Journal of Gerontology: BIOLOGICAL SCIENCES
1999, Vol. 54A, No. 4, B154–B159

Impaired Cholera Toxin Relaxation With Age in Rat Aorta

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Beta-adrenergic–mediated vasorelaxation declines with maturation and aging. Available data suggest that impaired stimulatory G-protein function could explain this deficit. We have previously found a loss of cholera toxin (CT)-stimulated adenosine diphosphate (ADP) ribosylation with age in rat aortic membrane preparations, without evidence for loss of the stimulatory alpha subunit of G protein (Gsα) by immunoblotting. The purpose of this investigation was to determine if cholera toxin–mediated vasorelaxation was also impaired with age. Aortic ring segments from 6 weeks, 6 months, 12 months, and 24 months old male F-344 rats were used. Contraction to KCl and phenylephrine was assessed along with relaxation to cholera toxin (azide-free), isoproterenol, and forskolin. There were no age-related changes to KCl or phenylephrine contraction. There was a significant decrease with age in relaxation to isoproterenol. This loss with age was significantly greater with KCl-preconstricted vessels than phenylephrine-preconstricted vessels. There were no age-related changes in the relaxation to forskolin. There was a significant decrease with age in the maximal relaxation to cholera toxin as well as a rightward shift in the dose-response curve. Cholera toxin–stimulated adenosine 3', 5'-cyclic phospate (cAMP) levels were measured and there was no increase in cAMP levels surrounding the time period associated with relaxation induced by cholera toxin. These data suggest that different preconstricting agents markedly affect the age-related changes in beta-adrenergic–mediated vasorelaxation. Furthermore, they suggest that the mechanism of cholera toxin–mediated vasorelaxation may not be mediated through increases in cAMP concentration.

RESPONSIVENESS to beta-adrenergic receptor (β-AR) stimulation has been shown to be decreased with aging in a variety of tissues including the heart, brain, parotid gland, and lung (1). Age-related changes have also been studied in vascular tissue. The loss of beta-adrenergic–mediated vasodilation with aging has been reported in rat aorta, mesenteric artery, and pulmonary artery (2,3), rabbit and guinea pig aorta (4,5), and human dorsal hand vein (6). Vasodilation to non–β-AR stimuli, including endothelium-dependent agonists such as acetylcholine, and endothelium-independent agonists such as nitrates, do not appear to decline with aging (7,8). Vasoconstriction appears to be generally unaffected by aging (9). The diminished responsiveness to β-AR agonists coupled with the maintenance of vasoconstrictor mechanisms, may contribute to the rise in peripheral resistance, orthostatic hypotension, and arterial insufficiency prevalent in older patients. The cellular mechanisms involved in the loss of β-AR vasodilation with aging have been studied; however, the mechanism of the impairment has yet to be characterized.

In the beta-adrenergic system, β-AR agonists bind to vascular smooth muscle cells transmembrane receptors which are coupled to adenylyl cyclase via interaction with a stimulatory G protein alpha subunit (Gsα). This activated Gsα activates adenylyl cyclase which converts adenosine triphosphate (ATP) to the cyclic nucleotide second messenger, cyclic adenosine monophosphate (cAMP). The resultant rise in intracellular cAMP induces vasorelaxation through the activation of cAMP-dependent protein kinase (3,10,11). This signal cascade has been explored in an attempt to determine the site or sites of the age-dependent declines in responsiveness. There appears to be no loss (12) or only a slight decrease (2) in β-AR density with aging. However, the percentage of β-ARs in the high-affinity state does appear to decline with age (2). Also, we (13) and others (14) found no changes in the Gi or Gs expression levels with age. Direct activation of adenylyl cyclase by forskolin (FSK) in vessels causes vasodilation that is unaffected with aging. Finally, relaxation by the membrane permeable analog of cAMP, dibutyryl cAMP, is also unaffected with age (3). Taken together, these data point to either a receptor or stimulatory G protein defect as a potential site of age-related alterations involved in the loss of β-AR responsiveness.

Cholera toxin (CTX) has been used to specifically explore changes of stimulatory G proteins. CTX is a bacterial toxin which irreversibly adenosine diphosphate (ADP)-ribosylates the arginine 201 residue of Gsα, preventing hydrolysis of guanosine triphosphate (GTP), and therefore constituitively activates Gsα. Kazaniez and Enero (15) found that Cholera toxin treatment of aorta from aged rats produced significantly less cAMP accumulation as compared to younger rats. Our laboratory has shown that CTX-induced labeling of Gsα decreases with age in rat aortic membranes, whereas western blotting showed no difference in Gsα protein levels (13). These data suggest a loss of Gsα function with age. CTX has been shown to produce slow-onset and long-lived inhibition of contractile responses to α-AR stimulation by increasing cAMP levels in some arteries (16,17), and also relatively immediate relaxant effects on precontracted rabbit and rat aorta (18). However, there are no reports of age-dependent direct relaxation of precontracted arteries by CTX.

Therefore, the present study was undertaken to further characterize the loss of β-AR–stimulated vasorelaxation with aging, and to investigate the direct vasodilatory effects of CTX in terms of aging utilizing the Fisher 344 rat model.

METHODS

Tension Experiments

Male Fischer 344 rats in four age groups (6 weeks, 6 months, 12 months, and 24 months old) were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Rats were sacrificed by pentobarbital sedation and decapitation. The thoracic aorta was

quickly removed and cleaned of loosely adhering fat and connective tissue in ice cold Krebs-Henseleit Solution (KHS) gassed with 95% O_2/5% CO_2 (19). The composition of KHS (in mM) was: NaCl, 115; KCl, 5; $CaCl_2$, 2; $MgSO_4$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 25; and dextrose, 11. To measure isometric tension development, the arteries were cut into rings approximately 2–3 mm wide and suspended between a fixed support and a Grass Instruments (Quincy, MA) FT.03 force transducer in a muscle bath system containing KHS at 37°C. The rings were stretched to their optimal length (l_0) (20), and allowed to equilibrate for 90 minutes. After equilibration, the arteries were challenged three times with 45 mM KCl to confirm responsiveness prior to testing.

Dose responses to phenylephrine (PE) (1 nM–10 μM) and KCl (10–100 mM) were performed by cumulative addition of the appropriate agonist. Following washout, vessels were precontracted to 70% of maximum level by addition of appropriate dose of either agonist (approximately 0.3 μM PE or 30 mM KCl by NaCl/KCl substitution). Once a stable level of precontraction was achieved, dose-dependent relaxations were performed with isoproterenol (ISO) (1 nM–10 μM), FSK (1 nM–10 μM), or azide-free CTX (0.5 μg/mL–10 μg/mL). KCl precontracted vessels were relaxed in the presence of 1 μM prazosin to block nonspecific action of ISO on α-adrenergic receptors.

Measurement of cAMP Production

Aortas from 6-week-old rats were prepared and mounted in the muscle bath system as described above. After equilibration, the effect of CTX on cAMP production was assessed as described by Kazanietz and Enero (15), except that the phosphodiesterase (PDE) inhibitor isobutylmethylxanthine (IBMX) was not used because it was not present in the vascular reactivity studies. The arteries were preconstricted with 0.3 μM PE and CTX (5 μg/mL) was added to the bath for 3.5 minutes. Basal levels of cAMP were obtained with vessels in the presence of the appropriate vehicle. The reaction was stopped by rapidly submerging the vessels into ice cold 5% trichloroacetic acid (TCA). The tissue was homogenized in a motor-driven Kontes glass homogenizer in 2 mL 5% TCA on ice. After homogenizing, samples were centrifuged at 3000*g* for 15 minutes at 4°C. The supernatant was removed and 1.5 mL of supernatant was washed three times with three volumes of water-saturated ether. The cAMP in the supernatant was determined using a commercially available radioimmunoassay (RIA) kit (PerSeptive Bio-systems, Framingham, MA). The protein concentration of the TCA-precipitated pellet was determined using the BCA method (Pierce Chemical Co., Rockford, IL). Values are expressed as pmol cAMP/mg protein.

Statistical Analysis

Results are expressed as mean values ± *SEM*. The experimental unit was the number of animals. In vascular reactivity studies, one or more rings from each animal was utilized for each test. Each test was repeated with tissue from six animals of each age group. For cAMP accumulation, tissues from five animals were used in the basal and 3.5-minute time points, and tissues from four animals were used for the 60-minute time point. Differences between groups in the vascular reactivity studies were assessed using one-way anlysis of variance (ANOVA) with $p < .007$ considered significant (to control for multiple comparisons). To determine age-related differences in cAMP production, one-way ANOVA with Bonferroni's post hoc comparison was performed with $p < .05$ considered significant. A two-way ANOVA using ED_{50} as the independent variable was performed to assess the interaction between drug (PE vs KCl) and age. The concentration of agonist which produced 50% of maximal response (ED_{50}) was determined by computer nonlinear regression using a four-parameter logistic equation (GraphPad Software, Inc, San Diego, CA).

Chemicals

Azide-free CTX was obtained from List Biologicals (Campbell, CA), FSK was obtained from Calbiochem-Novabiochem (San Diego, CA); all other chemicals were of the highest purity from Sigma (St. Louis, MO).

Results

Phenylephrine and KCl Contractile Response

All age groups of rats showed similar responsiveness to both constricting agents. Phenylephrine produced dose-dependent

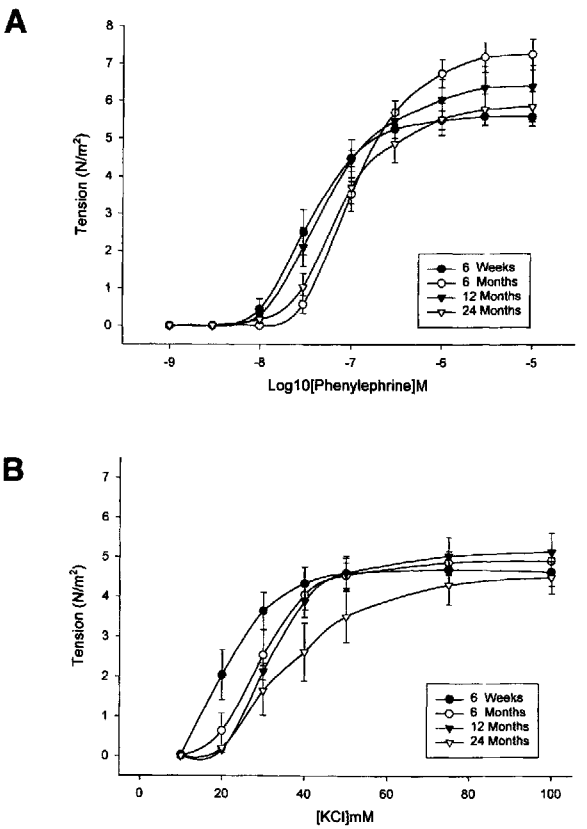

Figure 1. (A) The effects of phenylephrine (PE) on arteries from animals at 6 weeks, 6 months, 12 months, and 24 months of age. PE produced dose-dependent vasoconstriction with maximal response of 5.58 ± 0.25 N/m² for 6-week -old animals, 7.25 ± 0.41 N/m² for 6-month-old animals, 6.39 ± 0.57 N/m² for 12-month-old animals, and 5.85 ± 0.40 N/m2 for 24-month-old animals ($F = 3.05$, $p = .052$). Aortic rings from six animals were examined for each age group. (B) The effects of KCl on arteries from animals at 6 weeks, 6 months, 12 months, and 24 months of age. KCl produced dose-dependent vasoconstriction with maximum response of 4.64 ± 0.36 N/m² for 6-week-old animals, 4.92 ± 0.26 N/m² for 6-month-old animals, 5.14 ± 0.48 N/m² for 12-month-old animals, and 4.50 ± 0.41 N/m² for 24-month-old animals ($F = 0.55$, $p = 0.65$). Aortic rings from six animals were examined for each age group. N/m² = newtons per meter².

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Relaxation of Phenylephrine- and KCl-Preconstricted Arteries

The effects of ISO and FSK on PE-preconstricted arteries of 6 weeks, 6 months, 12 months, and 24 months old animals are shown in Figures 2a and 2b, respectively. The maximum response to ISO was reduced with aging in these arteries (F = 10.1, p < .001) and the sensitivity as indicated by ED_{50} value was also significantly reduced with age (F = 9.27, p < .001). The FSK response, however, was not different with age. All vessels relaxed fully to FSK and the ED_{50} values were not significantly different (F = 0.69, p = .53).

The effects of ISO and FSK on KCl-preconstricted arteries are shown in Figures 3a and 3b, respectively. Isoproterenol produced nearly complete relaxation in 6-week-old animals (94 ± 4%) and diminished relaxation in 6-month-old animals (41 ± 11%), while producing only minimal relaxation in 12 months and 24 months old animals (7 ± 2% and 4 ± 1%, respectively) (F = 49.7, p < .001). FSK-induced nearly complete relaxation of KCl-preconstricted arteries for all ages of animals (F = 4.1, p = .02). There was a tendency for an increased sensitivity in 6-week-old animals and a decreased sensitivity in 6-month-old animals which reached statistical significance.

Because PE and KCl elicit constriction via different mechanisms, the interaction between PE versus KCl and age was assessed. Isoproterenol-mediated vasorelaxation was significantly more impaired with age when KCl was used as a contracting agent (Figures 2a versus 3a; F = 12.69, p < .0001).

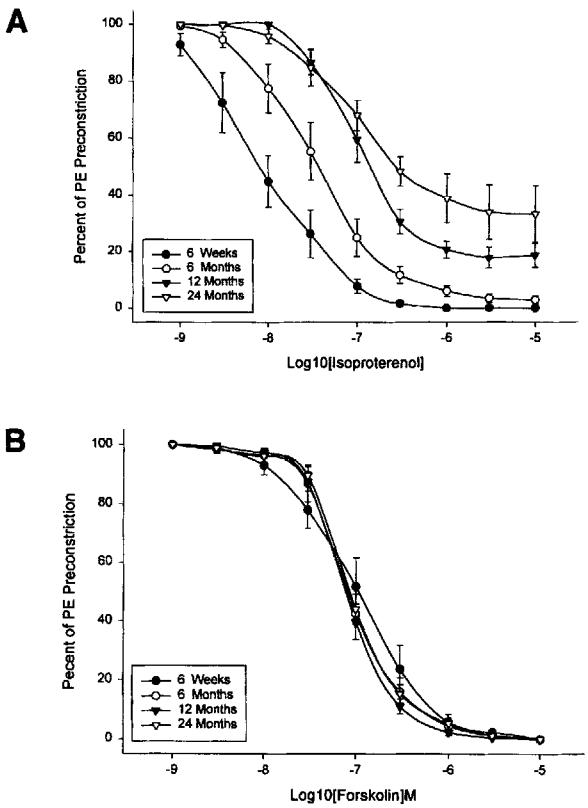

Figure 2. (A) The effects of isoproterenol (ISO) on phenylephrine (PE)-preconstricted arteries from animals at 6 weeks, 6 months, 12 months, and 24 months of age. The maximum response to ISO was reduced with aging in these arteries (F = 10.1, p < .001) and the sensitivity as indicated by ED_{50} value was also significantly reduced with age (F = 9.27, p < .001). Aortic rings from six animals were examined for each age group. (B) The effect of forskolin (FSK) on PE-preconstricted arteries from animals at 6 weeks, 6 months, 12 months, and 24 months of age. The FSK response was not different with age. All vessels relaxed fully to FSK and the ED_{50} values were not significantly different (F = 0.69, p = .53). Aortic rings from six animals were examined for each age group.

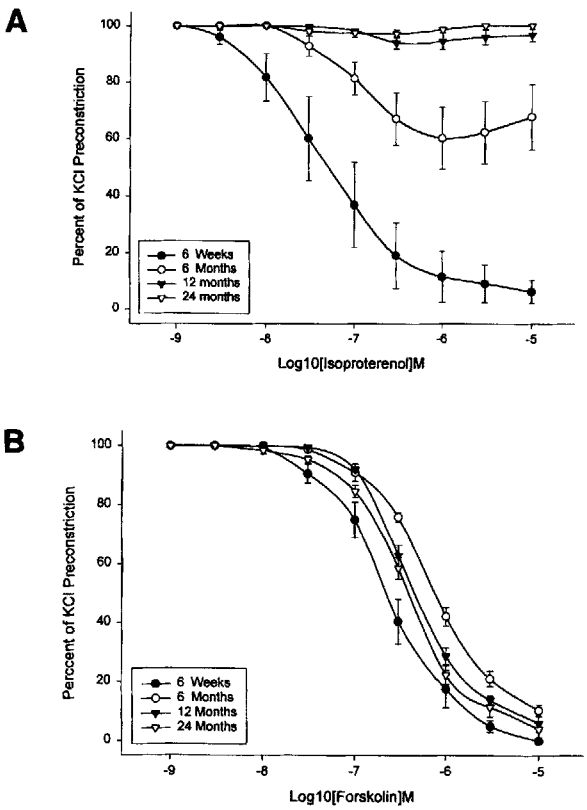

Figure 3. (A) The effects of isoproterenol (ISO) on KCl-preconstricted arteries. Isoproterenol produced nearly complete relaxation in 6-week-old animals (94 ± 4%) and diminished relaxation in 6-month-old animals (41 ± 11%), but produced only minimal relaxation in 12-month-old and 24-month-old animals (7 ± 2% and 4 ± 1% respectively) (F = 49.7, p < .001). Aortic rings from six animals were examined for each age group. (B) The effects of forskolin (FSK) on KCl-preconstricted arteries. FSK-induced nearly complete relaxation of KCl preconstricted arteries for all ages of animals (F = 4.1, p = .02). There was a tendency for an increased sensitivity in 6-week-old animals and a decreased sensitivity in 6-month-old animals which did not reach statistical significance. Aortic rings from six animals were examined for each age group.

Arteries preconstricted with PE and exposed to azide-free CTX showed almost immediate vasorelaxation. This age-related response is shown in Figure 4. Maximum response to CTX was significantly decreased with age ($F = 12.9$, $p < .001$), and sensitivity was also significantly decreased with age ($F = 9.66$, $p < .001$).

Data for ISO- and FSK-induced relaxation of PE- and KCl-preconstricted vessels and CTX-induced relaxation of PE-preconstricted vessels are summarized in Table 1 for all age groups.

cAMP Determination

Concentrations of cAMP were compared between CTX-treated and nonstimulated (vehicle) aorta in 6-week old animals because this age showed the greatest relaxation to CTX (Figure 5). Our preliminary data (not shown) found that 1 μM ISO or 1 μM FSK (in the absence of phosphodiesterase inhibition [PDI]) significantly increased cAMP concentrations compared to vehicle when stimulated for 2 or 3 minutes. These results were in agreement with those published by Deisher and colleagues (3). Arteries stimulated with 5 μg/mL CTX for 3.5 minutes had reached a stable level of relaxation (approximately 65%), whereas vehicle-treated vessels showed no discernible relaxation. In terms of cAMP production, there was a significant effect of CTX exposure over time in the three time points ($F = 31.97$; $p < .001$; Figure 5). Post hoc analysis revealed that there was not a concomitant cAMP rise in CTX-stimulated arteries at 3.5 minutes as compared to vehicle-treated vessels ($n = 5$, 1.63 ± 0.58 pmol/mg protein vs 1.32 ± 0.31 pmol/mg protein, $p > .05$). However, a significant CTX-stimulated rise in cAMP was detected after 60 minutes of exposure compared to vehicle-treated vessels ($n = 4$, 8.29 ± 1.08 pmol/mg protein vs 1.32 ± 0.31 pmol/mg protein; $p < .001$). No additional relaxation was observed after 3.5 minutes of exposure to CTX in these arteries (data not shown).

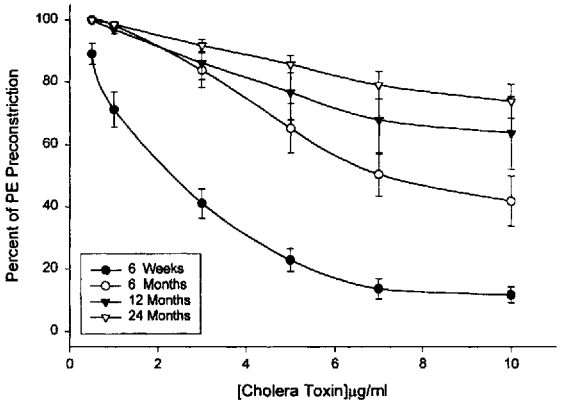

Figure 4. Arteries preconstricted with phenylephrine (PE) and exposed to azide-free cholera toxin are shown. Maximum response to cholera toxin was significantly decreased with age ($F = 12.9$, $p < .001$), and sensitivity was also significantly decreased with age ($F = 9.66$, $p < .001$). Aortic rings from six animals were examined for each age group.

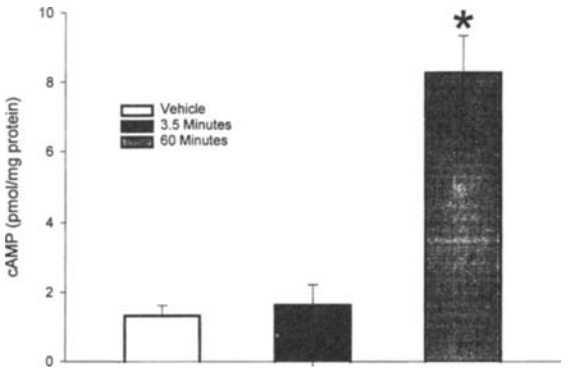

Figure 5. Cholera toxin (CTX) stimulated cAMP production in arteries from 6-week-old rats. Vessels were preconstricted with PE and exposed to vehicle ($n = 5$) or 5 μg/mL azide-free CTX for 3.5 ($n = 5$) or 60 ($n = 4$) minutes. After 3.5 minutes there was no significant increase in cAMP ($p = .673$), but after 60 minutes of exposure there was a significant rise in cAMP over basal (*$p < .001$).

Discussion

Using the male Fischer 344 rat aorta as a model, we have confirmed diminished β-AR–mediated vasorelaxation in rat aorta with aging that has been shown by multiple investigators. Aortic rings from 24-month-old rats preconstricted with the α-AR agonist, PE, required more than 10 times the concentration of ISO to achieve half-maximal relaxation compared to rings from 6-week-old animals. In arteries preconstricted with KCl,

Table 1. Vasorelaxation

Con/relax	Parameter	6 weeks ($n = 6$)	6 months ($n = 6$)	12 months ($n = 6$)	24 months ($n = 6$)	F Statistic, p value
PE/ISO	ED_{50}	13.1 ± 4.0 nM	46.5 ± 16.1 nM	124.5 ± 23.0 nM	153.6 ± 35.7 nM	$F = 9.3$, $p < .001$
	Max	100%	97 ± 1.4%	82 ± 8.0%	69 ± 8.2%	$F = 10.1$, $p < .001$
KCl/ISO	ED_{50}	93.6 ± 41.5 nM	102.7 ± 27.9 nM	N.A.	N.A.	$F = 0.03$, $p = .86$
	Max	94 ± 4.5%	41 ± 12.8%	7 ± 2.3%	4 ± 1.9%	$F = 49.7$, $p < .001$
PE/FSK	ED_{50}	151.8± 47.8 nM	118.4 ± 24.9 nM	98.3 ± 9.9 nM	109.1 ± 9.5 nM	$F = 0.69$, $p = .53$
	Max	100%	100%	100%	100%	
KCl/FSK	ED_{50}	328.3 ± 86.1 nM	829.2 ± 88.2 nM	548.0 ± 58.4 nM	422.0 ± 21.9 nM	$F = 9.9$, $p < .003$
	Max	100%	90 ± 2.0%	94 ± 2.6%	96 ± 2.8%	$F = 4.1$, $p = .02$
PE/CTX	ED_{50}	3.1 ± 0.33 μg/mL	5.0 ± 0.25 μg/mL	5.6 ± 0.89 μg/mL	7.6 ± 1.2 μg/mL	$F = 9.7$, $p < .001$
	Max	88 ± 2.5%	58 ± 8.0%	36 ± 11.5%	26 ± 5.4%	$F = 12.9$, $p < .001$

Notes: PE = phenylephrine; ISO = isoproterenol; KCl = potassium chloride; FSK = forskolin; CTX = cholera-toxin.

ISO response was all but abolished in 12- and 24-month-old animals, whereas vessels from 6-week-old animals relaxed fully. The direct activation of adenylyl cyclase with FSK resulted in similar responsiveness for all four ages of rat, suggesting that the signaling mechanisms downstream from the G protein are intact. This is the first study to relate vasorelaxation to ISO after preconstriction with two agents whose vasoconstrictor mechanisms are different.

It is well established that CTX stimulates ADP-ribosylation of arginine 201 residue of the Gsα, impairing its ability to hydrolyze GTP, and thus constituitive activation (13). Vascular relaxation to CTX has not been studied with aging (15). Our data demonstrate a significant decrease in relaxation to CTX with increasing age. These data support our previous findings of decreased CTX-stimulated [^{32}P-]ADP-ribosylation without a decrease in concentration of Gsα with increasing age in rat aorta (13).

In the current study, we did not find an increase in cAMP levels in tissue during the time period that vasorelaxation occurred. Increases in cAMP have previously been shown to be slow-onset (greater than 30 minutes) and long-lasting (up to 6 hours in duration) (16). Our data show similar results (Figure 5). Tsuru and colleagues (18) showed a similar "relatively immediate" relaxation effect of CTX in rat aorta. cAMP levels have been studied after CTX stimulation in rat aorta with age, and a decrement in cAMP generation was found (15). However, this study measured cAMP levels after stimulation with CTX with IBMX for 1 hour followed by 1 hour of IBMX alone. Therefore, the exact mechanism of action of CTX-induced relaxation on these arteries remains unclear. Tsuru and colleagues (18) did not report cAMP levels in their study; however, they did find that activation of protein kinase C (PKC) by phorbol ester inhibited the effect of CTX. They also found that neither propranolol, atropine, indomethacin, or the presence of endothelium influenced the CTX-stimulated relaxation, thus indicating that CTX most likely directly affects vascular smooth muscle without activating β-AR, muscarinic receptors, or by releasing prostanoids. Because it does not appear that cAMP is responsible for this CTX-stimulated vasorelaxation, and the effect is endothelium- and β-AR–independent, it is possible that a PKC/calcium-mediated pathway may be involved.

The difference in β-AR–mediated vasorelaxation when different vasoconstricting agents are used deserves comment. Both PE and KCl stimulate vasoconstruction via increased cytosolic calcium, promoting the interaction between actin and myosin in vascular smooth muscle cells. However, KCl stimulates vasoconstriction by a hyperpolarization of smooth muscle cells, which allows for the opening of calcium channels, entry of extracellular calcium, and increase in cytosolic calcium concentration. PE-induced vasoconstriction is caused by activation of α-1 adrenergic receptors, which stimulates release of calcium via inositol trisphosphate from intracellular stores. Therefore, the end-effects of PE and KCl are the same, an increase in cytosolic calcium. KCl-stimulated vasoconstriction is independent of receptor-mediated systems. PE-stimulated vasoconstriction is a receptor-mediated event and signal transduction pathways must be considered. PE stimulates both α-1b- and α-1d-AR subtypes (21). α-1b-ARs are linked to Gq, and there are recent data which suggest that activation of Gq-linked pathways enhance vasorelaxation and cAMP production in cultured vascular smooth muscle cells as well as in whole vascular tissue (22). Therefore, differences in β-AR-mediated relaxation after constriction by PE and KCl may be secondary to signal transduction cross-talk that may be occurring between Gs- and Gq-coupled receptors. Further study will be necessary to determine if this is correct.

In conclusion, β-AR–mediated vasodilation is impaired with aging. This difference is greater when the vasoconstricting agent is KCl rather than PE. Azide-free CTX also induces relaxation in aortic rings which decreases with age. This relaxation is not associated with a concomitant increase in cAMP levels during the time period in which vasorelaxation occurs. These data suggest that cross-talk between Gsα and other G protein-coupled receptors may play a role in the impaired relaxation seen with age. Furthermore, the mechanism of CTX-mediated vasorelaxation may not be through increases in cAMP concentration.

ACKNOWLEDGMENTS

This work was supported by the Research Service, Department of Veterans Affairs (SLM), and the National Alliance for Research on Schizophrenia and Depression, US Public Health Service grant T32 DA07262 (VJW).

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Received June 26, 1998
Accepted October 19, 1998