Cytosolic [Ca\(^{2+}\)] signaling pathway in macula densa cells

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**PETI-PETERDI, J.**, and P. DARWIN BELL. Cytosolic [Ca\(^{2+}\)] signaling pathway in macula densa cells. Am. J. Physiol. 277 (Renal Physiol. 46): F472–F476, 1999.—Previous micropuncture studies suggested that macula densa (MD) cells might detect variations in luminal sodium chloride concentration ([NaCl]\(_{l}\)) through changes in cytosolic calcium ([Ca\(^{2+}\)]). To test this hypothesis, MD [Ca\(^{2+}\)] was measured with fluorescence microscopy using fura 2 in the isolated perfused thick ascending limb with attached glomerulus preparation dissected from rabbit kidney. Tubules were bathed and perfused with a Ringer solution, [NaCl] was varied and isosmotically replaced with N-methyl-D-glucamine cyclamate. Control [Ca\(^{2+}\)], during perfusion with 25 mM NaCl and 150 mM NaCl in the bath, averaged 101.6 ± 8.2 nM (n = 21). Increasing [NaCl] to 150 mM elevated [Ca\(^{2+}\)], by 39.1 ± 5.2 nM (n = 21, P < 0.01). This effect was concentration dependent between zero and 60 mM [NaCl]. The presence of either luminal furosemide or basolateral nifedipine or 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), a potent Cl\(^{-}\)/H\(^{+}\)-exchanger and a Na\(^{-}\)-K\(^{+}\)-Cl\(^{-}\} cotransporter (6, 9, 10), the apical membrane contains a Na\(^{+}/H^{+}\) exchanger and a high density of K\(^{+}\) channels (6). At the basolateral membrane, NaCl exit may occur, at least in part, through Na\(^{+}-K^{+}\)-ATPase and Cl\(^{-}\) channels (6, 9, 11).

Previous micropuncture studies (4, 5) from our laboratory suggested that a cytosolic Ca\(^{2+}\) system might be involved in the MD cell signaling. In these studies, luminal perfusion of the Ca\(^{2+}\) ionophore, A23187, in the presence of perfusate Ca\(^{2+}\) enhanced feedback responses (4), whereas 8-(N,N-diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8), an inhibitor of intracellular release of Ca\(^{2+}\), reduced stop-flow pressure feedback responses (5). An MD cytosolic calcium ([Ca\(^{2+}\)]\(_{c}\)) signaling pathway, involved in TGF signal transmission, was appealing, since Ca\(^{2+}\) could have a number of potential effects including regulation of ionic transport processes (6) or stimulation of the release of a chemical mediator. However, more recent studies (7, 12; and unpublished observations) using fluorescence microscopy and the isolated perfused tubule technique to measure MD [Ca\(^{2+}\)], directly in response to changes in [NaCl], have produced equivocal results. It is therefore uncertain whether a rise in [NaCl] does in fact result in an elevation in MD [Ca\(^{2+}\)]. Part of the problem has been difficulties (at least in our laboratory) in using fura 2 to reliably measure changes in MD [Ca\(^{2+}\)].

Therefore, the purpose of this study was to reassess the effects of changes in [NaCl] on MD [Ca\(^{2+}\)]. Our work is the first to report that increases in [NaCl] do indeed produce significant increases in MD [Ca\(^{2+}\)]. We went on to then examine the mechanism by which increased [NaCl] induces elevations in MD [Ca\(^{2+}\)].

**METHODS**

**CTAL preparation.** Individual CTALs with attached glomeruli were dissected from rabbit kidney and perfused in vitro as described previously (13) with minor modifications. To prevent intracellular accumulation of NaCl prior to the experiment, the dissection solution was an isosmotic, low-NaCl Ringer solution consisting of (in mM) 25 NaCl, 120 N-methyl-D-glucamine (NMDG) cyclamate, 5 KCl, 1 MgSO\(_{4}\), 1.6 Na\(_{2}\)HPO\(_{4}\), 0.4 NaH\(_{2}\)PO\(_{4}\), 1.5 CaCl\(_{2}\), 5 d-glucose, and 10 HEPES. Temperature was maintained at about 4°C. An individual CTAL was transferred to a chamber mounted on an inverted microscope. The tubule was kept in the low-NaCl solution until it was cannulated and perfused with the isosmotic 25 mM NaCl solution. Then the bathing solution...
was changed to the 150 mM NaCl Ringer solution at 37°C. According to our experience, this maneuver greatly improved the responsiveness of MD cells, at least in terms of (Ca^{2+})
 dynamics. When we used a Ringer dissection solution containing 150 mM NaCl, we got the same equivocal results reported by others, i.e., increases, decreases, or no changes in (Ca^{2+}), with alterations in luminal composition.

Measurement of (Ca^{2+})
 of MD cells was measured with dual-excitation wavelength fluorescence microscopy (Photon Technologies, Princeton, NJ) using the fluorescent probe fura 2 (Teflabs, Austin, TX). Fura 2 fluorescence was measured at an emission wavelength of 510 nm in response to excitation wavelengths of 340 and 380 nm, alternated at a rate of 25 Hz by a computer-controlled chopper assembly. An adjustable photometer window was positioned over the MD plaque (consisting of ~10–15 cells), and emitted photons were detected by a Leitz photometer that was modified for photon counting. Magnification was ×400 using an Olympus ×40 UVFL lens. Autofluorescence-corrected ratios (340 nm/380 nm) were calculated at a rate of 5 points/s using PTI software. MD cells were loaded with the dye by adding the acetoxymethyl ester of fura 2 (fura 2-AM, 10 μM) dissolved in DMSO to the 25 mM NaCl containing luminal perfusate. Loading required ~15 min, after which fura 2-AM was removed from the lumen. A ~20-min incubation of the tubule with the control perfusion solution was allowed to permit stabilization of fluorescent signal.

Increases in (Ca^{2+})
 normally result in an increase in the fura 2 340-nm signal and a decrease in the 380-nm signal. In MD cells, progressing from a perfusate of 25 mM NaCl Ringer solution (=90 mosmol/kg) to 150 mM NaCl Ringer solution (=300 mosmol/kg), results in parallel changes in the 340-nm and 380-nm signals. This is the result of cell volume-induced changes in fura 2 concentration. We do not believe that it is valid to use ratio measurement as an index of changes in (Ca^{2+})
 in this situation. Thus we believe that fura 2 cannot be used for reliable measurements of (Ca^{2+})
, with variations in luminal osmolality. Baseline fura 2 ratios were measured under control conditions (25 mM [NaCl] and 150 mM NaCl in the bath) with increases in [NaCl], and after return to the low-NaCl perfusate. As indicated, all perfusates were maintained isosmotic.

The 340/380 ratios (R) were converted into (Ca^{2+})
 values using the equation of Grynkiewicz et al. (8) as follows

\[
[Ca^{2+}]_i = K_d \times \frac{(R - R_{min})}{(R_{max} - R)} \times \left(\frac{S_{380}}{S_{360}}\right)
\]

where R_{max} and R_{min} are R values under saturating and Ca^{2+}-free conditions, respectively, and S_{380} and S_{360} are the fluorescent signals (S) emitted by the Ca^{2+}-free (f) and Ca^{2+}-bound (b) forms, respectively, of fura 2 at 380 nm. In situ calibration was accomplished after permeabilizing MD cells with 5 μM ionomycin and measuring fluorescence at both wavelengths under Ca^{2+}-free (2 mM EGTA) or saturating Ca^{2+} (1.5 mM CaCl_2) conditions. R_{max}, R_{min}, and S_{380}/S_{360} values were 1.03, 0.4, and 1.97, respectively, K_d, the dissociation constant of fura 2 for Ca^{2+}, was taken as 224 nM (8).

Materials. The following pharmacological agents were used in these experiments: 50 μM of the Na^2-2Cl^-K^+ cotransport blocker furosemide; 500 μM TMB-8, a putative inhibitor of intracellular release of bound Ca^{2+}; 1 μM of the voltage-gated Ca^{2+}-channel blocker nifedipine (Pfizer, New York, NY); and 100 nM BAY K 8644, a voltage-gated Ca^{2+}-channel agonist, or 10 μM of 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB; RBI, Natick, MA), a potent chloride channel blocker. As denoted in the text, agents were added to the perfusate or bath. Furosemide was dissolved in methanol, BAY K 8644 in ethanol, and TMB-8, nifedipine, and NPPB in DMSO, with final solvent concentrations of less than 0.5%. Data were collected after an incubation of 2–3 min with these agents. Chemicals, if not indicated, were purchased from Sigma Chemical, St. Louis, MO. Vehicles used in these studies, such as DMSO, ethanol, and methanol, did not affect either the resting [Ca^{2+}], or the [NaCl]-induced MD [Ca^{2+}] responses. Also, NMDG cyclamate does not interfere with fura 2 fluorescence measurements.

Data analysis. Data are expressed as means ± SE. Statistical significance was tested using Student’s t-test for paired samples and analysis of variance. Significance was accepted at P < 0.05.

RESULTS

Basal and [NaCl]-dependent Ca^{2+} activity. Resting MD [Ca^{2+}]_i in the presence of 25 mM [NaCl], was 101.6 ± 8.2 nM (n = 21). As shown in a representative recording in Fig. 1, and summarized in Fig. 2, increasing [NaCl], from 25 to 150 mM caused a rapid and reversible increase in [Ca^{2+}], which averaged 39.1 ± 5.2 nM (n = 21, P < 0.01). Also, this [NaCl]-induced elevation in [Ca^{2+}], was NaCl concentration dependent between 0 and 60 mM [NaCl]. Normalized as percentage of the basal value of 65.1 ± 3.8 nM in the presence of zero [NaCl], increasing [NaCl] to 25, 40, 50 and 60

![Fig. 1. Representative recording of luminal sodium chloride concentration ([NaCl])-dependent changes in macula densa (MD) cytosolic calcium concentration ([Ca^{2+}]).](http://ajprenal.physiology.org/)

![Fig. 2. Effects of luminal furosemide, basolateral nifedipine and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) on [NaCl]-dependent increase in MD [Ca^{2+}]. Values are means ± SE; numbers in parentheses refer to number of experiments in each group. * P < 0.05, compared with control values with 25 mM [NaCl]. ** P < 0.05, compared with control values with 150 mM [NaCl].](http://ajprenal.physiology.org/)
mM increased [Ca\(^{2+}\)]\(_{\text{c}}\), by 21 \(\pm\) 4, 29 \(\pm\) 4, 37 \(\pm\) 4, and 49 \(\pm\) 3%, respectively (\(n = 5\), \(P < 0.05\) compared with each preceding value). Also, as shown in Fig. 2, addition of 50 \(\mu\)M furosemide to the luminal perfusate significantly reduced resting [Ca\(^{2+}\)]\(_{\text{c}}\) and abolished [NaCl]-induced elevations in [Ca\(^{2+}\)]\(_{\text{c}}\).  

Mechanism of increase in [Ca\(^{2+}\)]\(_{\text{c}}\). To determine the source for the increase in [Ca\(^{2+}\)]\(_{\text{c}}\), we first examined the effect of TMB-8, a putative inhibitor of intracellular release of bound Ca\(^{2+}\). Addition of 500 \(\mu\)M TMB-8 to the bath had no effect on the [Ca\(^{2+}\)]\(_{\text{c}}\) response; increasing [NaCl], from 25 to 150 mM increased [Ca\(^{2+}\)]\(_{\text{c}}\), by 35.5 \(\pm\) 12.3 nM (\(n = 5\), \(P < 0.05\)), a value not significantly different from control.  

It is well established that progressive increases in [NaCl], between 20 to 60 mM result in parallel depolarization of the MD cell basolateral membrane (3, 6). Therefore, the presence of a membrane voltage-associated Ca\(^{2+}\) pathway seemed plausible. To examine the role of putative voltage-gated Ca\(^{2+}\) channels in the MD [Ca\(^{2+}\)]\(_{\text{c}}\) response to increased [NaCl], experiments were performed utilizing the dihydropridine Ca\(^{2+}\) antagonist nifedipine. As shown in Fig. 2, the increase in MD [Ca\(^{2+}\)]\(_{\text{c}}\), normally elicited by 150 mM [NaCl], was abolished in the presence of 1 \(\mu\)M nifedipine added to the bath. Furthermore, bath nifedipine caused a significant reduction of the MD resting [Ca\(^{2+}\)]\(_{\text{c}}\). In contrast, nifedipine failed to produce similar inhibitory effects when added exclusively to the luminal perfusate. Increasing [NaCl], from 25 to 150 mM in the presence of 1 \(\mu\)M luminal nifedipine increased [Ca\(^{2+}\)]\(_{\text{c}}\), by 37.3 \(\pm\) 6.2% (\(n = 6\), \(P < 0.05\)) from a resting value of 100.3 \(\pm\) 10.7 nM, an increase that was not significantly different from that found in the absence of luminal nifedipine.  

Basolateral Cl\(^{-}\) channels are thought to be responsible for the depolarization of MD cells (3, 6, 11, 16). To further evaluate the existence of voltage-gated Ca\(^{2+}\) channels at the basolateral membrane of MD cells and the role of Cl\(^{-}\) channels in the membrane depolarization-Ca\(^{2+}\) response, we performed experiments using BAY K 8644, a voltage-gated Ca\(^{2+}\) channel agonist, and NPPB, a potent chloride channel blocker. Added to the bath, 100 nM BAY K 8644 increased [Ca\(^{2+}\)]\(_{\text{c}}\), by 32.2 \(\pm\) 8.1 nM (\(n = 5\), \(P < 0.05\)) from a basal value of 75.2 \(\pm\) 9.6 nM. As shown in Fig. 2, 10 \(\mu\)M NPPB added to the bath significantly decreased resting [Ca\(^{2+}\)]\(_{\text{c}}\), and abolished the increase in MD [Ca\(^{2+}\)]\(_{\text{c}}\), normally elicited by 150 mM [NaCl]. Finally, it is interesting to note that MD [Ca\(^{2+}\)]\(_{\text{c}}\) values were not statistically different under the experimental conditions of luminal furosemide treatment (57.6 \(\pm\) 3.7 nM), zero [NaCl] (65.1 \(\pm\) 3.8 nM), and basolateral nifedipine (60.0 \(\pm\) 8.4 nM) treatment.

**DISCUSSION**

In the present studies, we measured [Ca\(^{2+}\)]\(_{\text{c}}\) in MD cells with fluorescence microscopy using the isolated perfused CTAL-MD preparation. Resting [Ca\(^{2+}\)]\(_{\text{c}}\), during perfusion with 25 mM NaCl and 150 mM NaCl in the bath averaged 101.6 \(\pm\) 8.2 nM, a value within the range that has been reported previously (2, 7, 14). Increases in [NaCl], from 25 to 150 mM resulted in consistent increases in MD [Ca\(^{2+}\)]\(_{\text{c}}\). The reason why we have now obtained consistent MD [Ca\(^{2+}\)]\(_{\text{c}}\) responsiveness may be the result of two technical modifications. First was the use of a low-NaCl dissection solution, which should minimize luminal NaCl entry and prevent prolonged high intracellular [Na\(^{+}\)] and [Ca\(^{2+}\)]. Second, was the fact that all solutions were isosmotic, which eliminated cell volume-related changes in fura 2 fluorescence (unpublished observations). Salomonsson et al. (7, 14) reported no consistent changes in MD [Ca\(^{2+}\)]\(_{\text{c}}\); however, these experiments were performed using hypotonic luminal perfusates, which may have interfered with the detection of changes in [Ca\(^{2+}\)]\(_{\text{c}}\).  

[NaCl]-induced Ca\(^{2+}\) responses were also found to be concentration dependent between zero and 60 mM [NaCl], the most sensitive range for changes in TGF responses (2, 6, 17). These findings are consistent with the conclusion of previous micropuncture experiments (4, 5) that MD cells might detect variations in tubular fluid composition and transmit vasoconstrictor feedback signals to the afferent arteriole through a cytosolic Ca\(^{2+}\) system.  

It is generally accepted that generation of feedback signals involves, at least in part, changes in [NaCl]. At the apical membrane, the primary NaCl entry site is through the Na\(^{+}\)-2Cl\(^{-}\)-K\(^{+}\) cotransporter (6, 10), whereas NaCl exit may occur, at least in part, through Na\(^{+}\)-K\(^{+}\)-ATPase and a Cl\(^{-}\) conductance (6, 9, 11). Secondary changes in cell [Cl\(^{-}\)], as the result of alterations in NaCl transport by MD cells, acting at the basolateral Cl\(^{-}\) channel (9) may be responsible for the changes in basolateral membrane potential (\(V_{\text{bl}}\)) that are observed in these cells (3, 10, 15, 16). The regulation of \(V_{\text{bl}}\) by intracellular Cl\(^{-}\) was established in previous microelectrode and ion substitution studies (3, 6, 10, 11). These studies indicated that apical Na\(^{+}\)-2Cl\(^{-}\)-K\(^{+}\) cotransporter by MD cells would result in an elevation in intracellular Cl\(^{-}\) activity, electrogenic Cl\(^{-}\) efflux across the basolateral membrane, and depolarization of \(V_{\text{bl}}\). This general notion has been supported by Schlatter et al. (15, 16), who showed that addition of NPPB to the bathing solution hyperpolarized MD cells. Thus progressive increases in [NaCl], from 20 to 60 mM result in parallel changes in TGF responses (vasoconstriction) and membrane depolarization (6).  

Shown in Fig. 3 is a schematic drawing of how such transport-related events may increase MD [Ca\(^{2+}\)]. In support of this model, we found that luminal furosemide abolished elevations in MD [Ca\(^{2+}\)], in response to an increase in [NaCl]. From this observation, we conclude that the apical Na\(^{+}\)-2Cl\(^{-}\)-K\(^{+}\) cotransporter is involved in the MD Ca\(^{2+}\) response. In previous work measuring MD \(V_{\text{bl}}\) (3, 10), we found that progressive increases in [NaCl], from 20 to 60 mM resulted in progressive depolarization of the MD \(V_{\text{bl}}\) and that furosemide abolished [NaCl]-induced MD depolarization. Schlatter et al. (15, 16) likewise found that furosemide hyperpolarized MD cells.  

It was therefore reasoned that this large depolarization of \(V_{\text{bl}}\), in the presence of high [NaCl], could serve to promote calcium entry across the basolateral membrane. We therefore examined the presence of putative voltage-gated Ca\(^{2+}\) channels in MD cells. Addition of
the dihydropyridine Ca$^{2+}$ antagonist nifedipine to the bathing solution abolished the [NaCl]-induced increase in MD [Ca$^{2+}$], and significantly reduced resting [Ca$^{2+}$]. Also, BAY K 8644, a voltage-gated Ca$^{2+}$ channel agonist, added to the bath significantly increased MD [Ca$^{2+}$]. These data strongly support the functional expression of voltage-gated Ca$^{2+}$ channels at the basolateral membrane of MD cells.

Since basolateral Cl$^-$ channels are responsible for the depolarization of MD $V_{bl}$ with increases in [NaCl], (6, 9, 11, 16), another test of this model was to determine the effect of inhibiting the basolateral Cl$^-$ channels. [NaCl]-induced changes in MD [Ca$^{2+}$], were evaluated in the presence of the Cl$^-$ channel blocker NPPB. Addition of NPPB to the bathing solution, significantly reduced resting [Ca$^{2+}$], and abolished the elevation in MD [Ca$^{2+}$], in response to increasing [NaCl]. Thus these results further support a role for depolarization-induced Ca$^{2+}$ entry across the MD basolateral membrane.

In most cell types, sustained activation of a cytosolic calcium system requires both Ca$^{2+}$ mobilization as well as Ca$^{2+}$ entry. In earlier micropuncture studies (5), a role for Ca$^{2+}$ entry across the luminal membrane was tested by removal of luminal Ca$^{2+}$ and addition of a Ca$^{2+}$ chelator. In the presence of an isotonic solution, depletion of luminal Ca$^{2+}$ did not influence feedback responses, eliminating a role for Ca$^{2+}$ entry into the MD cells across the luminal membrane. However, Naruse et al. (12) found alterations in the afferent arteriolar diameter in response to changes in luminal [Ca$^{2+}$]. Based on this finding, they suggested the presence of an apical dihydropyridine-sensitive Ca$^{2+}$ conductance. In contrast, the present studies showed that administration of nifedipine to the luminal perfusate had no effect on either the resting [Ca$^{2+}$], or the [NaCl]-induced Ca$^{2+}$ response of MD cells. Thus our data support the conclusion of earlier micropuncture experiments, indicating the absence of apical voltage-gated Ca$^{2+}$ channels in MD cells.

In previous micropuncture experiments (5), luminal administration of TMB-8, a putative inhibitor of intracellular release of bound Ca$^{2+}$, resulted in a substantial reduction in the magnitude of feedback responses. This inhibition was specific for Ca$^{2+}$, since inhibition by TMB-8 could be overcome by simultaneous addition of a Ca$^{2+}$ ionophore. In the present studies, TMB-8 had no effect on MD [Ca$^{2+}$] dynamics, suggesting that Ca$^{2+}$ entry via basolateral voltage-gated Ca$^{2+}$ channels may be the primary source for the [NaCl]-induced Ca$^{2+}$ signal. However, these studies cannot entirely rule out the possibility that Ca$^{2+}$ mobilization may occur in response to elevated [NaCl]. It should be noted that previous work from our laboratory (2, 4) has reported that changes in luminal osmolality can also influence feedback responses. It is possible that this occurs through alterations in cell volume, which could have an effect on [Ca$^{2+}$], and possibly on Ca$^{2+}$ mobilization. Unfortunately, due to technical constraints, it was not possible to evaluate the effects of changes in perfusate osmolality on MD [Ca$^{2+}$]. Nevertheless, the current studies support a model in which Ca$^{2+}$ entry across the basolateral membrane plays an important role in MD Ca$^{2+}$ signaling.

It remains to be shown that MD Ca$^{2+}$ signaling is directly involved in TGF signal transmission. However, because of the nature of the cell signaling properties of this divalent ion, we believe that it is very likely that MD [Ca$^{2+}$] plays a crucial role in TGF$^{2+}$ signaling. Ca$^{2+}$ could subserve this function by activating transport processes, or by stimulating the release of a chemical mediator, or through some other signaling cascade that has yet to be discovered. Thus these studies suggest that the coupling between transport-related events at the apical membrane and the signal propagation by MD cells may involve an MD Ca$^{2+}$ signaling system.

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