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Ca^{2+} SIGNALING IN FOWL AORTIC SMOOTH MUSCLE INCREASES DURING MATURATION BUT IS IMPAIRED IN NEOINTIMAL PLAQUES

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Summary

Many bird species show the spontaneous development of high arterial pressure and vascular lesions in the aorta and other large arteries. In chickens, arterial pressure tends to increase with age/maturation (particularly in males), and subendothelial hyperplasia (neointima) in the abdominal aorta is often seen prior to sexual maturation. The mechanisms involved, however, are not known. Our aim, therefore, was to determine (1) whether cytosolic Ca^{2+} signaling (CCS) responses to vasoactive substances in fowl aortic smooth muscle differ among chickens at different maturation stages and (2) whether CCS responses to Ca^{2+} channel agonists in neointimal plaques differ from those in normal aortic smooth muscle.

K^+ increased CCS in a dose-dependent manner in isolated and superfused abdominal aortic smooth muscle tissue from chicks (5–9 weeks old), pullets (11–18 weeks old) and adult hens (20 weeks and older); CCS responses increased as chickens matured. The addition of Bay K 8644 (10^{-6} mol l^{-1}) to Ringer's solution containing 50 mmol l^{-1} K^+ further increased CCS, and this response was reduced to half by nifedipine (10^{-6} mol l^{-1}). Norepinephrine did not alter CCS in chicks, whereas marked dose-dependent increases in CCS were noted in pullets. In contrast to the CCS responses to K^+, the norepinephrine-induced CCS responses became smaller in adult hens. Isolated neointimal plaques showed only slight increases in CCS in response to 50 mmol l^{-1} K^+ plus Bay K 8644, whereas clear responses were noted in aortic smooth muscle tissue underlying the plaques. These results suggest (1) that CCS responses to Ca^{2+} channel agonists increased with sexual maturation in fowl, but (2) that CCS responses to norepinephrine were low in mature hens and to K^+ plus Bay K 8644 were low in spontaneously developed neointima, suggesting that phenotypic modulation of Ca^{2+} channel/norepinephrine receptors may have occurred during maturation/aging and in neointima.

Key words: vascular smooth muscle, neointimal plaque, Ca^{2+} signalling, age-dependent response, voltage-gated Ca^{2+} channel, chicken.

Introduction

Many bird species have a higher blood (arterial) pressure than most mammals, and they also spontaneously develop vascular lesions in the aorta and other large arterioles that partly resemble mammalian atherosclerosis (Grollman *et al.* 1963; Rymaszewski *et al.* 1976; Gupta and Grewal, 1980). In fowl, the increase in arterial pressure appears to be age-dependent and greater in males than in females (Girard, 1973; Madison and Nishimura, 1994; Weiss *et al.* 1957). Our previous study indicates that the mean aortic pressure of female chickens (White Leghorn, 30–35 weeks old) was 138±2 mmHg (18.4 kPa) (mean ± S.E.M., N=45), whereas that of males (54–57 weeks old, N=27) was 176±5 mmHg (23.5 kPa) (Kamimura *et al.* 1995; Nishimura *et al.* 1981). Vascular plaques, comprising proliferating neointimal cells and abundant matrix, are frequently seen in the abdominal aortae of chickens prior to sexual maturation. The mechanisms that result in high arterial pressure and vascular lesions in birds, however, are not clear.

A variety of evidence suggests that cellular Ca^{2+} is causally involved in the pathogenesis of atherosclerosis in which cytosolic Ca^{2+} may act as a pathological second messenger (Phair, 1988). In general, arterial [Ca^{2+}] is elevated in atherosclerotic vessels or arteries from hypertensive animals (Fleckenstein-Grün and Fleckenstein, 1991; Kwan, 1985), and many of the cellular and molecular processes that function abnormally in atherosclerosis, such as plasma lipoprotein processing, growth factor responses and endothelial function, are known to be Ca^{2+}-regulated (Phair, 1988). Furthermore, the responsiveness of atherosclerotic arteries to vasoactive agents is variably augmented, depending on the stage of

atherosclerosis (Heistad *et al.* 1984). However, neither the role of cellular Ca^{2+} metabolism in neointimal formation nor the time course of changes in cytosolic Ca^{2+} signal responses to vasoactive substances during the advancement of atherosclerotic vascular lesions is known.

Because arterial pressure in fowl appears to increase with age/maturation, we examined (1) cytosolic Ca^{2+} signaling (CCS) responses of abdominal aortic smooth muscle to voltage-gated (K^+ plus Bay K 8644) and hormone-mediated (norepinephrine) Ca^{2+} channel agonists in chicks, pullets and adult hens to determine whether changes in Ca^{2+} channels are causally related to elevated arterial pressure; and (2) CCS responses of aortic smooth muscle in spontaneously developed neointimal plaques to determine whether vascular smooth muscle phenotypes are modulated during neointima formation.

Materials and methods

Animals and maintenance

Female domestic fowl *Gallus gallus* (White Leghorn breed, DK DeKalb strain), 3–43 weeks of age (body mass 163–1837 g; *N*=71) were used for the present studies. One-day-old chicks were kindly provided by DeKalb Poultry Research, Inc. (Dr Larry F. Vint). Chicks were maintained in temperature-controlled brooders (25–37 °C) for 4 weeks and thereafter kept in groups (5–25 birds per cage) in large indoor pens (1.10×1.02×1.88 m) (length × width × height) in temperature-controlled rooms (22–24 °C) with a photoperiod of 12 h:12 h light:dark. Chickens under 20 weeks old were fed with Start and Grow (Purina, St Louis, MO, USA; 17 % protein, 1 % Ca^{2+}) and thereafter with Layena (Purina; 16 % protein, 3.5 % Ca^{2+}). Multiple vitamins (Vita-Start, GQF, Savannah, GA, USA; 0.23 g l^{-1}) were added to drinking water for chickens in brooders.

Drugs and reagents

Norepinephrine, epinephrine, phenylephrine, nifedipine, digitonin, Triton X-100, bovine serum albumin (BSA) and EGTA were purchased from Sigma Chemical Company (St Louis, MO, USA). Acetoxymethyl ester of Fura-2 (Fura-2 AM, 1 mmol l^{-1} in dry dimethylsulfoxide, DMSO; Molecular Probes, Inc., Eugene, OR, USA), Pluronic F-127 (Molecular Probes, Inc.), Ca^{2+} ionophore (Bromo-A23187), minimum essential medium with Hank's balanced salt solution (MEM Hank, Gibco) and Bay K 8644 (Calbiochem Corporation, San Diego, CA, USA) were purchased commercially. Avian Ringer's solution (referred to as Ringer) contains (in mmol l^{-1}): 115.0 NaCl, 10.0 sodium acetate, 5.0 KCl, 2.5 $CaCl_2$, 0.5 $MgCl_2$, 0.8 NaH_2PO_4, 0.2 Na_2HPO_4, 25.0 $NaHCO_3$, 8.3 glucose, 5.0 alanine, 0.11 ascorbic acid and 0.025 % BSA (pH 7.4, 307.8±2.0 mosmol kg^{-1}). Ringer's solutions containing 10–50 mmol l^{-1} K^+ were prepared by replacing NaCl with KCl; we confirmed that there was no change in osmolality. Mannitol (50 mmol l^{-1}) was added to the Ringer's solution used for tissue dissection (dissection Ringer; osmolality 358±2 mosmol kg^{-1}; *N*=61); a slightly hypertonic solution helps to isolate vascular smooth muscle tissue quickly. Bromo-A23187 (10^{-3} mol l^{-1}, −70 °C), Bay K 8644 (10^{-3} mol l^{-1}, −70 °C) and Pluronic F-127 (20 %, room temperature) were dissolved in DMSO and stored as shown. Nifedipine (10^{-3} mol l^{-1}) was dissolved in DMSO on the day of an experiment. Stock solutions were diluted with Ringer to appropriate concentrations immediately before use. All Ringer's solutions were aerated with 95 % O_2 and 5 % CO_2.

Preparation of aortic smooth muscle tissue

For aortic smooth muscle tissue preparations, abdominal aortae were excised from decapitated chickens (one 10 mm segment from 9-week-old or younger chicks; two 10 mm segments from birds 11 weeks old or older) and cleared of surrounding adipose and connective tissues in MEM Hank supplemented with an additional 10 mmol l^{-1} NaCl to simulate avian serum. The endothelium was removed by rubbing the luminal surface with a cotton swab. The aortic smooth muscle layers were carefully isolated from the adventitia using fine forceps under a dissecting microscope in aerated chilled dissection Ringer's solution. We used avian Ringer's solution containing 50 mmol l^{-1} mannitol because the adventitia can be easily peeled off, without damaging the aortic smooth muscle layers, in a slightly hyperosmotic solution. Hyperosmotic solution has been used for dissecting renal tubules without altering transport properties (Nagineni *et al.* 1984). Isolated abdominal aortic smooth muscle layers (final area 4 mm×8 mm) were placed on a glass coverslip, filling the third quarter of space from the top (which includes the entire beam path) and were glued at the corners with Super Glue (Super Glue Co., Hollis, NY, USA). Various methods of adhering tissues to the glass coverslip were tested, including mechanical attachment, cell tack, gelatin, etc. Super Glue consistently provided a good mounting on the coverslip. We used a minimal amount of glue, restricting it the corners, to avoid possible interference between the glue and the fluorescence. Similarly, neointimal proliferative plaque (if any) in the abdominal aorta (most frequent location) was removed and mounted onto a coverslip as described above (Fig. 1). Histological examination of the plaque showed marked subintimal thickening, consisting of a proliferation of spindle-shaped fibromuscular cells and abundant matrix (Fig. 1). The time necessary for aortic smooth muscle dissection was 10–15 min, and all tissue specimens were equilibrated in avian Ringer's solution (pH 7.4) at 37 °C for at least 30 min after being mounted on the coverslip.

Cytosolic Ca^{2+} measurement

The cytosolic Ca^{2+} signals of aortic smooth muscle cells were determined using a fluorescent indicator, Fura-2 AM, and a dual-wavelength spectrofluorophotometer (Shimadzu, RF-5000) (Wang *et al.* 1992; Yu *et al.* 1989). The isolated aortic smooth muscle tissues, mounted on the glass coverslips as described above, were placed in a flow-through fluorescence cuvette (approximately 1.8 ml) and superfused with prewarmed aerated avian Ringer's solution by a peristaltic pump (3 ml min^{-1}); the cuvette was placed in a water-jacketed holder

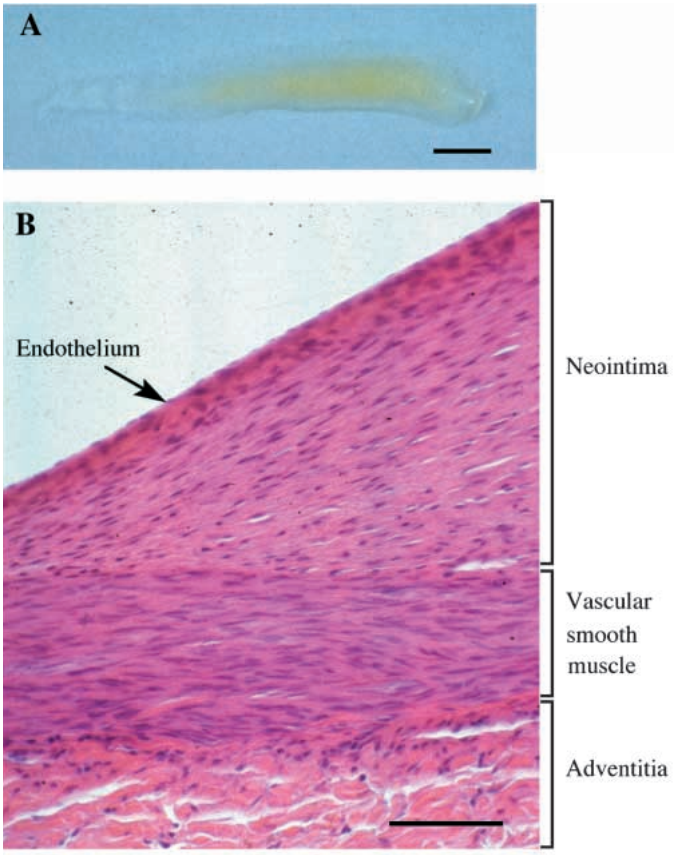

Fig. 1. Macroscopic (A) and histological (B) examination of a neointimal plaque (cross section of approximately one-third of the neointima) spontaneously developed on the ventral side of the abdominal aorta immediately above the ischiadic bifurcation (17-week-old male chicken). Neointimal plaques were isolated from the aortae and used for cytosolic [Ca^{2+}] measurement. Neointimal growth comprises fibromuscular cells and matrix under a layer of endothelium. Smooth muscles are compressed to thin layers under the plaque. Scale bars: A, 2 mm; B, 50 μm.

maintained at 37 °C. After measurement of autofluorescence, the tissue preparations were incubated with Fura-2 AM (4 μmol l^{-1}) and Pluronic F-127 (0.02 %) for 60 min. In preliminary studies, we incubated aortic smooth muscle tissues for 30–120 min with 2–8 μmol l^{-1} Fura-2 AM. Prolonging incubation beyond 60 min did not increase Fura-2 loading, suggesting that Fura-2 had penetrated throughout the aortic smooth muscle layers (126.7±16.5 μm, N=7) within 60 min. Excitation spectra before and after Fura-2 loading were superimposed to confirm sufficient uptake of the fluorescent indicator (approximately two- to threefold increase in 340 nm fluorescence) by the aortic smooth muscle tissue. The excitation wavelength was alternated at 8 s intervals between 340 and 380 nm, while fluorescence at 510 nm was recorded continously. The tissues were excited by a light source perpendicular to them, and emission was measured at 90 ° to the light. When a stable baseline of Fura-2 fluorescence ratio at 340 nm/380 nm had been obtained, the aortic smooth muscle tissue preparations were superfused first with Ringer's solution for another 5–10 min to measure control levels and then with Ringer containing a test drug.

Initially, we attempted to conduct an *in situ* calibration for each tissue at the end of each experiment by saturating the tissue with cytosolic Ca^{2+}. To attempt saturation, we used a Ca^{2+} ionophore in the presence of extracellular Ca^{2+}, depolarization induced by K^+ plus Bay K 8644 or digitonin-induced cell membrane lysis. We could not, however, obtain reliable calibrations to assess absolute concentrations for cytosolic Ca^{2+}. We therefore expressed the CCS of aortic smooth muscle tissues by the ratio of fluorescence emission at 510 nm evoked by the two excitation wavelengths (340 nm and 380 nm) as an index for cytosolic Ca^{2+} levels. Levels of autofluorescence were subtracted prior to calculation of the fluorescence ratio. To ensure that the acetoxymethyl ester form of Fura-2 was hydrolyzed in the cells by cellular enzymes to bind to cytosolic Ca^{2+}, we released loaded Fura-2 from the tissue/cell preparation by cell membrane lysis with digitonin (5–10 min). Digitonin (10–40 μg ml^{-1}) increased fluorescence emission, with the excitation maximum at a wavelength of approximately 340 nm. The addition of EGTA (20 mmol l^{-1}, 10 min) to chelate Ca^{2+} decreased fluorescence and shifted the peak towards 380 nm, indicating that the changes in fluorescence are likely to reflect changes in cytosolic Ca^{2+} levels. Furthermore, addition of Triton X-100 (1 %) caused no specific membrane lysis and increased the fluorescence intensity only slightly, suggesting that Fura-2 compartmentalization, if any, is minimal.

Experimental protocols

We defined the maturation stages of chickens as follows: (1) chicks (5–9 weeks of age), (2) pullets (11–18 weeks of age) and (3) hens (20 weeks old and over).

CCS responses to 50 mmol l^{-1} K^+, K^+ plus Bay K 8644 and nifedipine in aortic smooth muscle (protocol 1)

We examined whether K^+-induced depolarization and Bay K 8644 increase the CCS. The isolated aortic smooth muscle tissues were superfused in the following order: Ringer (for 10 min), 50 mmol l^{-1} K^+ Ringer (12 min), 10^{-6} mol l^{-1} Bay K 8644 in 50 mmol l^{-1} K^+ Ringer (12 min), 10^{-5} mol l^{-1} nifedipine in 50 mmol l^{-1} K^+ Ringer plus Bay K 8644 (15 min) and Ringer (10 min).

K^+-induced CCS responses in aortic smooth muscle tissues from chickens at different maturation stages (protocol 2)

We examined whether CCS responses to K^+ increase in a dose-related manner and are altered by the stage of maturation. The effects of 10, 20, 30 and 50 mmol l^{-1} K^+ on the fluorescence ratio were tested in chicks, pullets and hens. The tissues were superfused with Ringer's solutions containing 10–50 mmol l^{-1} K^+, each for 8 min, in a cumulative fashion. Only birds that exhibited no macroscopically visible neointima in the aorta were used for this protocol.

Norepinephrine-induced CCS responses in aortic smooth muscle tissues from chickens at different maturation stages (protocol 3)

We examined whether CCS responses to norepinephrine increase in a dose-dependent manner and are altered by age/maturation. The effects of 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} mol l^{-1} norepinephrine were tested in chicks, pullets and hens. Only birds that exhibited no macroscopically visible neointima in the aorta were used for this protocol. The tissues were superfused with Ringer's solutions containing 10^{-8} to 10^{-5} mol l^{-1} norepinephrine for 8 min each in a cumulative fashion.

CCS signaling responses to 50 mmol l^{-1} K^+ and 50 mmol l^{-1} K^+ plus Bay K 8644 in isolated neointimal plaques and underlying aortic smooth muscle tissues (protocol 4)

Since it has been postulated that medial smooth muscle cells are mobilized and proliferate in the subendothelial region to form neointima, we intended to determine whether the CCS responses of neointimal plaques to Ca^{2+} channel agonists are similar to those in intact aortic smooth muscle tissues. The neointimal plaque and aortic smooth muscle tissues underlying and cephalad to the plaque were removed from the same chicken, and CCS responses to 50 mmol l^{-1} K^+ and 50 mmol l^{-1} K^+ plus Bay K 8644 (10^{-6} mol l^{-1}) were examined. To eliminate a possible effect of time on the viability of tissue preparations, the order of measurements of cytosolic Ca^{2+} in the plaque, aortic smooth muscle tissue underlying the plaque and aortic smooth muscle tissue cephalad to the plaque were altered randomly. Aortae that did not show spontaneous development of neointima were not used in this protocol.

Data analysis

The autofluorescence at 340 and 380 nm was subtracted from the experimental fluorescence level prior to the calculation of the fluorescence ratio at 340 nm/380 nm. The mean values and standard errors (S.E.M.) of fluorescence ratios calculated at 8 s intervals during the treatment were considered to represent CCS responses to the designated treatments. The mean of the fluorescence ratios from the initial 3 min was used to represent the norepinephrine effect. A two-factor analysis of variance (ANOVA) followed by, when applicable, the Newman–Keuls multiple-comparisons test was used to evaluate the results of protocols 1, 2 and 3. Paired *t*-tests and independent *t*-tests were used for statistical analysis of protocol 4.

Results

Cytosolic Ca^{2+} signaling responses to 50 mmol l^{-1} K^+, K^+ plus Bay K 8644 and nifedipine in aortic smooth muscle tissues (protocol 1)

The mean age of the birds used for this protocol was 18.0±4.0 weeks, and the mean body mass was 1251±249 g (N=5). The time courses of changes in the fluorescence ratio following various treatments are shown in Fig. 2. The mean fluorescence ratio (mean value of designated treatment period) was increased (by 20.3±2.2 % from the control Ringer level; P<0.05, ANOVA) after superfusion with 50 mmol l^{-1} K^+ (Fig. 2). The addition of Bay K 8644 (10^{-6} mol l^{-1}) to 50 mmol l^{-1} K^+ further increased the fluorescence ratio (by 39.7±6.7 % from the control Ringer level; P<0.01, ANOVA). The fluorescence ratio was partially reduced by nifedipine (10^{-5} mol l^{-1}) (P<0.05 compared with fluorescence ratio changes evoked by Bay K 8644 plus 50 mmol l^{-1} K^+; ANOVA). The fluorescence ratio became even lower when the aortic smooth muscle tissue was again superfused with Ringer's solution.

K^+-induced cytosolic Ca^{2+} signaling responses in aortic smooth muscle tissues from chickens at different maturation stages (protocol 2)

Fig. 3 shows representative examples of K^+-induced CCS changes in a young and a mature bird, while Fig. 4 gives a summary of the dose–response curves from chicks (5–9 weeks of age, 535±64 g, N=6), pullets (11–18 weeks of age, 1144±91 g, N=6) and mature hens (31–40 weeks of age, 1439±26 g, N=6). In chicks, the fluorescence ratio increased significantly in response to 50 mmol l^{-1} K^+ (by 18.9±1.9 %; P<0.05 compared with Ringer, ANOVA). In pullets, the fluorescence ratio increased (compared with Ringer, P<0.01 at all doses, ANOVA) in a dose-dependent fashion (20 mmol l^{-1} K^+, 10.3±4.2 %; 30 mmol l^{-1} K^+, 29.1±15.0 %; 50 mmol l^{-1} K^+, 30.5±11.8 %). In hens, the fluorescence ratio also increased markedly (compared with Ringer, P<0.01 at all doses, ANOVA) (20 mmol l^{-1} K^+, 23.8±2.1 %; 30 mmol l^{-1} K^+, 30.6±3.3 %; 50 mmol l^{-1} K^+, 36.7±6.1 %). Furthermore, the

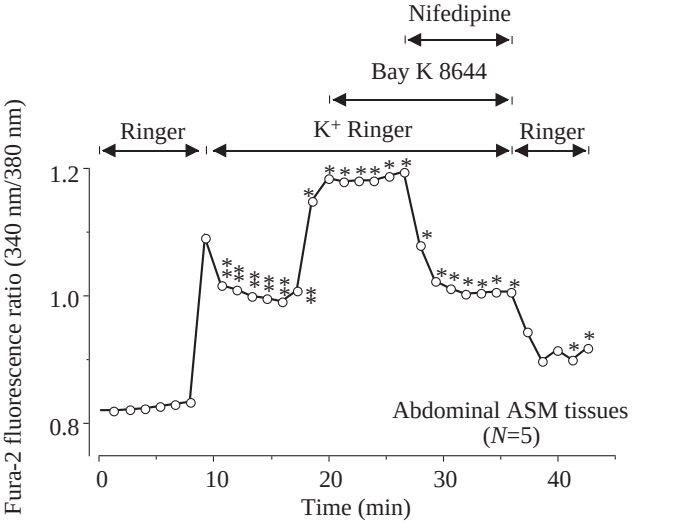

Fig. 2. Time course of the increase in cytosolic Ca^{2+} signaling (expressed as the Fura-2 fluorescence ratio at 340 nm/380 nm) induced by 50 mmol l^{-1} K^+ and 50 mmol l^{-1} K^+ plus Bay K 8644 (10^{-6} mol l^{-1}) in abdominal aortic smooth muscle (ASM) tissues. Addition of nifedipine (10^{-5} mol l^{-1}) (in Ringer containing 50 mmol l^{-1} K^+ plus Bay K 8644) partially reduced the fluorescence ratio. The figure shows the mean of five experiments (four pullets and one mature hen, 18.0±4.0 weeks old). *P<0.05, **P<0.01, compared with the mean level of the control period (Ringer superfusion) by a paired *t*-test.

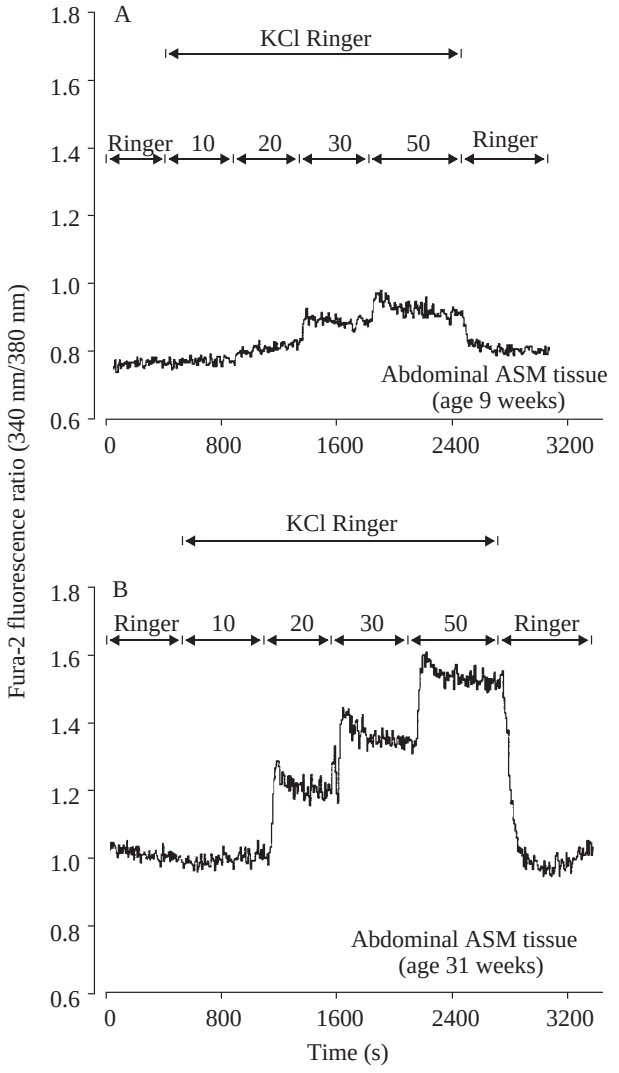

Fig. 3. Representative recordings of cytosolic Ca^{2+} responses (expressed as the Fura-2 fluorescence ratio at 340 nm/380 nm) to Ringer's solution containing 10–50 mmol l^{-1} K^+ in aortic smooth muscle (ASM) tissues from a chick (A) and a mature hen (B). The fluorescence ratio increased in a dose-related fashion, and the increases in fluorescence ratio were much larger in the mature bird.

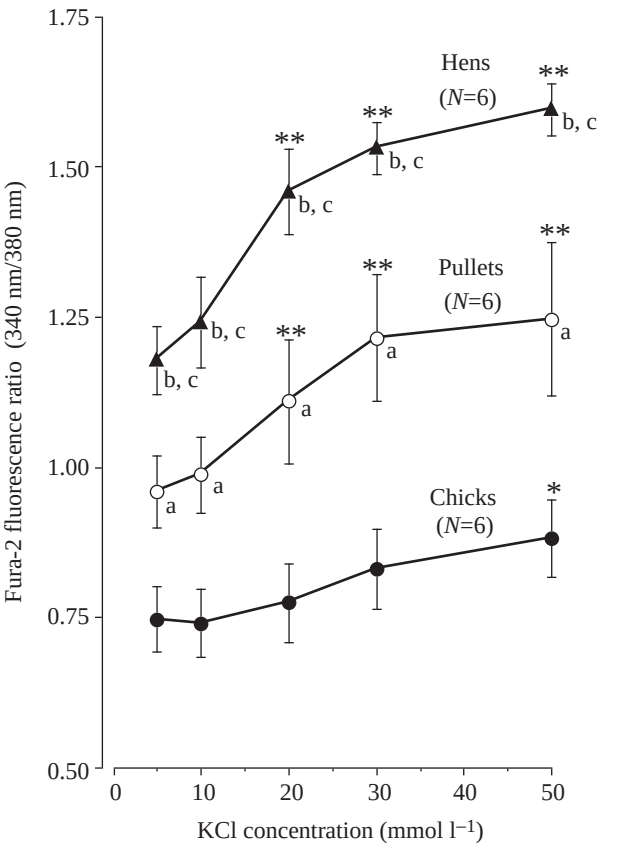

Fig. 4. Cumulative dose–response curves of cytosolic Ca^{2+} signaling (expressed as the Fura-2 fluorescence ratio at 340 nm/380 nm) evoked by 5–50 mmol l^{-1} K^+ in superfusate were compared among abdominal aortic smooth muscle tissues from chicks (5–9 weeks of age), pullets (11–18 weeks of age) and adult hens (31–40 weeks of age). The K^+-induced fluorescence ratio increased in a dose-related fashion in pullets and hens. The K^+-induced responses also increased progressively with age (maturation) at all doses. **P*<0.05, ***P*<0.01 compared with basal (5 mmol l^{-1} K^+) fluorescence ratio within the group; $^{a,b}P$<0.01 compared with chicks; ^{c}P<0.01 compared with pullets at respective K^+ concentration (ANOVA). See text for percentage change at each dose. Values are means ± S.E.M., *N*=6.

Fura-2 fluorescence ratio prior to K^+ application (chicks 0.747±0.054; pullets 0.960±0.061; hens 1.178±0.056) and the fluorescence ratio evoked by K^+ increased progressively with age/maturation at all doses (*P*<0.01, ANOVA) (Fig. 4). The percentage increases in the fluorescence ratio at 20 mmol l^{-1} K^+ (the steepness of the curve) were also significantly higher in the order chicks<pullets<hens. There were no significant differences in autofluorescence levels at 340 nm (arbitrary units) (chicks 34.0±5.8; pullets 56.2±11.9; hens 54.1±10.6) or in the 340 nm/380 nm autofluorescence ratios (chicks 1.15±0.03; pullets 1.16±0.04; hens 1.13±0.04) among the three groups. After incubation with Fura-2 AM and Pluronic F-127, the fluorescence level at 340 nm increased approximately twofold (chicks 68.4±9.4; pullets 129.4±24.3; hens 97.0±23.9).

Norepinephrine-induced cytosolic Ca^{2+} signaling responses in aortic smooth muscle tissues from chickens in different age groups (protocol 3)

Fig. 5 shows representative recordings of norepinephrine-induced CCS changes in a chick and a pullet, and Fig. 6 gives the summary dose–response studies from chicks (5–8 weeks of age, 437±43 g, *N*=7), pullets (12–18 weeks of age, 1283±66 g, *N*=4) and adult hens (20–37 weeks of age, 1513±78 g, *N*=4). In chicks, the fluorescence ratio during the control Ringer period was low (0.828±0.088), and the fluorescence ratio did not increase in response to norepinephrine. In pullets, the fluorescence ratio prior to drug application was higher (1.300±0.119) than in chicks and increased significantly (ANOVA), responding to norepinephrine in a dose-dependent

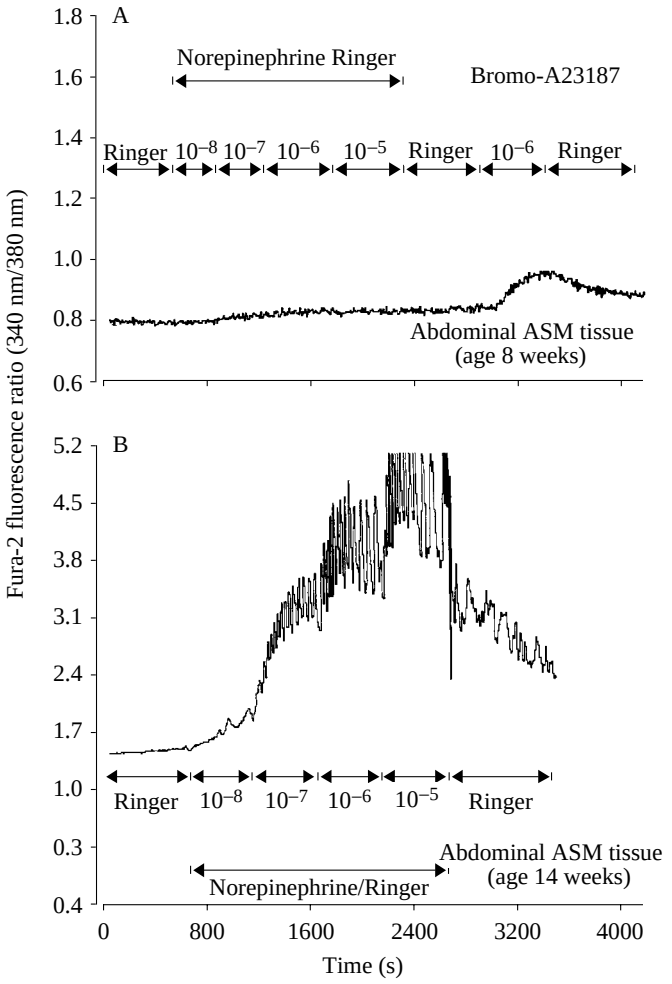

Fig. 5. Representative recordings of cytosolic Ca^{2+} responses (expressed as the Fura-2 fluorescence ratio at 340 nm/380 nm) to Ringer's solution containing norepinephrine (10^{-8} mol l^{-1} to 10^{-5} mol l^{-1}) by aortic smooth muscle (ASM) tissues from a chick (A) and a pullet (B). Norepinephrine increased the fluorescence ratio only slightly in the chick, whereas the fluorescence ratio was increased by the Ca^{2+} ionophore Bromo-A23187 (10^{-6} mol l^{-1}). Dose-dependent increases in fluorescence ratio in response to norepinephrine occur in the pullet.

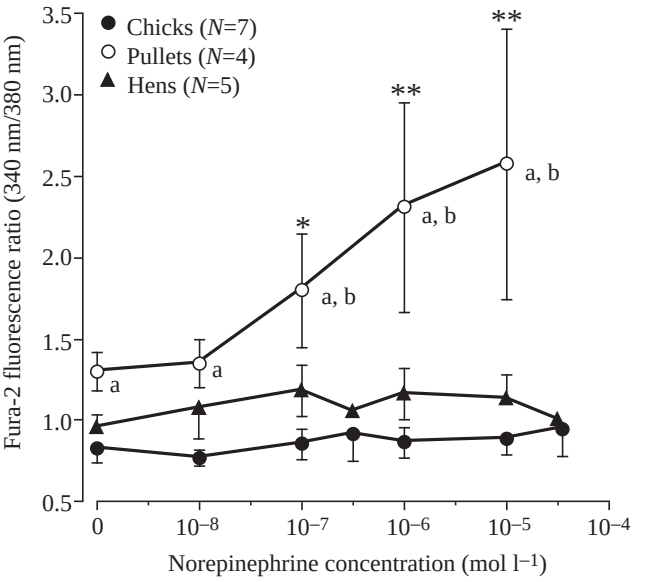

Fig. 6. Cumulative dose–response curves to 10^{-8} to 5×10^{-5} mol l^{-1} norepinephrine of cytosolic Ca^{2+} signals (expressed as the Fura-2 fluorescence ratio at 340 nm/380 nm) were compared among abdominal aortic smooth muscle tissues from chicks (5–8 weeks of age), pullets (12–18 weeks of age) and hens (20–37 weeks of age). The norepinephrine-induced fluorescence ratios increased in a dose-related fashion in pullets, but not in chicks and hens. The fluorescence ratios evoked by norepinephrine were higher in pullets than in chicks at all doses (P<0.01; ANOVA), whereas no significant differences in fluorescence ratio were noted between chicks and hens. *P<0.05, **P<0.01 compared with basal fluorescence ratio within each group; ^{a}P<0.01 compared with chicks; ^{b}P<0.01 compared with hens (ANOVA). Values are means ± S.E.M.

manner (Fig. 6) (percentage increase from Ringer: 10^{-8} mol l^{-1}, 3.6±3.4; 10^{-7} mol l^{-1}, 34.8±15.4; 10^{-6} mol l^{-1}, 71.4±34.7; 10^{-5} mol l^{-1}, 91.3±47.4). In hens, however, the fluorescence ratio (0.956±0.076) showed only small increases in response to norepinephrine, and there was no clear dose-related response. There was a significant increase in norepinephrine responses in pullets (P<0.01 at all doses, ANOVA) compared with chicks, whereas no significant differences in CCS responses were noted between chicks and hens at any dose of norepinephrine. There were no significant differences in autofluorescence levels at 340 nm (arbitrary units) (chicks 41.3±5.9; pullets 53.1±8.4; hens 52.8±13.3) or in 340 nm/380 nm autofluorescence ratios (chicks 1.10±0.03; pullets 1.12±0.02; hens 1.17±0.04) among the three groups. After incubation with Fura-2 AM and Pluronic F-127, the fluorescence level at 340 nm increased two- to threefold (chicks 152.0±59.5; pullets 179.5±50.5; hens 188.8±74.6).

Cytosolic Ca^{2+} signaling responses to 50 mmol l^{-1} K^+ and 50 mmol l^{-1} K^+ plus Bay K 8644 in isolated neointimal plaques and underlying aortic smooth muscle tissues (protocol 4)

Fig. 7 shows recordings from a single bird and Fig. 8 summarizes the CCS responses to 50 mmol l^{-1} K^+ and to the combination of 50 mmol l^{-1} K^+ plus Bay K 8644 in isolated neointimal plaques, aortic smooth muscle tissues underneath the plaques and aortic smooth muscle tissues cephalad to the plaques (macroscopically intact area) from hens 30±2.6 weeks of age (1509±63 g, N=15). In most cases, we excised these three tissues from the same birds for examination of each type of Ca^{2+} response. In neointimal plaques, both 50 mmol l^{-1} K^+ and K^+ plus Bay K 8644 increased the fluorescence ratio only slightly from the basal level (Fig. 7). In aortic smooth muscle tissues underlying the plaque, the fluorescence ratio (0.899±0.091) was increased significantly (14.1±3.5 %, P<0.05, paired t-test) by 50 mmol l^{-1} K^+ compared with the Ringer control. The aortic smooth muscle tissue cephalad to

the plaque (macroscopically intact) showed two types of responses: (i) marked responses and (ii) poor responses. In Fig. 8, we show the average of all cases (i and ii), whereas Fig. 7 represents a bird that showed marked CCS responses in the aortic smooth muscle underlying and cephalad to the plaques. In aortic smooth muscle tissues that showed marked responses, the fluorescence ratio prior to drug application was high (1.537±0.120) and it increased further (P<0.05, N=4) after application of 50 mmol l^{-1} K^+ (by 35.3±9.1 %) and after application of K^+ plus Bay K 8644 (by 54.2±15.7 %). In aortic smooth muscle with poor responses, the basal fluorescence ratio was low (0.694±0.065) but was increased significantly (P<0.01) (9.4±1.9 % and 19.8±2.8 %, respectively) by the superfusion of 50 mmol l^{-1} K^+ and K^+ plus Bay K 8644. Furthermore, the magnitude of responses to 50 mmol l^{-1} K^+ was significantly higher (independent t-test) in aortic smooth muscle under plaques (P<0.05) or cephalad to plaques (P<0.01) than in neointimal plaques.

There were no significant differences among plaques (Pl, N=12), aortic smooth muscle tissues underneath the plaques (UP, N=13) and aortic smooth muscle tissues cephalad to the plaques (CP, N=12) in autofluorescence levels at 340 nm (arbitary units) (Pl, 78.8±15.7; UP, 60.1±10.6; CP, 84.4±30.3). The autofluorescence ratio at 340 nm/380 nm was slightly higher (independent t-test) in Pl (1.40±0.07) than in UP (1.18±0.05, P<0.05) or CP (1.12±0.03, P<0.01). After incubation with Fura-2 AM and Pluronic F-127, the fluorescence level at 340 nm increased two- to threefold (Pl, 220.3±40.0; UP, 141.0±34.2; CP, 203.4±58.8).

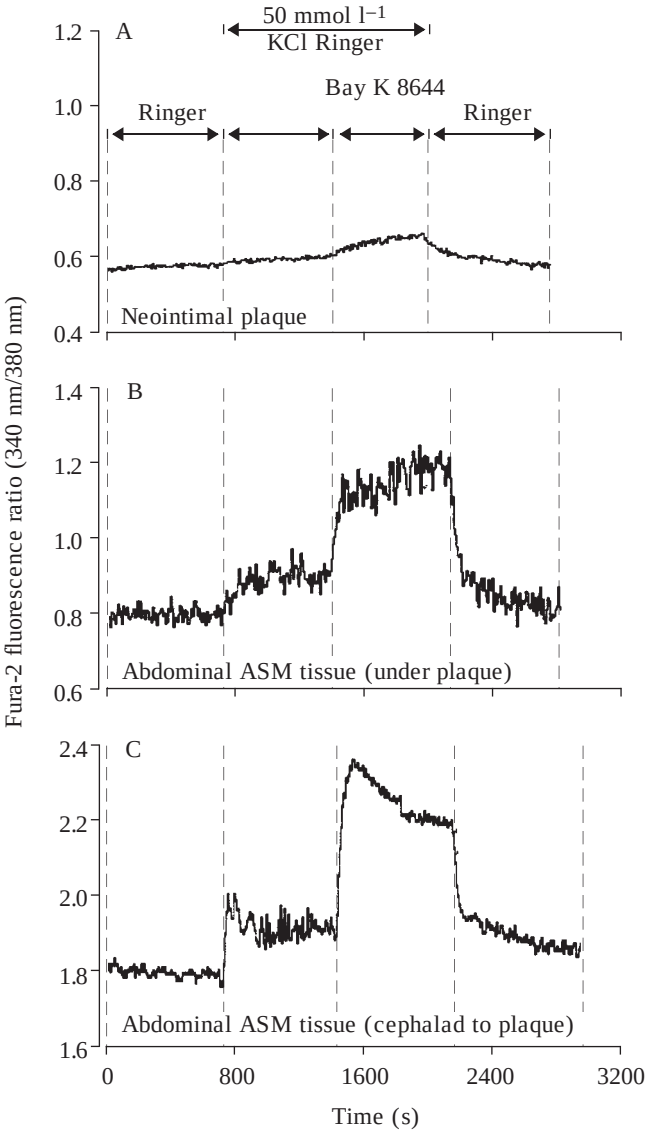

Fig. 7. Time course recordings of cytosolic Ca^{2+} signaling responses (expressed as the Fura-2 fluorescence ratio at 340 nm/380 nm) to 50 mmol l^{-1} K^+ and 50 mmol l^{-1} K^+ plus Bay K 8644 (10^{-6} mol l^{-1}) in neointimal plaque (A), abdominal aortic smooth muscle (ASM) tissue under the plaque (B) and abdominal aortic smooth muscle tissue cephalad to the plaque (C) from an 18-week-old pullet. Neointimal plaque was removed from the underlying abdominal aortic smooth muscle tissue under a dissecting microscope and placed on a coverslip (see Materials and methods). The macroscopically intact abdominal aortic smooth muscle tissue cephalad to the plaque was selected. Note that the ordinate scale differs in B and C.

Discussion

Ca^{2+} channels in fowl aortic smooth muscles

Freshly isolated aortic smooth muscle preparations responded to K^+, Bay K 8644 and nifedipine, indicating that fowl aortic smooth muscle cells possess voltage-gated and hormone-mediated Ca^{2+} channels. Bay K 8644 is structurally

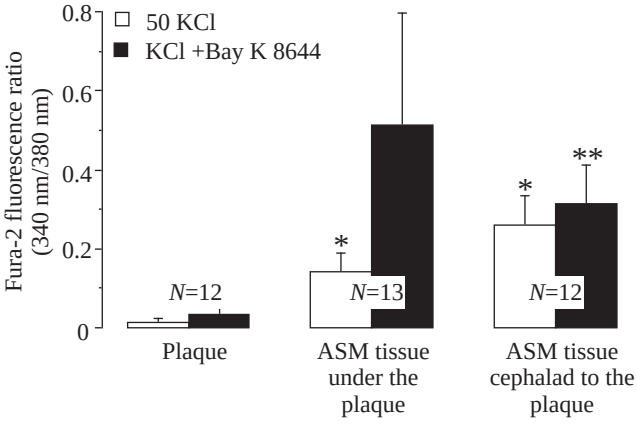

Fig. 8. Changes in cytosolic Ca^{2+} signaling responses (expressed as the Fura-2 fluorescence ratio at 340 nm/380 nm) to 50 mmol l^{-1} K^+ (open columns) and 50 mmol l^{-1} K^+ plus Bay K 8644 (10^{-6} mol l^{-1}) (filled columns) in neointimal plaque, abdominal aortic smooth muscle tissue (ASM) under the plaque and aortic smooth muscle tissue cephalad to the plaque. The three types of tissue were collected simultaneously from the same bird in 12 of 15 birds. The basal fluorescence ratios during control Ringer superfusion were: plaque, 0.640±0.074; aortic smooth muscle underlying the plaque, 0.899±0.091; and aortic smooth muscle cephalad to the plaque, 0.975±0.132. *P<0.05, **P<0.01 compared with the respective basal fluorescence ratio by paired t-test. Values are means + S.E.M.

similar to the dihydropyridine-type Ca^{2+} entry blockers and, in general, Bay K 8644 and high $[K^+]$ are considered to activate identical voltage-sensitive Ca^{2+} channels (Dubé *et al.* 1985). In the present study, however, the addition of Bay K 8644 to a high dose of K^+ further increased CCS, and nifedipine did not completely abolish their effects, suggesting that part of the CCS changes evoked by K^+ and Bay K 8644 may be mediated *via* a Ca^{2+} channel other than the L-type voltage-gated channel or may interact with the same channel but *via* different mechanisms. An additive effect of K^+ and Bay K 8644 has been reported in rat renal mesangial cells (Yu *et al.* 1989) and 'aged' pig coronary arterial rings (Dubé *et al.* 1985). It has been postulated that depolarization by high $[K^+]$ may restore the coupling mechanism between the Bay K 8644 receptor and the Ca^{2+} channel and activate the Ca^{2+} channel to the threshold level for Bay K 8644 (Dubé *et al.* 1985).

Age-related changes in cytosolic Ca^{2+} signaling responses

We found (1) that in fowl aortic smooth muscle tissues, CCS increases in response to extracellular application of K^+ and norepinephrine in a dose-related manner, and (2) that CCS responsiveness (the steepness of the dose–response curves) to K^+ and norepinephrine is low in chicks and increases with maturation, but that norepinephrine responses are reduced in adult birds. The mechanism for these age (maturation)-dependent changes in Ca^{2+} signaling is unclear at present, but voltage-sensitive or hormone-operated Ca^{2+} channels (or α-adrenoceptors) may not be fully expressed in chicks; as the birds mature, the number and sensitivity (or opening time) of Ca^{2+} channels (or norepinephrine receptors) may increase, leading to the increase in Ca^{2+} conductance. Marked differences in response to KCl suggest that the maturation/age-related changes occur primarily in the handling of extracellular Ca^{2+} *via* changes in Ca^{2+} channels, whereas the reduced CCS responses to norepinephrine in the aortae from adult birds may reflect phenotypic modulation of norepinephrine receptors or/and post-receptor signal pathways, such as the G-protein-mediated receptor–Ca^{2+} channel coupling mechanism (Nebigil and Malik, 1993).

Previously, it was reported that endothelium-dependent relaxation induced by acetylcholine and angiotensin II in chickens (Yamaguchi and Nishimura, 1988) is also maturation/age-dependent (Hasegawa *et al.* 1993). The relaxation responses were not clearly seen in chicks, whereas marked relaxation responses developed with maturation of the birds. Furthermore, angiotensin-II-induced or acetylcholine-induced relaxation diminished in mature birds when arterial pressure reached a plateau and neointimal lesions developed on the abdominal aorta. The time course of maturation-dependent changes in CCS responses to norepinephrine observed in the present study agrees approximately with the time course of age-dependent changes in endothelium-dependent vasorelaxation, suggesting that impaired vascular endothelium–smooth muscle communication may alter vascular function phenotypes such as Ca^{2+} channel activities. Furthermore, since diffuse subendothelium thickening often develops in adult chickens, it is possible that the presence of microscopic vascular lesions may reduce the responsiveness to norepinephrine, although we selected for the present studies (except protocol 4) abdominal aortae that showed no macroscopically visible vascular plaques.

There were no significant differences among the three groups in either autofluorescence levels or the fluorescence at 340 nm after Fura-2 loading, so that the observed maturation (age)-dependent changes in CCS responses are not due to age-related differences in autofluorescence or Fura-2 loading. Furthermore, in all experiments, autofluorescence levels were subtracted prior to the calculation of fluorescence ratios. The laboratory chow for adult hens contains a higher Ca^{2+} concentration than the chow given to chicks and pullets (to compensate for Ca^{2+} loss from laying eggs). It is unlikely, however, that the enhanced CCS responses with maturation/age can be ascribed to the difference in Ca^{2+} concentration in the diet since (i) CCS responses to K^+ and norepinephrine are higher in pullets than in chicks, although both were maintained on the same food; (ii) the plasma Ca^{2+} levels of laying hens (Laverty and Wideman, 1985) are similar to those of pullets (Kissell and Wideman, 1985); and (iii) the Ca^{2+} gradient across the cell membrane is so great that if a slight change in extracellular Ca^{2+} concentration were to occur it would not alter the rate of Ca^{2+} influx or the responsiveness of CCS to Ca^{2+} channel agonists.

In the present study, the fluorescence ratio of aortic smooth muscle tissues during the control period (presumably reflecting basal cytosolic Ca^{2+} signal levels) was higher in pullets than in chicks, but the difference between pullets and hens was not consistent. Overall, age and basal fluorescence ratio showed a weak positive correlation (r=0.421, N=72). Chickens have a higher arterial pressure than mammals, and arterial pressure tends to increase with maturation, particularly in males, reaching a plateau (Girard, 1973; Kamimura *et al.* 1995; Madison and Nishimura, 1994; Nishimura *et al.* 1981). Plasma catecholamine levels are also higher, particularly in males (Kamimura *et al.* 1995; Madison and Nishimura, 1994; Nishimura *et al.* 1981), and fowl arterial pressure is reduced by blockade of the adrenergic nervous system, suggesting that sympathetic activities may be elevated. Although it is not certain whether there is a causal relationship between cytosolic Ca^{2+} levels and elevated arterial pressure in chickens, it has been reported that cytosolic Ca^{2+} levels and muscle tension are higher in aortae from spontaneously hypertensive rats than in those from control rats or from spontaneously hypertensive rats treated with angiotensin-converting enzyme inhibitors (Sada *et al.* 1990). Similarly, increased intracellular Ca^{2+} levels and enhanced Ca^{2+} signal transduction have been demonstrated in freshly isolated aortic smooth muscle cells from hypertensive rats (modified coarctation) (Papageorgiou and Morgan, 1991) and in cultured vascular smooth muscle cells from spontaneously hypertensive rats (Bendhack *et al.* 1992), suggesting that abnormal Ca^{2+} handling may exist in hypertensive vessels (Kwan, 1985). No difference was observed, however, between spontaneously hypertensive rats

and Wistar-Kyoto rats (normotensive) in intracellular Ca^{2+} levels of either intact mesenteric arteries or primary or first subcultures of myocytes, whereas passaged myocytes from the same arterioles of spontaneously hypertensive rats exhibited elevated basal Ca^{2+} levels and enhanced responsiveness to angiotensin (Bukoski, 1990). Similarly, resting cytosolic [Ca^{2+}] and responses to K^+ and K^+ plus Bay K 8644 in pancreatic arterioles did not differ between spontaneously hypertensive rats and control rats (Storm *et al.* 1992).

Altered cytosolic Ca^{2+} signaling in impaired aortic smooth muscles

The present study indicates that aortic smooth muscle tissues from chickens that developed neointima showed variable responses to K^+ and K^+ plus Bay K 8644. In general, the CCS responses to K^+ in aortic smooth muscle in these chickens (Fig. 8) were lower than those of the group showing no macroscopic neointima (Fig. 4, hens) and showed only small increases after additional Bay K 8644, suggesting that the properties of voltage-gated Ca^{2+} channels may be altered in the neointima and underlying (or adjacent) vascular smooth muscle cells. Recent evidence suggests that cellular Ca^{2+} metabolism may be causally related to atherogenesis (Fleckenstein-Grün and Fleckenstein, 1991; Phair, 1988). Orimo and Ouchi (1990) reported that the cytosolic [Ca^{2+}] in cultured vascular endothelial cells was elevated by cardiovascular risk factors (such as free radicals) *in vitro*, whereas pretreatment with nifedipine inhibited the increases in both cytosolic [Ca^{2+}] and low-density lipoprotein transport across endothelial cells. It is thus possible that the increased influx of Ca^{2+} into vascular endothelial cells caused by various risk factors may further damage the function of endothelial cells, resulting in platelet aggregation and increased uptake of low-density lipoprotein and other macromolecules from the plasma (Orimo and Ouchi, 1990), whereas inhibition of Ca^{2+} influx may prevent the damage. Similarly, Ca^{2+} channel antagonists inhibited balloon-catheter-induced vascular smooth muscle proliferation (Jackson *et al.* 1988) and phenotypic modulation to 'immature' cell types (Pauletto *et al.* 1992). These findings appear to suggest that dysfunction of the Ca^{2+} channels or of Ca^{2+} handling at cell membranes may underlie altered vascular function.

Neointimal lesions develop spontaneously in young chickens prior to sexual maturation as well as in blood vessels in which endothelial injury was evoked by a balloon catheter (Madison and Nishimura, 1994; A. B. Madison and H. Nishimura, unpublished results). The spontaneously developed neointimal plaque in fowl is unique in that it develops at an early stage in life without excess cholesterol/fat in the diet. The present study indicates that CCS responses to voltage-gated Ca^{2+} channel agonists are low but demonstrable in spontaneously developed neointima. The low CCS responses in neointima are not due to damage to the tissues during dissection, since neointimal cells grow well in culture medium and incorporate [^{3}H]thymidine into DNA (Shimada *et al.* 1996; T. Shimada and H. Nishimura, unpublished results). The small but demonstrable CCS responses to Ca^{2+} channel agonist and the presence of myofilament bundles in neointima (Madison and Nishimura, 1994; A. B. Madison and H. Nishimura, unpublished results) suggest that neointimal cells are of smooth muscle origin. Neointimal cells in fowl contain abundant endoplasmic reticulum, Golgi bodies, vesicle structures and extracellular matrix, showing the features of a 'synthetic state' (Moss and Benditt, 1970; Madison and Nishimura, 1994; A. B. Madison and H. Nishimura, unpublished results). The vascular smooth muscle cells change their phenotypes from a contractile to a synthetic state (or dedifferentiated state) in culture and in fetal and atherosclerotic vessels of humans and other mammals (Gabbiani *et al.* 1984; Glukhova *et al.* 1991; Schwartz *et al.* 1995). In addition to morphological characteristics, altered cytoskeletal protein expressions, such as the ratio of α-actin and its isoforms and the ratio of vimentin to desmin, have been reported in atherosclerotic vessels (Gabbiani *et al.* 1984; Schmid *et al.* 1982) and may affect the responsiveness of vascular smooth muscle cells to Ca^{2+} channel agonists and vasoactive substances.

Avian aortae possess two phenotypically different cell populations; in adult chickens, the tunica media shows regularly alternating layers of tracts of classical smooth muscle cells (lamellar cells) separated by layers of fibroblast-like interlamellar cells (Schmid *et al.* 1982). Lamellar cells express α-actin, myosin, desmin and other contractile smooth-muscle-specific proteins, but interlamellar cells lack smooth muscle characteristics (Benson and Yablonka-Reuveni, 1992), suggesting that these two types of cells may represent two different cell lineages. We assume that the lamellar cells that constitute the major population of intact aortic smooth muscle cells are primarily responsible for the CCS responses to K^+, Bay K 8644 and norepinephrine. The relative contribution of lamellar cells and interlamellar cells to age/maturation-dependent alteration in cytosolic Ca^{2+} responsiveness, however, is not clear.

It remains uncertain which factors determine phenotypic modulation from the contractile to the synthetic type (or fetal type) of vascular smooth muscle cells. It has been reported that cultured smooth muscle cells from human atherosclerotic plaques secrete a mitogenically active substance that immunologically resembles platelet-derived growth factors (Libby *et al.* 1988). Furthermore, mRNA for platelet-derived growth factor and for the platelet-derived growth factor receptors is localized to specific cell types within the human atherosclerotic plaque; mesenchymal-like intimal cells dominantly express the platelet-derived growth factor A chain (Schwartz *et al.* 1995; Wilcox *et al.* 1988). Therefore, enhanced levels of growth factors in the local environment may evoke phenotypic modulations of vascular smooth muscle cell functions. It has been shown that incubation of cultured pituitary cells with epidermal growth factor reduces the activity of voltage-gated L-type Ca^{2+} channels (Hinkle *et al.* 1993). Similarly, platelet-derived growth factor reduces vascular smooth muscle cell populations that respond to voltage-

dependent and inositol-trisphosphate-mediated Ca^{2+} channel agonists (Masuo *et al.* 1991). Furthermore, the fact that the change in the time course of endothelium-dependent relaxation approximately agrees with that of CCS responses to norepinephrine in chickens suggests that endothelium–smooth muscle communication plays an important role in the maintenance of normal vascular function phenotypes.

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