels may also promote altered gene transcription. In other systems, such as skeletal muscle where aging produces a decline in muscle performance, MaxiK channels are increased in aged animals. However, their physiological role in skeletal muscle is unclear; therefore, the impact of MaxiK increased expression in the musculoskeletal system is difficult to predict.

In contrast to skeletal muscle, the role of MaxiK channels in the vasculature is much more defined, where they are thought to be key regulators of vascular tone. Despite their relevance in vascular physiology, and that aging is the main risk factor for coronary artery disease, changes in MaxiK channel expression during aging had not been previously addressed. In this work, we demonstrate for the first time that aging induces a reduction in MaxiK channel expression in coronary myocytes. Because this class of channels regulates both [Ca^{2+}], and membrane potential, their reduction in aged subjects is likely to produce a deleterious physiological impact, leading to vascular disease. In addition, MaxiK channels are important therapeutic targets that mediate, at least in part, the vascular effects of nitric oxide and nitroglycerin. Thus, the age-dependent decrease in density of MaxiK channels has implications in the treatment of cardiovascular disorders predominantly affecting the older population. The finding that MaxiK channels are reduced during aging prompts the question whether other coronary ion channels are affected during aging.

In human coronary vessels, MaxiK channels are mainly formed by the pore-forming α subunit and regulatory β subunits (likely β₁). Recently, lacZ gene expression in a β₁ gene–targeted mice demonstrated that β₁ is also expressed in coronary vessels of this animal species. β₁ subunits do not form MaxiK channels themselves but increase their Ca^{2+}/V sensitivity and slow down their activation kinetics. The increased Ca^{2+} sensitivity conferred by the β₁ subunit is thought to allow the MaxiK α subunit to open in response to local Ca^{2+} changes and maintain vascular tone. In agreement, cerebral arteries of β₁ knockout mice have higher tone and lose their ability to constrict in response to the pore blocker IbTx. In this work, we have demonstrated the reduction in the number of functional MaxiK channels (Figures 1 and 2) and a reduction of the MaxiK α-subunit protein in both rat and human coronary arteries from old subjects (Figures 3 through 5). At present, we do not know the nature of the MaxiK β subunit(s) in rat coronary arteries or whether its (their) expression changes with aging. Nevertheless, it is likely that MaxiK channels from rat coronary myocytes are assembled by αβ₁ subunits because they do constrict in the presence of IbTx (Figure 1). Moreover, it seems that the ratio of αβ₁ subunits is unaltered in aged
coronary arteries because MaxiK current activation kinetics were practically identical in both old and young populations (not shown).

It is interesting to note that aging seems to produce tissue-specific changes in channel expression. As mentioned above, in skeletal muscle, aging (5- to 7- versus 24- to 26-month-old rats) produced an increased MaxiK expression,29 whereas aging produced a reduction of channel density in coronary arteries (Figures 2 through 5; 3 versus 25 to 30 months old). In corporal smooth muscle, MaxiK channels also seem to diminish with age because rat erectile dysfunction of retired breeders (>9 months old) can be restored by MaxiK gene transfection to normal levels.38 Moreover, the age-dependent change is not uniform among different vascular beds, because in pulmonary artery MaxiK expression is not altered significantly when old versus young vessels were compared (Figure 3). The mechanism for this tissue-specific aging-related change in channel density is unknown. It would be interesting to determine whether sexual hormones that diminish during aging39 trigger these changes.

The number of functional channels present in single coronary myocytes from young rats (3 months old) was estimated at ≈1000 channels/cell. Considering a rather even distribution of MaxiK channels, as observed in immunochemical experiments (Figure 4A), the channel density in rat coronary myocytes is ≈0.5 channels/μm². This channel density is smaller than that calculated for human coronary arteries (4 channels/μm²),7 toad stomach (1 channel/μm²),40 and for rabbit jejunum (2 channels/μm²).41 The variation in channel density across species and smooth muscles may reflect differences in the relative contribution of MaxiK channels in the regulation of muscle tone. In this respect, it is noteworthy that humans possess ≈8 times more MaxiK channels than rats. However, other factors that alter MaxiK channel activity, such as resting membrane potential, intracellular pathways, and Ca²⁺ may also contribute to the regulatory role of these channels in different species and vascular beds.

The decrease in channel density of young versus old coronary arteries, as measured in single cells with electrophysiological methods (Figure 2), was corroborated with immunochemical experiments (Figures 3 through 5). The use of intact coronary vessels eliminated the possibility that variations in the enzyme treatments used to isolate old and young coronary cells were differentially affecting MaxiK density.

In summary, our results, using several independent measurements, demonstrate that MaxiK channels are diminished in aging coronary arteries in rats and in humans. We propose that a diminution in the numbers of MaxiK channels leads to a decrease in the normal tonic hyperpolarizing force provided by the activity of these channels in coronary arteries and, thus, may contribute to the increased risk of coronary spasm in older people. In general, modifications in expression of ion channels that regulate cell excitability most likely contribute to a disrupted (impaired) cell (organ) function (viability) in older people. Our results also reveal MaxiK channels as important therapeutic targets to alleviate vascular disease.

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