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ACTIVATION OF CALCIUM CHANNEL BY SHEAR-STRESS IN CULTURED RENAL DISTAL TUBULE CELLS

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Using the patch-clamp technique and the fura-2 fluorescence measurement, we found that flow of a normal solution simultaneously increased both the inward cation (Ca) currents and the cytosolic Ca activity (Ca_i) in cultured renal distal tubule cells (A6 cells). The activation of these signals was voltage-independent and required a lag period of about 30 s. Flow of a Ca free solution (plus 0.1-0.5 mM EGTA) failed to increase these signals. The Ca current increased and saturated with increasing extracellular Ca concentrations (apparent Km, 1 mM Ca; maximum Ca current, 43 pA). Ni (1 mM) and La (1 mM) inhibited the flow-induced Ca_i-increase, but nicardipine (50 μM) did not. These results strongly suggest that in A6 cells flow increases Ca-influx through a shear-stress activated Ca-channel and may regulate the cellular transport functions.

The renal tubule cells change their cellular transport functions in response to tubular flow. When the tubule perfusion rate was increased from 6 to 26 nl/min, K secretion by the distal tubule increased from 35 to 65 pmol/min (1). Since more K was secreted at higher flow rates without decreasing luminal K concentrations (2), tubular flow probably increases the K permeability in the luminal membrane. In the rabbit descending limb of Henle, Miwa and Imai (3) found that water permeability was increased as the perfusion rate was increased. However, little is known about the cellular mechanism how the tubule cells respond to flow and regulate these transport functions. Recently, in JTC-12 cells, a cell line from monkey kidney which possesses characteristics of the proximal tubule, Chang et al. (4) reported that a Ca-activated K conductance was increased by mechanical forces including flow. Moreover, in vascular endothelial cells Olesen et al. (5) described a shear-stress induced K current and Ando (6,7) showed a flow-dependent cytosolic Ca-increase. These

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results suggest a possibility that mechanosensitive ion channels (8,9) could be involved in the response to flow-induced mechanical forces and may be responsible for Ca entry.

In the present study we report simultaneous recordings of the patch-clamp technique (10) and the fura-2 fluorescence signal (11) in A6 cells. We found that flow (20-40 nl/min) activated a Ca current from bath to cell which increased a cytosolic Ca concentration (Ca_i). The increased Ca_i, as a second messenger, may play an important role in the cellular transport systems.

METHODS

Cells from A6 line derived from Xenopus kidney distal tubules were used in passage numbers 70-80. The cells were seeded at low density (1-2 x $10^3/cm^2$) and were incubated in Dulbecco's Modified Eagle Medium diluted by 15% (in volume) with H_2O, containing 10% fetal bovine serum (12), at 26 °C for 2-4 days. Single cells and a group of 2-20 cells cultured on glass coverslips were provided for a fluorescence-microscopical study. Only single cells were used for simultaneous recordings of the whole-cell patch-clamp and the fura-2 fluorescence. Experiments were conducted at 26-28 °C.

The ion composition of the extracellular solution was as follows (in mM): 120 NaCl or NMDGCl (N-methyl-D-glucamine), 3 KCl, 1 $MgCl_2$, 0.5 $CaCl_2$, 10 HEPES, 1 Napyruvate, and 5.5 D-glucose; pH was adjusted to 7.6 with NaOH. A Ca free solution was prepared by removing $CaCl_2$ and adding 0.1-0.5 mM EGTA.

Whole-cell currents were recorded using the patch-clamp technique (10). Recording techniques were similar to those described previously (13): the current signals were recorded with a patch-clamp amplifier (Nihon-Kohden CEZ2200, Tokyo, Japan) and stored on VCR tape through a pulse-code modulator (Sony PCM-501ES, Tokyo). The patch-pipette contained (in mM): 120 KCl or 100 Nagluconate and 20 NaCl, 1 $MgCl_2$, 10 HEPES, 0.01 EGTA; pH was adjusted to 7.6 with NaOH. The tip resistance of the patch-electrodes filled with 120 mM KCl was 5-8 MOhm.

Intracellular free Ca concentrations were recorded by means of fura-2 fluorescence (11). Cells were loaded with fura-2 by incubating coverslips in a normal solution containing 3 μM fura-2/AM (Dotite) for 60 min at 23-24 °C. Using a Nihon-bunko diffraction grating spectroscope (CAM 230, Tokyo, Japan) equipped with an inverted microscope (Nikon TMD, Tokyo), the fluorescence signals through an emission filter of 500 nm (bandwidth, 11 nm) were measured with altering excitation wavelengths (bandwidth, 10 nm) of 340 and 380 nm every 10 ms. Background fluorescence from coated collagen on the coverslip was negligible. Through the experiments "in vitro" calibration was made at pCa 8-5 in the calibration media composed of (in mM) 120 KCl, 1 $MgCl_2$, 10 HEPES-NaOH, 5 EGTA, and appropriate amounts of $CaCl_2$ as well as 15 μM fura-2 free acids (Dotite)(14). The sigmoidal ratio-pCa curve (15) was consistently observed and provided for the calibration.

Cells were exposed to flow of about 20 and 40 nl/min which was made by applying pressure of 1.005 and 1.01 atm., respectively, to a perfusion-pipette. Intensity of shear stress (T) was calculated for laminar flow in cylindrical tubes according to the equation (5):

$$T = 4uQ/\pi r3 \qquad (1)$$

where u represents fluid viscosity (0.0085 dyn·s/cm2), Q, the flow rate (ml/s) and r, the internal radius of the tip of tubes (0.00075 cm). The equation only approximates shear stress as the effects of the tapered end of the tube on flow-rate was not corrected. Calculated shear stress caused by flow rate of 20 and 40 nl/min was 8.6 and 17.3 dyn/cm2, respectively. These values must slightly decline on the cell surface, because the pipette-tip was about 50 μm distant from the cell.

RESULTS

A6 cells loaded with fura-2 showed stable resting signals and responded to flow. When they were exposed to flow of a normal solution (40 nl/min), a cytosolic Ca concentration (Ca_i) increased after a lag period of about 30 s and was sustained (Fig. 1a, upper trace). Ca_i returned to the original level and similarly increased when flow was stopped and restarted. Flow of a Ca-free solution failed to increase a resting level of Ca_i (Fig. 1a, lower trace), suggesting that flow stimulates a Ca entry process across the cell surface membrane rather than Ca-release from an internal Ca store. Unlike electrically excitable cells, depolarization of voltage-gated Ca channels did not stimulate Ca entry. Exposure of A6 cells to 24 mM K had no detectable effect on Ca_i in the absence and presence of flow (Fig. 1b). Flow of a Ca-free solution (dashed line in Fig. 1b) failed to keep the elevated level of Ca_i, confirming the hypothesis that flow activates the Ca-entry process which allows Ca ions to move into the cell. As shear stress increases in

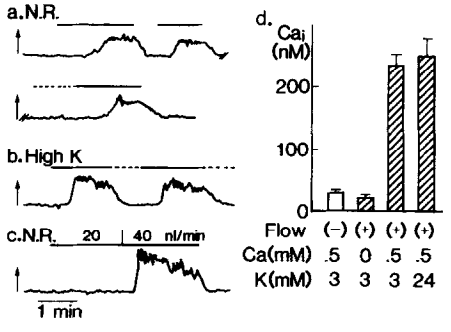

Figure 1. Representative traces of Ca_i-change induced by flow of normal Ringer (N.R.)(a) and 24 mM K (high K)(b) solutions. Perfusates of Ca-containing and Ca-free solutions are indicated by solid and dashed lines, respectively. Arrows indicate the ratio of 500 nm fura-2 fluorescence excited at 340 nm to that excited at 380 nm (see Fig. 3a). c, Ca_i-change in response to flow of 20 and 40 nl/min (Flow rate was 40 nl/min unless otherwise mentioned). d, Quantitative results of a resting level of Ca_i (open column) and flow-induced Ca_i (hatched columns). From left to right, 26 +/- 1.8 (n=20), 19 +/- 4.7 (n=7), 235 +/- 16 (n=18), and 251 +/- 30 nM (n=8)(mean values +/- S.E.).

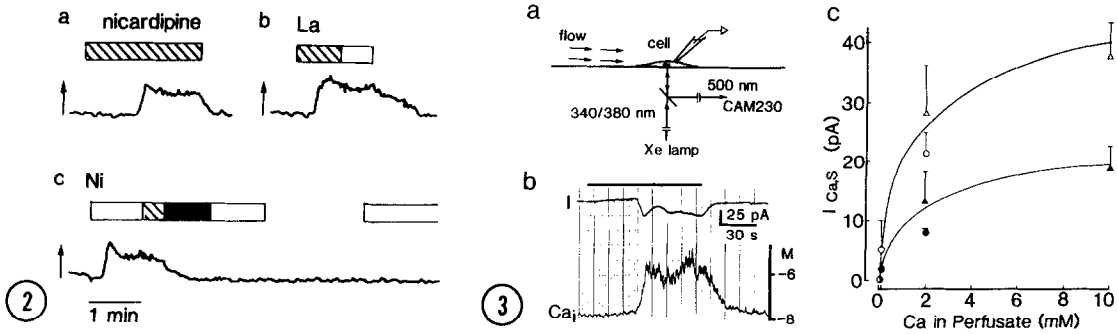

Figure 2. Effects of potent blockers of voltage-dependent Ca channels on flow-induced Ca_i-increase. Open columns indicate flow of a normal solution. a, Normal solutions containing 50 μM nicardipine (hatched); b, 0.1 mM La^{3+} (hatched); c, 0.1 and 1 mM Ni^{2+} (hatched and filled). The cells were bathed in a nominally Ca free solution (0 EGTA) in this and following experiments.

Figure 3. a, Experimental design showing simultaneous recordings of the whole-cell patch-clamp and the fura-2 fluorescence on a single-cell. b, Representative traces showing whole-cell currents (I) and fura-2 signals (Ca_i) at E_{Cl} (-48 mV), before and after flow (solid line). Note time courses of the inward current (downward) and Ca_i-increase (upward) evoked by flow. c, $I_{Ca,S}$ are plotted as a function of Ca concentrations in perfusates: peak (open symbols) and plateau (filled symbols). A major monovalent cation in perfusates was Na (circles) or NMDG (triangles)(n=3-7). Solid lines are calculated by use of Michaelis-Menten equation with a K_m of 1 mM and maximum currents of 43 (peak) and 19 (plateau) pA. $I_{Ca,S}$ (peak) were 5 +/- 5, 21 +/- 4, 28 +/- 8, and 37 +/- 6 pA at 0.1, 2 (○), 2 (△), and 10 mM Ca, respectively.

proportion to flow-rate, more cells responded to flow at higher flow-rates; 53 and 94% of A6 cells (n=17) were activated by flow of 20 and 40 nl/min, respectively (Fig. 1c). The quantitative results of Fig. 1a,b are shown in Fig 1d. Flow of normal and high K solutions significantly increased a resting level of Ca_i, but no significant difference between the two (Student's t test).

Ca_i-increase was not specifically inhibited by extracellular perfusion containing Ca-channel blockers (Fig. 2): mean values of Ca_i-increase were 244 +/- 45 nM (50 μM nicardipine, n=10), 228 +/- 88 nM (0.1 mM La^{3+}, n=2), and 257 +/- 83 nM (0.1 mM Ni^{2+}, n=3). Ni^{2+} (1 mM) irreversibly blocked Ca_i-increase (Fig. 2c). La^{3+} reduced percentage of the cells responded to flow: 100% (control, n=14), 75% (0.01 mM La^{3+}, n=4), 22% (0.1 mM La^{3+}, n=9), and 0% (1 mM La^{3+}, n=3).

Simultaneous recordings of both the whole-cell current and the fura-2 fluorescence signal were made on the single-cell (Fig. 3a). When a pipette-filling solution was 100 mM Nagluconate and 20 mM NaCl, flow of a NMDGCl solution containing 10 mM Ca simultaneously increased the inward current and Ca_i at the

equilibrium potential for Cl (E_{Cl})(Fig. 3b). Since the inward current at E_{Cl} (-48 mV) is carried by cations including Ca and a major monovalent cation was a large organic cation (NMDG), Ca-influx must represent the inward current and the source of elevated Ca_i. The currents (designated $I_{Ca,S}$) and Ca_i-increase induced by flow returned to the original levels when flow was stopped. Unlike voltage-gated Ca channel, $I_{Ca,S}$ was evoked by flow at cell membrane potentials (Vm) between -40 and -80 mV. Figure 3c illustrates the relationships between $I_{Ca,S}$ and extracellular Ca concentrations (Ca_o). A major monovalent cation in the perfusate was Na or NMDG. $I_{Ca,S}$, initial peaks and sustained levels, increased and saturated as Ca_o was increased, indicating that contribution of monovalent cations to $I_{Ca,S}$ was negligible, i.e. Na was also impermeant to the shear-stress induced channel in A6 cells. Therefore, Ca ions may enter the cell through a Ca-selective channel rather than a Ca-permeable cation channel (16). Using the Michaelis-Menten equation (17), the maximum values of $I_{Ca,S}$ were 43 (peak) and 19 (plateau) pA. K_m, a half maximum concentration of Ca_o, was 1 mM (peak and plateau). However, we have failed to observe single Ca-channels in numerous outside-out patches. This could be due to low channel density or too small single channel conductance.

DISCUSSION

The present study demonstrates for the first time that flow simultaneously increases the inward Ca current ($I_{Ca,S}$) and cytosolic Ca concentration (Ca_i) in cultured renal tubule cells (A6 cells). Since time course of the Ca_i-increase showed a transient peak and plateau, two possible mechanisms can be proposed to explain the Ca_i-increase: first, Ca-influx through Ca permeable channels in the cell membrane; second, Ca-release from the internal Ca store. Several lines of evidence support the mechanism of Ca-influx as previously speculated by Chang et al (4): first, flow of a Ca-free solution failed to increase Ca_i and did not keep the elevated level of Ca_i. Second, Ni^{2+} (1 mM) and La^{3+} (1 mM) inhibited the flow-induced Ca_i-increase. Third, flow simultaneously increased both $I_{Ca,S}$ and Ca_i in the single A6 cells. We do not exclude a possibility that a part of the inward current ($I_{Ca,S}$, Fig. 3c) may be carried through the Ca-activated cation channel as previously described by Yellen (18), however, this channel is practically impermeable to Ca (18). It should be

noted that in JTC-12 cells (4) and in our present study flow did increase Ca_i in the absence of extracellular ATP as previously reported by Ando (6). In contrast, Ando (7) and Mo (19) reported that flow did not increase Ca_i in the absence of extracellular ATP. The reason for the discrepancy between the results of the cultured renal tubule cells and the recent works in vascular endothelial cells is unknown.

According to the results of potent voltage-dependent Ca-channel blockers (Fig. 2), the shear-stress activated Ca channel in A6 cells was identified which is unlike previously described voltage-dependent Ca channels in excitable membranes (20), a parathyroid hormone induced Ca channel in cultured mouse distal tubule cells (21), and a cell-swelling induced Ca channel in A3 cells (of rabbit medullary thick ascending limb cells (22)). Following results also support this idea: flow-induced Ca_i-increase was independent of extracellular K concentrations and $I_{Ca,S}$ was observed at Vm=-40 - -80 mV.

We have previously described a stretch-activated (S-A) cation channel in these cells (23), however, it is unlikely that in the present study the S-A channel was activated by flow of 20-40 nl/min and allowed Ca ions to move into the cell. Because we have found that 1) the S-A channel was similarly activated by membrane-stretch with or without Ca_o; 2) membrane-stretch activated the S-A channel without a lag-time of several ten seconds; 3) Na currents through the S-A channel was decreased as Ca_o was increased (Kawahara et al., manuscript in preparation).

The lag-time of about 30 seconds before flow-induced Ca_i-increase was consistently observed and was not shortened by flow of a Ca free solution in advance (lower trace of Fig. 1a), suggesting that extracellular Ca, as well as flow, plays a key role in the activation of a shear-stress activated Ca channel. Therefore, we examined one possibility that Ca-induced Ca-release mechanism (24) may be involved in the process of flow-induced Ca_i-increase. However, pretreatment of A6 cells with 10 μM ryanodine, a potent inhibitor of Ca-induced Ca-release mechanism (25,26), has no detectable effect on the flow-induced Ca_i-increase (n=6). In JTC-12 cells bathed in a Na-free solution (4), flow hyperpolarized cell membrane potentials by increasing a Ca-activated K conductance with a lag-time of about 6 s. These results suggest that the lag-time may be necessary for activation of Ca entry evoked by flow.

Shear-stress activated Ca current is not artifact caused by dislocation of patch-pipettes. Because we found that 1) induction of $I_{Ca,S}$ and Ca_i-increase was reversible; 2) flow of a Ca-free solution failed to induce $I_{Ca,S}$; 3) the lag-time of the flow-induced Ca_i-increase was almost unchanged whether A6 cells were under the whole-cell clamp condition or not.

As Ca is well known to regulate a number of cellular transport functions, these studies bring a new aspect of flow-dependent ion and water transports in renal tubules. Since Ca-dependent K channels have been found in the luminal membrane of the proximal (16,27,28) and distal (29-32) tubules, flow-induced Ca_i-increase may regulate the K efflux across the luminal membrane. Verkman et al. (33) reported the endosomes containing vasopressin-sensitive water channel in rats kidney collecting tubules. Thus, the elevated levels of Ca_i induced by flow may change the membrane water permeability by regulating the fusion rate of the endosomes to the luminal membrane. Further studies should be needed to evaluate these hypotheses.

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