Increased intracellular Ca^{++} in the macula densa regulates tubuloglomerular feedback

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Increased intracellular Ca^{++} in the macula densa regulates tubuloglomerular feedback.

Background. Tubuloglomerular feedback is initiated by an increase in NaCl at the macula densa lumen, which in turn increases intracellular Ca^{++}. In the present study, we examined the role of increased intracellular Ca^{++} in tubuloglomerular feedback and the source of the increased Ca^{++}. We hypothesized that an increase in intracellular Ca^{++} at the macula densa via the basolateral Na^+/Ca^{++} exchanger, caused by an increase in luminal NaCl, initiates Ca^{++}-mediated Ca^{++} release from intracellular stores, which is essential for tubuloglomerular feedback.

Methods. Rabbit afferent arterioles and attached macula densas were simultaneously microperfused in vitro. Tubuloglomerular feedback was induced by increasing macula densa Na^+/Cl^- from 11/10 mmol/L (low) to 81/80 mmol/L (high) and was measured before and after treatment.

Results. To investigate whether elevations in intracellular Ca^{++} are required for tubuloglomerular feedback, the calcium ionophore A23187 or the Ca^{++} chelator BAPTA-AM was added to the macula densa lumen. During the control period, tubuloglomerular feedback decreased afferent arteriole diameter from 18.1 ± 1.1 μm to 15.3 ± 0.8 μm. Adding 2×10^{-6} mol/L A23187 to the low NaCl macula densa perfusate induced tubuloglomerular feedback; diameter decreased from 18.0 ± 1.0 μm to 15.4 ± 0.9 μm ($N = 6$; $P < 0.01$). After adding BAPTA-AM (25 μmol/L) to the macula densa lumen, tubuloglomerular feedback response was completely eliminated. We next studied the source of increased macula densa Ca^{++} in response to increased NaCl concentration. During the control period, tubuloglomerular feedback decreased afferent arteriole diameter from 18.5 ± 1.6 μm to 15.3 ± 1.2 μm ($N = 6$; $P < 0.01$). After adding the Na^+/Ca^{++} exchanger inhibitor 2′4′-dichlorobenzamil (10 μmol/L) or KB-R7943 (30 μmol/L) to the bath, the tubuloglomerular feedback response was blocked; however, the afferent arteriole response to angiotensin II or adenosine was not altered. Next, we tested the Ca^{++}-adenosine triphosphatase (ATPase) inhibitor thapsigargin (0.1 μmol/L), which has been reported to inhibit sarcoplasmic reticulum Ca^{++}-ATPase activity and prevent restoration of intracellular Ca^{++} stores. When thapsigargin was added to the macula densa lumen, it reduced the first tubuloglomerular feedback response by 33% and completely eliminated the second and third tubuloglomerular feedback responses. In the absence of thapsigargin, there was no significant decrease in the tubuloglomerular feedback responses ($N = 6$). Neither the L-type Ca^{++} channel blocker nifedipine (25 μmol/L), nor the T-type Ca^{++} channel blocker pimozide (10 μmol/L), inhibited tubuloglomerular feedback when added to the macula densa lumen.

Conclusion. We concluded that (*1*) increased intracellular Ca^{++} at the macula densa is required for the tubuloglomerular feedback response; (*2*) Na^+/Ca^{++} exchange appears to initiate Ca^{++}-mediated Ca^{++} release from intracellular stores; and (*3*) luminal L-type or T-type Ca^{++} channels are not involved in tubuloglomerular feedback.

Micropuncture studies have shown that increased NaCl concentration at the distal tubule lowers the single-nephron glomerular filtration rate (SNGFR) [1], a phenomenon called tubuloglomerular feedback. Tubuloglomerular feedback is initiated by enhanced $Na^+/K^+/2\,Cl^-$ cotransport through the apical membrane when the NaCl concentration at the macula densa lumen is increased [2], thereby leading to an increase in intracellular sodium [3] and chloride [4]. There have been earlier studies using fluorescence microscopy and isolated perfused tubules to measure macula densa cytosolic Ca^{++} in response to changes in luminal NaCl; however, results have been equivocal [3–5]. More recent studies suggest that the increased macula densa NaCl concentration and depolarization brought about by enhanced Cl^- efflux cause intracellular Ca^{++} to increase [6]. Elevations in cytosolic Ca^{++} concentration result in further signal transmission to the glomerular vessels, eliciting vasoconstriction and decreasing the glomerular filtration rate (GFR). In the present study, we examined the role of increased intracellular Ca^{++} in tubuloglomerular feedback and the source of the increased Ca^{++}.

Previous reports demonstrated that luminal perfusion of the Ca^{++} ionophore A23187 in the presence of Ca^{++} increased tubuloglomerular feedback responses [7], whereas an inhibitor of intracellular Ca^{++} release [8-(diethylamino)octyl3,4,5-trimethoxybenzoate hydrochloride (TMB-8)] reduced stop-flow pressure (SFP) re-

sponses in micropuncture preparations [8]. Basolateral [6] or luminal [9] voltage-gated Ca^{++} channels, the Na^{+}/Ca^{++} exchanger [10], and release of bound intracellular Ca^{++} stores [8] have all been suggested as potential sources of the increase in intracellular Ca^{++}. We hypothesized that an increase in intracellular Ca^{++} at the macula densa is essential for tubuloglomerular feedback and evaluated the source of increased macula densa Ca^{++} concentration in response to increased luminal delivery of NaCl. In vitro perfusion of a microdissected afferent arteriole and adherent tubular segment containing the macula densa was used to assess feedback responses. This technique allowed us to add pharmacologic probes to different compartments (vessel, macula densa lumen, and interstitial space) while avoiding the influence of systemic factors.

METHODS

Afferent arterioles with the macula densa attached were isolated and microperfused as described previously [11, 12]. Briefly, young male New Zealand white rabbits were fed standard rabbit chow and given tap water ad libitum. The rabbits were anesthetized with ketamine (50 mg/kg, intramuscularly) and given an intravenous injection of heparin (500 U). Kidneys were sliced along the corticomedullary axis, and slices were placed in ice-cold minimum essential medium (MEM) (Gibco, Grand Island, NY, USA) containing 5% bovine serum albumin (BSA) (Intergen, Purchase, NY, USA). A single superficial afferent arteriole and its intact glomerulus from each rabbit were microdissected together with adherent tubular segments consisting of portions of the thick ascending limb, macula densa, and early distal tubule. Samples were transferred to a temperature-regulated chamber mounted on an inverted microscope (Olympus IMT-2) with Hoffmann modulation. Both the afferent arteriole and the end of either the distal tubule or thick ascending limb were cannulated with an array of glass pipettes as described previously [13]. Intraluminal pressure of the afferent arteriole was measured by Landis' technique, using a fine pipette introduced into the lumen through the perfusion pipette. The afferent arteriole was perfused with oxygenated MEM supplemented with 5% BSA, 5 mmol/L NaHCO$_3$, 10 mmol/L NaCl, 10 mmol/L HEPES, and 10 mmol/L NaOH. Intraluminal pressure was maintained at 60 mm Hg throughout the experiment. The macula densa was perfused with physiologic saline consisting of 10 mmol/L HEPES, 1.0 mmol/L CaCO$_3$, 0.5 mmol/L K$_2$HPO$_4$, 4.0 mmol/L KHCO$_3$, 1.2 mmol/L MgSO$_4$, 5.5 mmol/L glucose, 0.5 mmol/L Na acetate, 0.5 mmol/L Na lactate, and either 80 mmol/L NaCl (high) or 10 mmol/L NaCl (low). MEM was gassed with air, and physiologic saline was oxygenated with 100% O$_2$. The pH of each solution was 7.4. The bath was similar

to the arteriolar perfusate except that it contained 0.15% BSA, and was exchanged continuously at a rate of 1 mL/min. Microdissection and cannulation were completed within 90 minutes at 8°C, after which the bath was gradually warmed to 37°C for the rest of the experiment. Once the temperature was stable, a 30-minute equilibration period was allowed before taking any measurements. Images were displayed at magnifications up to 1980× and recorded with a video system. Afferent arteriole diameter was measured with a MetaMorph image analysis system (Universal Imaging, West Chester, PA, USA).

The Ca^{++} ionophore A23187, the cell membrane-permeant Ca^{++} chelator BAPTA-AM, and the Na^{+}/Ca^{++} exchanger inhibitor 2'4'-dichlorobenzamil were obtained from Molecular Probes (Eugene, OR, USA). The selective Na^{+}/Ca^{++} exchanger inhibitor KB-R7943 was obtained from Tocris Cookson (Ellisville, MO, USA). The Ca^{++} channel blockers nifedipine and pimozide, and the calcium-adenosine triphosphatase (ATPase) inhibitor thapsigargin, were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Statistics

Values are expressed as mean ± SEM. A paired *t* test was used to examine whether the diameter at a given concentration was different from control. ANCOVA was used to examine whether dose-response curves differed between groups, and a two-sample *t* test was used to examine whether the changes in diameter at a given concentration differed between groups. $P < 0.05$ was considered significant using Bonferroni's correction for multiple comparisons.

RESULTS

In the first series of experiments, we investigated the ability of elevated macula densa intracellular Ca^{++} to induce a tubuloglomerular feedback response. The Ca^{++} ionophore A23187 was added to the macula densa lumen together with the low NaCl perfusate. As shown in Figure 1, when the macula densa perfusate was changed from low to high NaCl, afferent arteriole diameter decreased from 18.1 ± 1.1 μm to 15.3 ± 0.8 μm ($P < 0.01$). When the luminal solution was switched back to low NaCl, diameter returned to baseline. When increasing concentrations of A23187 were added to the low NaCl macula densa perfusate, tubuloglomerular feedback was induced at 2 μmol/L and 5 μmol/L A23187; afferent arteriole diameter decreased from 18.0 ± 1.0 μm to 15.4 ± 0.9 μm and 14.5 ± 0.9 μm, respectively ($N = 6$; $P < 0.01$).
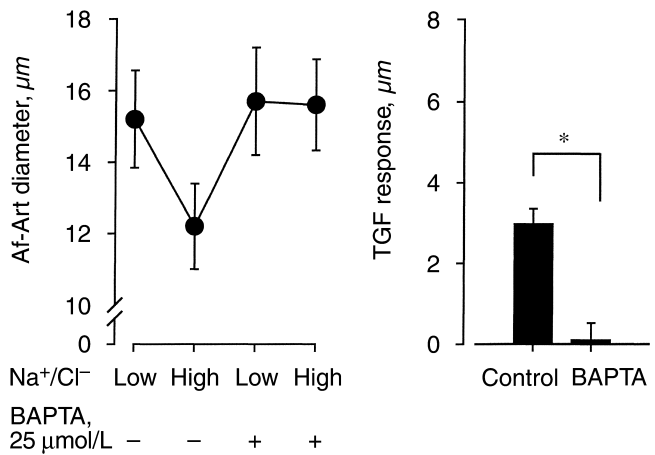
We next conducted experiments with the cell membrane-permeant Ca^{++} chelator BAPTA-AM to determine whether chelating intracellular Ca^{++} would prevent the tubuloglomerular feedback response. When the macula densa perfusate was changed from low to high
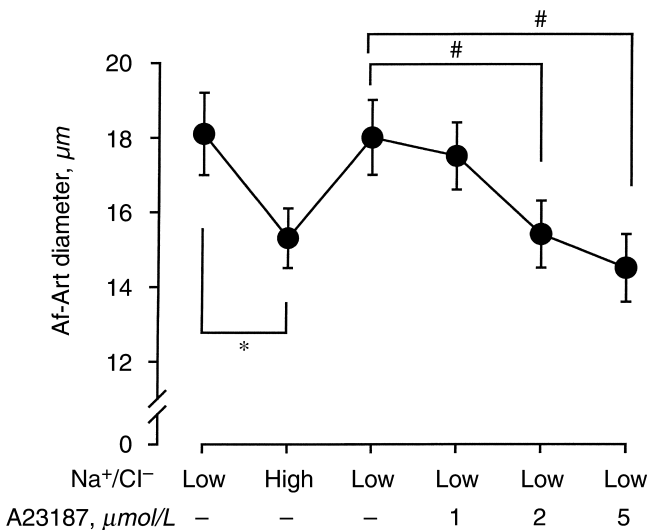
Fig. 1. Effect of the Ca^{++} ionophore A23187 on afferent arteriole (Af-Art) diameter during perfusion with low NaCl. Addition of increasing concentrations of A23187 (2 and 5 μmol/L) induced tubuloglomerular feedback. The magnitude of the decrease in diameter was no different from high NaCl-induced tubuloglomerular feedback. *$P < 0.01$, low vs. high; #$P < 0.01$, with vs. without A23187 ($N = 6$).

Fig. 2. Effect of the intracellular Ca^{++} chelator BAPTA-AM on high NaCl-induced tubuloglomerular feedback (TGF). In the presence of BAPTA-AM at the macula densa lumen, high NaCl-induced tubuloglomerular feedback was completely blocked. Af-Art is afferent arteriole. *$P < 0.01$ ($N = 7$).

NaCl, afferent arteriole diameter decreased from 15.2 ± 1.4 μm to 12.2 ± 1.2 μm ($N = 7$; $P < 0.01$). When the solution was changed to low NaCl, diameter returned to 15.4 ± 1.2 μm. Adding BAPTA-AM (25 μmol/L) to the macula densa lumen did not alter afferent arteriole diameter when the macula densa was perfused with low NaCl (15.4 ± 1.2 μm vs. 15.7 ± 1.5 μm), but inhibited constriction when the macula densa perfusate was changed to high NaCl (15.7 ± 1.5 μm vs. 15.6 ± 1.3 μm) (Fig. 2). The afferent arteriole response to angiotensin II was the same whether BAPTA-AM was present at the macula densa or not. Doses of 1 and 10 nmol/L angiotensin II decreased diameter from 17.7 ± 1.0 μm to 14.8 ± 1.5 μm and 11.0 ± 1.5 μm, respectively. With BAPTA-AM at the macula densa, diameter decreased from 17.5 ± 0.6 μm to 15.0 ± 1.3 μm and 10.0 ± 1.2 μm at 1 and 10 nmol/L angiotensin II, respectively ($N = 5$). Taken together, these data suggest that increased macula densa intracellular Ca^{++} is essential for tubuloglomerular feedback.

To make sure that the constriction we observed when we added A23187 to the macula densa lumen was not due to a direct influence on the afferent arteriole, we first treated the macula densa with BAPTA-AM and then added 5 μmol/L A23187 to the macula densa lumen together with low NaCl. We found that when BAPTA-AM was present at the macula densa, A23187 no longer decreased diameter (19.6 ± 1.4 μm vs. 19.7 ± 1.3 μm; $N = 6$), suggesting that A23187-induced afferent arteriole constriction is mediated by the macula densa and is not the result of a direct action on the afferent arteriole.

The next series of experiments was conducted to determine the source of increased intracellular Ca^{++} in response to increased NaCl concentration at the macula densa. Although the exact mechanism by which Ca^{++} enters the macula densa is not known, Na$^+$/Ca^{++} exchange has been suggested [14]. Consequently, we first tested whether blocking the Na$^+$/Ca^{++} exchanger would inhibit tubuloglomerular feedback. During the control period, afferent arteriole diameter decreased in response to increased NaCl concentration (from 18.5 ± 1.6 μm to 15.3 ± 1.2 μm; $N = 6$; $P < 0.01$). Adding the Na$^+$/Ca^{++} exchanger inhibitor 2'4'-dichlorobenzamil (10 μmol/L), to the bath did not alter afferent arteriole diameter when the macula densa was perfused with low NaCl (18.4 ± 1.6 μm vs. 18.7 ± 1.6 μm), but inhibited constriction when the macula densa perfusate was changed to high NaCl (from 18.9 ± 1.6 μm to 18.5 ± 1.6 μm) (Fig. 3). To confirm that the effects of 2'4'-dichlorobenzamil are strictly due to blockade of Na$^+$/Ca^{++} exchange, we used the selective Na$^+$/Ca^{++} exchanger blocker KB-R7943. We found that in the presence of 3 × 10^{-5} mol/L KB-R7943 in the bath, high NaCl-induced tubuloglomerular feedback was completely blocked. In the absence of KB-R7943, afferent arteriole diameter decreased in response to increased NaCl concentration (from 19.5 ± 1.7 μm to 16.2 ± 1.6 μm; $N = 5$; $P < 0.01$), while in the presence of KB-R7943 diameter remained unchanged when the macula densa NaCl concentration increased from low to high (19.5 ± 1.4 μm vs. 18.7 ± 1.4 μm) (Fig. 3). These data suggest that Ca^{++} entry into the macula densa via the basolateral Na$^+$/Ca^{++} exchanger is essential for tubuloglomerular feedback.
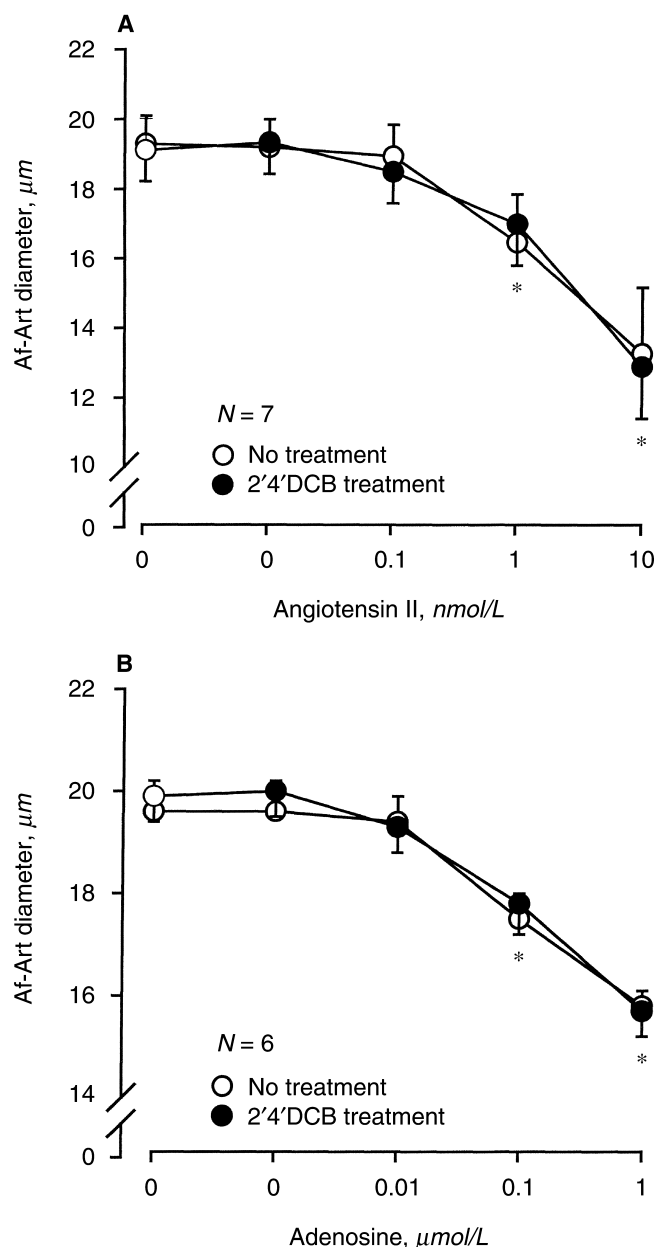
The Na$^+$/Ca^{++} exchanger is expressed not only in the distal nephron but also in the renal afferent arterioles.
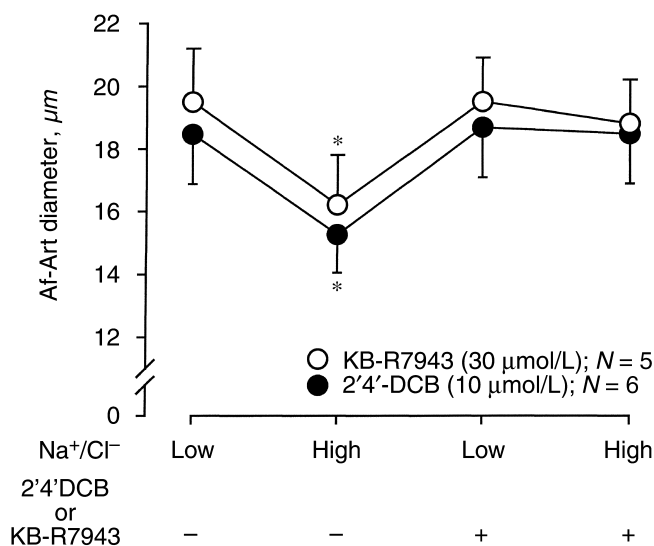
Fig. 3. Effect of the Na$^+$/Ca^{++} exchanger blocker 2′4′-dichloroben-zamil (2′4′-DCB) or KB-R7943 on tubuloglomerular feedback. Addition of 2′4′-dichlorobenzamil or KB-R7943 to the bath inhibited high NaCl-induced tubuloglomerular feedback. Af-Art is afferent arteriole. *P < 0.01.

Fig. 4. Afferent arteriole response to angiotensin II (*A*) or adenosine (*B*) when the Na$^+$/Ca^{++} exchanger blocker 2′4′-dichlorobenzamil (2′4′-DCB) was added to the bath. The afferent arteriole (Af-Art) exhibited the same response to angiotensin II or adenosine whether 2′4′-dichlorobenzamil was present or not, suggesting that it does not alter afferent arteriole contractility or basal diameter. *P < 0.01.

To test the possibility that the inhibitory effect of 2′4′-dichlorobenzamil on tubuloglomerular feedback is due to direct blockade of the Na$^+$/Ca^{++} exchanger at the afferent arteriole and the resultant alteration in arteriolar contractility, we perfused the afferent arteriole alone and studied its response to angiotensin II or adenosine in the presence of 2′4′-dichlorobenzamil. We first generated a dose-response curve with angiotensin II as a control, then added 2′4′-dichlorobenzamil (10 μmol/L) to the bath and generated a second angiotensin II dose-response curve. Angiotensin II constricted afferent arterioles in a dose-dependent manner. Doses of 1 and 10 nmol/L angiotensin II decreased diameter from 19.2 ± 0.8 μm to 16.5 ± 1.4 μm and 13.3 ± 1.9 μm, respectively. Adding 2′4′-dichlorobenzamil to the bath did not alter basal diameter, and angiotensin II constricted 2′4′-dichlorobenzamil–treated afferent arterioles to the same extent as those that were not treated (Fig. 4A). Figure 4B illustrates the afferent arteriole response to adenosine in the absence or presence of 2′4′-dichlorobenzamil. Adenosine constricted the afferent arteriole in a dose-dependent manner. Doses of 0.1 and 1 μmol/L adenosine reduced afferent arteriole diameter from 19.6 ± 0.5 μm to 17.5 ± 0.5 μm and 15.8 ± 0.3 μm, respectively. After we treated arterioles with 2′4′-dichlorobenzamil, adenosine-induced constriction was unchanged. Taken together, these data suggest that 2′4′-dichlorobenzamil at 10 μmol/L does not alter the response of afferent arteriole diameter to two different vasoconstrictors.

To see whether Na$^+$/Ca^{++} exchange mediates the entire increase in Ca^{++} or whether other processes are involved, we evaluated the role of intracellular Ca^{++} in mediation of feedback signals. Thapsigargin, which has been reported to inhibit sarcoplasmic reticulum Ca^{++}-ATPase activity and prevent restoration of intracellular Ca^{++} stores [15], was added to the macula densa perfusate and multiple tubuloglomerular feedback responses were induced to deplete intracellular Ca^{++} stores. During the control period, tubuloglomerular feedback decreased afferent arteriole diameter from 18.2 ± 0.9 μm
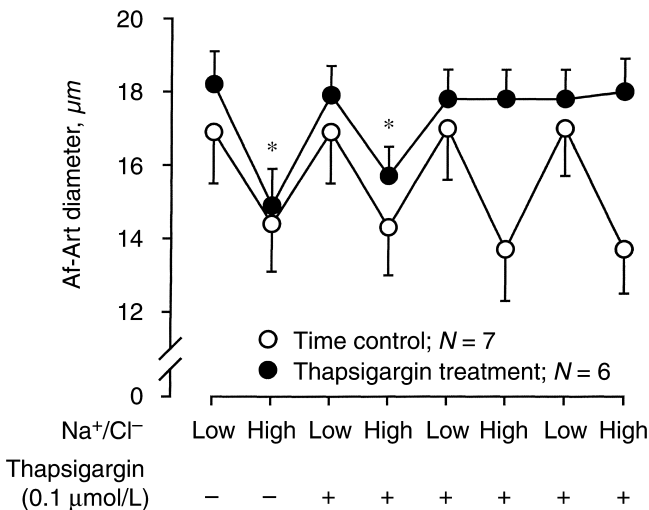
Fig. 5. Effect of the Ca^{++}-adenosine triphosphatase (ATPase) inhibitor thapsigargin on high NaCl-induced tubuloglomerular feedback. In the presence of thapsigargin, multiple tubuloglomerular feedback responses were induced. The first tubuloglomerular feedback response was inhibited 33% by thapsigargin and the second and third tubuloglomerular feedback responses were completely blocked. Af-Art is afferent arteriole. *P < 0.01, low vs. high (N = 6).

Fig. 6. Effect of adding Ca^{++} channel blockers to the macula densa lumen on high NaCl-induced tubuloglomerular feedback. Addition of either the L-type Ca^{++} channel blocker nifedipine or the T-type Ca^{++} channel blocker pimozide to the macula densa lumen did not alter tubuloglomerular feedback. Af-Art is afferent arteriole. *P < 0.01.

to 14.8 ± 1.0 μm (N = 6; P < 0.01). Adding thapsigargin (0.1 μmol/L) to the macula densa lumen along with low NaCl did not alter basal diameter (17.9 ± 0.9 μm vs. 17.9 ± 0.8 μm), but reduced the first tubuloglomerular feedback response by 33% when the macula densa perfusate was changed to high NaCl and completely eliminated the second and third tubuloglomerular feedback responses (second, from 17.8 ± 0.8 μm to 17.8 ± 0.8 μm; third, from 17.8 ± 0.8 μm to 18.0 ± 0.9 μm) (Fig. 5). In the absence of thapsigargin, there was no significant decrease in tubuloglomerular feedback responses. Tubuloglomerular feedback decreased afferent arteriole diameter by 2.5 μm (from 16.9 ± 1.4 μm to 14.4 ± 1.3 μm) when the macula densa perfusate was first changed from low to high NaCl. When a second, third, and fourth tubuloglomerular feedback response was tested, diameter decreased by 2.6 μm (from 16.9 ± 1.4 μm to 14.3 ± 1.3 μm), 3.3 μm (from 17.0 ± 1.4 μm to 13.7 ± 1.4 μm) and 3.3 μm (from 17.0 ± 1.3 μm to 13.7 ± 1.2 μm), respectively.

We next investigated whether Ca^{++} entry via L-type and T-type Ca^{++} channels at the apical side of the macula densa normally participates in the transmission of feedback signals. Figure 6 shows the tubuloglomerular feedback response during luminal perfusion with the L-type Ca^{++} channel blocker nifedipine (25 μmol/L) or the T-type Ca^{++} channel blocker pimozide (10 μmol/L), as these concentrations effectively blocked Ca^{++} channels in the microperfused macula densa and afferent arteriole [6] [abstract; Feng, Li, Navar, *Hypertension* 40:428, 2002]. We found that tubuloglomerular feedback responses ob-

tained with nifedipine or pimozide in the macula densa lumen were not significantly different from control.

DISCUSSION

We evaluated the role of macula densa intracellular Ca^{++} in mediation of the tubuloglomerular feedback response. In addition, we attempted to identify the source of the increase in macula densa intracellular Ca^{++} in response to increased luminal delivery of NaCl. We found that (*1*) the increase in macula densa intracellular Ca^{++} caused by adding the Ca^{++} ionophore A23187 was sufficient to induce a tubuloglomerular feedback response, while chelation of intracellular Ca^{++} by BAPTA-AM inhibited tubuloglomerular feedback; (*2*) 2′4′-dichlorobenzamil blocked tubuloglomerular feedback by inhibiting Ca^{++} entry via the macula densa basolateral Na^{+}/Ca^{++} exchanger, which in turn appeared to initiate Ca^{++}-mediated Ca^{++} release from these stores; and (*3*) luminal L-type or T-type Ca^{++} channels were not involved in the tubuloglomerular feedback response.

The mechanism whereby the macula densa cells detect changes in luminal fluid composition and transmit feedback signals to the vascular smooth muscle has previously been suggested by Bell et al [7, 16]. Raising the NaCl concentration in the macula densa perfusate increased intracellular Ca^{++} [6]. When the Ca^{++} ionophore A23187 was added to a tubular perfusate containing 4 mEq/L Ca^{++} in micropuncture preparations, it markedly increased SFP feedback responses. In addition, when Ca^{++}

was completely removed from the perfusion solution, feedback responses were significantly attenuated [8]. Our in vitro microperfusion studies confirmed these earlier observations. When the macula densa was perfused with a low NaCl solution containing 2 or 5 μmol/L A23187 to increase cell membrane permeability to Ca^{++}, the reduction in afferent arteriole diameter was not different from the tubuloglomerular feedback induced by high NaCl. Chelating macula densa intracellular Ca^{++} with BAPTA-AM blocked the tubuloglomerular feedback response induced by high NaCl. These results suggest that increased macula densa intracellular Ca^{++} is essential for transmission of tubuloglomerular feedback signals.

Since Ca^{++} plays an important role in mediation of the tubuloglomerular feedback response, it was of interest to determine the source of the increase in macula densa intracellular Ca^{++} during the tubuloglomerular feedback response. One possibility is that the Na$^+$/Ca^{++} exchanger at the basolateral membranes of epithelial tissues may participate in regulation of the cellular Ca^{++} system in response to increased luminal NaCl concentration. Studies in a variety of tissues, including proximal tubular cells [17–19], indicate that a Na$^+$/Ca^{++} exchange mechanism depends upon a large inward Na$^+$ electrochemical gradient to provide the driving force for Ca^{++} to exit from the cell to the extracellular fluid. Normally, Ca^{++} is extruded by the basolateral Na$^+$/Ca^{++} exchanger. Na$^+$/Ca^{++} exchanger activity is strongly affected by the transmembrane Na$^+$ concentration gradient and differences in electrical potential. Evidence suggests that when the NaCl concentration at the macula densa lumen is increased, activation of the Na/K/2 Cl cotransporter at the macula densa leads to an increase in intracellular sodium and chloride as well as depolarization of the membrane potential. Both increased intracellular Na$^+$ and membrane depolarization could be driving forces that move calcium into the macula densa cells via the Na$^+$/Ca^{++} exchanger [20]. To test the role of macula densa Na$^+$/Ca^{++} exchange in the mediation of tubuloglomerular feedback, we used 2′4′-dichlorobenzamil, which has been reported to inhibit the Na$^+$/Ca^{++} exchanger in other cells [21, 22]. We found that when 2′4′-dichlorobenzamil was added to the bath, it completely inhibited tubuloglomerular feedback. Since the Na$^+$/Ca^{++} exchanger is expressed not only in the distal nephron but also in the renal afferent arterioles [23], and since inhibition of the Na$^+$/Ca^{++} exchanger has recently been reported to increase renal vascular resistance [24], we perfused the afferent arteriole alone and studied its response to two vasoconstrictors (angiotensin II and adenosine) in the presence of 2′4′-dichlorobenzamil. Adding 2′4′-dichlorobenzamil to the bath did not alter either basal diameter or the vasoconstrictor effect of angiotensin II and adenosine. It seems unlikely that inhibition of tubuloglomerular feedback by 2′4′-dichlorobenzamil is due to a direct effect on the afferent arteriole. Results of selective Na$^+$/Ca^{++}-exchanger inhibition with KB-R7943 are consistent with the effects of 2′4′-dichlorobenzamil. We believe these are the first data to show that Ca^{++} entry *via* the macula densa basolateral Na$^+$/Ca^{++} exchanger is necessary for tubuloglomerular feedback. These results are consistent with immunohistochemical studies showing that the renal distal convoluted tubule cells at the basolateral membrane contain numerous Na$^+$/Ca^{++} exchangers [10]. Early patch clamp studies of macula densa cells also support functional expression of the Na$^+$/Ca^{++} exchanger at the basolateral membrane of the macula densa [25].

Although the Na$^+$/Ca^{++} exchanger causes at least part of the increase in macula densa Ca^{++} required for the tubuloglomerular feedback response, it is not clear whether Na$^+$/Ca^{++} exchange mediates the entire increase in Ca^{++} or whether other processes are involved. We considered the possibility that activation of a cytosolic Ca^{++} system could involve mobilization of Ca^{++} from internal storage sites. It has been reported that in epithelial cells as well as macula densa cells, inositol 1,4,5-triphosphate (IP$_3$)-dependent agonists such as adenosine triphosphate (ATP) [26, 27] and bradykinin [28] increase intracellular Ca^{++} by releasing Ca^{++} from the endoplasmic reticulum. To see how release of intracellular Ca^{++} contributes to mediation of feedback signals, we used thapsigargin, which has been reported to inhibit the endoplasmic reticulum Ca^{++} pump and prevent restoration of intracellular Ca^{++} stores [15]. In our studies, adding thapsigargin to the macula densa perfusate led to progressive inhibition of tubuloglomerular feedback responses, consistent with previous micropuncture studies by Bell and Reddington [8] who found that addition of TMB-8 inhibited release of Ca^{++} from the sarcoplasmic reticulum and markedly decreased the magnitude of SFP feedback responses. Although the mechanism that causes macula densa intracellular Ca^{++} signaling is not fully understood, our data suggest that Na$^+$/Ca^{++} exchange appears to initiate Ca^{++}-mediated Ca^{++} release from intracellular stores. While we are aware of the limitations of this approach, it must be noted that this and analogous calcium-ATPase inhibitors have been used extensively in various cell lines and isolated arteriole preparations. To our knowledge, most of the effects of thapsigargin have been shown to be related to depletion of Ca^{++} from intracellular stores. However, it should be noted that thapsigargin only causes transient increases in cytoplasmic Ca^{++}. When release of Ca^{++} from intracellular stores is stimulated in the presence of thapsigargin, intracellular Ca^{++} increases and then usually returns to baseline. The increase in the presence of thapsigargin is only slightly greater than in its absence. Other mechanisms return cytoplasmic Ca^{++} to basal levels. Thus prolonged elevation

of cytoplasmic Ca^{++} that could disrupt other cellular functions is not a concern.

Finally, we tested whether increases in NaCl at the macula densa lumen increase Ca^{++} entry from the tubular fluid into the macula densa cells. Neither the L-type Ca^{++} channel blocker nifedipine nor the T-type Ca^{++} channel blocker pimozide altered the tubuloglomerular feedback response induced by high NaCl. Bell and Reddington [8], using micropuncture techniques, found that supplementing the perfusate with verapamil, which reportedly blocks voltage-dependent Ca^{++} channels, had no effect, and that SFP feedback responses were similar in magnitude to those obtained with the isotonic solution. Recently, Peti-Peterdi and Bell [6] used fluorescence microscopy and isolated perfused tubules to measure macula densa intracellular Ca^{++} in response to changes in luminal NaCl. They found that adding nifedipine to the bath abolished the increase in macula densa cytosolic Ca^{++} induced by high NaCl, suggesting functional expression of L-type Ca^{++} channels at the basolateral membrane of the macula densa. We did not test adding a voltage-dependent Ca^{++} channel blocker to the bath, because it would directly interfere with contractility of the smooth muscle cells. It has been demonstrated that systemic or intrarenal arterial administration of Ca^{++} channel blockers can increase both whole-kidney blood flow and GFR [29]. Taken together, increased macula densa intracellular Ca^{++} in response to increased NaCl concentration does not appear to be dependent on Ca^{++} entry from the luminal fluid via voltage-dependent Ca^{++} channels.

Changes in intracellular Ca^{++} in the macula densa during tubuloglomerular feedback are controversial; various reports indicate that increasing luminal NaCl induces a decrease, no change or an increase in macula densa calcium [5, 6]. It appears that much of the controversy may be due to the technical difficulty of measuring intracellular Ca^{++} in the macula densa when luminal NaCl is increased. First, many of the measurements were made in preparations in which the glomerulus was not attached. This leads to the possibility that the macula densa was damaged. Second, as recently reported by Salomonsson et al [5] and Peti-Peterdi and Bell [6], macula densa cell volume changes significantly when luminal NaCl concentration is altered. Even with ratiometric dyes such as Fura-2 or Indo-1, large changes in cell volume and thus concentration of the dye can make calibration and interpretation of the data difficult. Finally, the reported R_{max}/R_{min} ratios for measurements of Ca^{++} using Fura-2 have been relatively low, bringing into question the accuracy of the measurements. It is not possible to simultaneously measure tubuloglomerular feedback and macula densa Ca^{++} in our preparation using current technology. Thus, one is left with an indirect experimental design to investigate how intracellular Ca^{++} changes in the macula densa during tubuloglomerular feedback. Our data using BAPTA-AM indicate that a change in Ca^{++} is necessary for tubuloglomerular feedback. By themselves, these data do not necessarily provide support for either an increase or a decrease. However, the $2'4'$-dichlorobenzamil, KB-R7943, and thapsigargin data appear to support the hypothesis that an increase in luminal NaCl at the macula densa leads to an increase in intracellular Ca^{++}.

CONCLUSION

The present studies suggest that macula densa intracellular Ca^{++} plays an essential role in the tubuloglomerular feedback response. Increased cytosolic Ca^{++} induced tubuloglomerular feedback, whereas chelated cytosolic Ca^{++} inhibited tubuloglomerular feedback. Furthermore, the macula densa basolateral Na$^+$/Ca^{++} exchanger appears to be involved in increases in intracellular Ca^{++} and Ca^{++}-mediated Ca^{++} release from intracellular stores, whereas luminal L-type or T-type Ca^{++} channels do not.

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