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Nuclear and Cytosolic Calcium Signaling Induced by Extracellular ATP in Rat Kidney Inner Medullary Collecting Duct Cells

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Using a laser scanning confocal microscopy of fluorescent Ca^{2+} indicator (Fluo-3-AM) the spatiotemporal Ca^{2+} dynamics in cultured kidney inner medullary collecting duct cells was investigated. In response to extracellular ATP (100 μM), nuclear (Fl_n) and cytosolic (Fl_c) fluorescence intensity increased simultaneously. UTP similarly increased Fl_n and Fl_c, but ADP and AMP did not. A ratio between Fl_n and Fl_c was about 1.06 ± 0.03 at rest and increased 1.71 ± 0.02 at the peak of stimulation (n=74). In Ca^{2+}-free condition, ATP increased Fl_n and Fl_c with a smaller peak intensity, but the peak ratio was similar (1.52 ± 0.03, n=70). Faster time resolution of 100 ms in line scanning mode did not detect the delay between nuclear and cytosolic Ca^{2+} responses. Our results indicate that nuclear Ca^{2+} was not diffused from the cytoplasm and that it may be directly released from the nuclear envelope, a possible Ca^{2+} store.

Extracellular ATP and related nucleotides increase intracellular Ca^{2+} concentrations in many cell types (1), including the renal tubules (2-6). They can modulate the renal tubular functions, such as activation of membrane ion channels (7,8) and may control fundamental nuclear processes, such as gene transcription (9). In the renal inner medullary collecting duct (IMCD), intracellular Ca^{2+} was mainly released from the internal store via the P_2 purinergic receptor (P_{2u}) (6).

Recently, it became possible to measure subcellular Ca^{2+} dynamics with the Ca*2*+-indicator using a confocal microscopy. Difference in Ca^{2+} concentration between the nucleoplasm and the cytoplasm has been observed, particularly after stimulation: nuclear Ca^{2+} concentrations were higher than cytosolic one in hepatocyte (10), smooth muscle (11), sympathetic neuron (12), and epithelial LLC-PK_1 cells (13). Gerasimenko et al. (14) showed that nuclear Ca^{2+} was directly released from the isolated nuclear envelope of mouse liver cells in response to IP_3 and cyclic ADP ribose. However, the issue of intracellular Ca^{2+} distribution is controversial. Some workers have proposed that nuclear Ca^{2+} is diffused from cytoplasm with a short delay and is not independently controlled (15-18). Higher nuclear Ca^{2+} may be due to an artifact (15).

We have therefore investigated the fluorescence intensity of the Ca*2*+-sensitive dye (Fluo-3-AM) at rest and during stimulation using a laser scanning confocal microscopy, which eliminates the influences of unequal cell geometry and dye distribution on Ca*2*+ fluorescence images. It is also an advantage that the nucleus of the IMCD cells grown in monolayers was recognized without any staining in a phase contrast microscopy (Fig.1a). The goal of the present study is to determine the resting and stimulated cellular Ca^{2+} distribution in the nucleus and cytosol to better understand the regulation of intracellular Ca^{2+} homeostasis and a role of the nuclear envelope. We found that a simultaneous increase in nuclear and cytosolic Ca^{2+} after the exposure to ATP and AVP (IP_3 producing agonists (1,19,20)), with or without Ca^{2+} in the bath, and that Fl_n was always larger than Fl_c during stimulation.

METHODS

Neonatal Wistar rat (1-3 days of age) was anesthetized by ethel. The kidney was removed and its papillary region was incubated for 20 min at 33 °C in a standard solution containing collagenase (1 mg/ml). Softened tissue was minced by sterile small scissors and was centrifuged down twice at 1200 rpm for 3 min. Pellet was suspended in the culture medium (a mix of Dulbecco's Modified Eagle's Medium and Ham's F12 (1:1)) containing 10% FBS (fetal bovine serum, Gibco,

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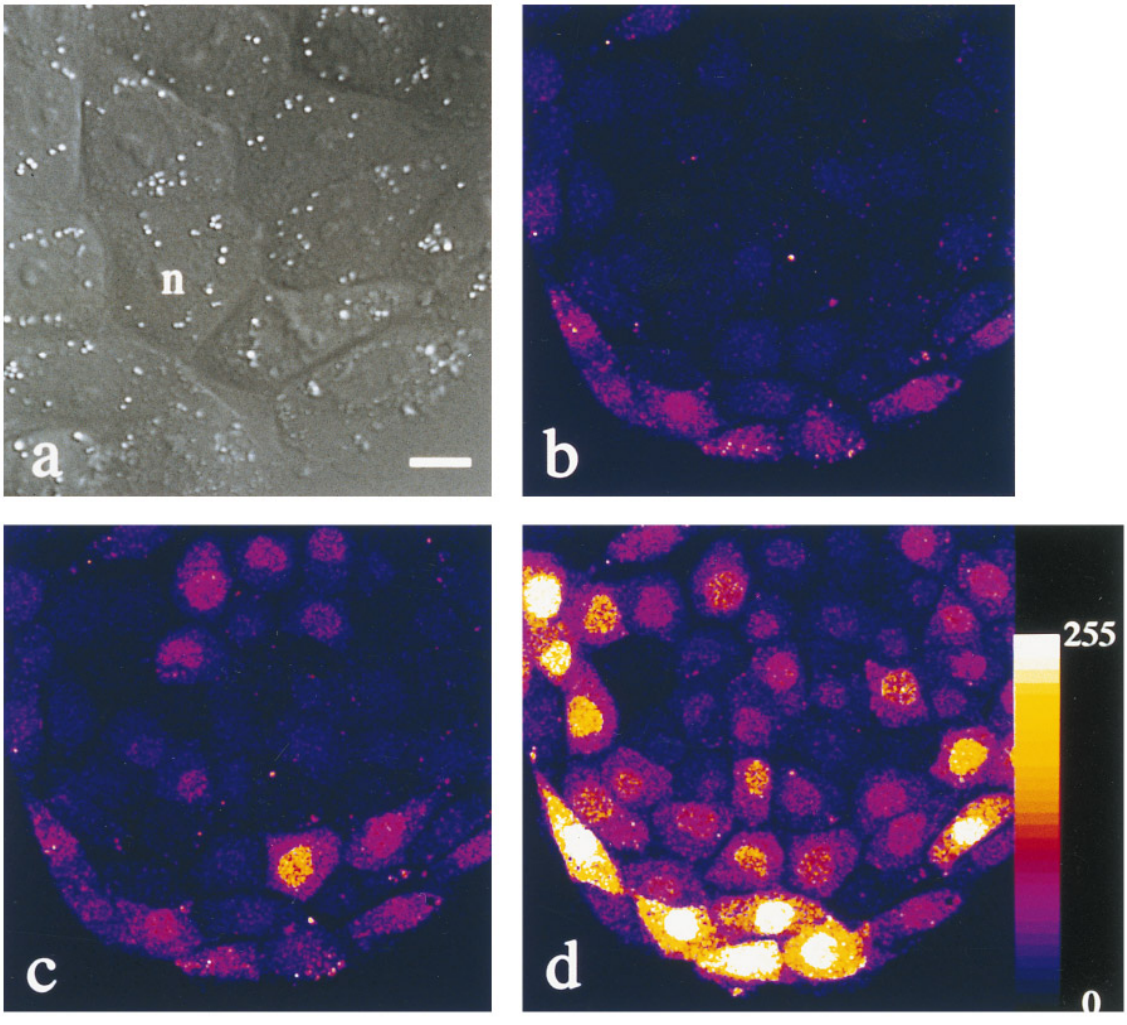

FIG. 1. Nuclear and cytosolic Ca^{2+} distribution of a monolayer of kidney IMCD cells. (**a**) A phase contrast microscopy image. Nuclear area, round in the middle of the cell, can be recognized from cytoplasm. ***n* indicates the nucleus being highly stimulated by ATP in Fig.1c. (b-c**) Laser scanning confocal microscopy images (512 × 512 pixels). Fluo-3 fluorescence at rest (b) and 8 s after application of ATP (2 μM (c) and 100 μM (d)). Pseudo-color sequence in proportion to Ca^{2+} concentrations (from low to high) was black-violet-purple-yellow-white. Scale bar is 20 μm.

USA) and was seeded on a glass coverslip coated by rat tail collagen Type 1 (Sigma, USA) which covered the center hole of 10 mm in culture dish (chamber). After incubation of 24-48 h at 37 °C in a humidified atmosphere of 5% CO_2, the tubules and clusters, which were stained with fluorescence-labeled Ab-AVP_2 receptors (21), made monolayer colonies consisting of >20 cells (Fig.1a).

Ion composition of the extracellular solution was as follows (in mM): 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 5.5 D-glucose, and 10 Hepes; pH was adjusted to 7.40 ± 0.02 by NaOH. Ion composition of a Ca^{2+}-EGTA buffer system for "in vivo" calibration was the same as a standard solution except various concentrations of Ca^{2+} and 5 mM EGTA (10^{-6}, 10^{-7}, and 10^{-8} M Ca^{2+} solutions contained 4.45, 2.70, and 0.55 mM Ca^{2+}, respectively). ATP and its analogues were purchased from Sigma (USA).

For fluorescence measurements, the cells were incubated in a standard solution containing 3 μM Fluo-3-AM (Ester form of Ca^{2+} indicator dye, Molecular probes, USA) at room temperature for 40 min. After being rinsed three times with a Hepes solution, the chamber was transferred and mounted on the stage of Axiovert 135M. The cells were scanned through a glass coverslip (160 μm in thickness) by argon laser of 488 nm of confocal microscopy system (Carl Zeiss LSM410, Germany). Confocal plane was set about 5 μm above the bottom, where nucleus seemed to be maximum. The lateral and Z resolution was 0.2 and 1.2 μm, respectively, with a pin hole of 25 μm in diameter (× 63 oil objective Zeiss, NA1.4). Pseudo-color images (512 × 512 pixels) of fluorescence (>515 nm) were taken every 5 s and were stored on the computer memory (8-bit digital images). When relative fluorescence intensity was converted into arbitrary units between 0 and 255, a level of background fluorescence was 12.4 ± 0.04 either in the presence or absence of Ca^{2+} in the bath. This value was subtracted from the measurements. Perinuclear cytosolic fluorescence (Fl_c) was determined as the difference between perinuclear fluorescence including nucleus and nuclear fluorescence (Fl_n). For faster resolution some cells were scanned every 100 ms including the intervals in the line scan mode. The acquisition, processing, and measurement of fluorescence images was accomplished by LSM software package (Carl Zeiss) with a combination of NIH image (1.55).

All experiments were conducted at the room temperature (26-28 °C). Values were expressed as mean ± S.E. Statistical analysis was done with Student t-test (paired and unpaired). $P<0.05$ was accepted

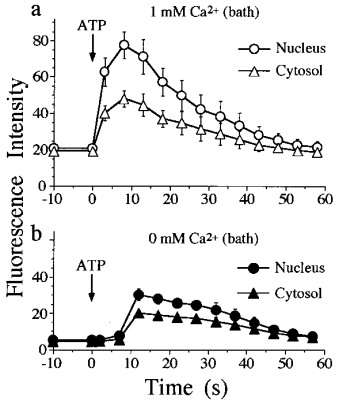

FIG. 2. Time courses of nuclear and cytosolic fluorescence changes due to cellular Ca^{2+} signals of the representative colony, with (**a**) or without Ca^{2+} (**b**) in the bath. Bars indicate standard errors (n>30).

for statistical significance. Unless otherwise mentioned, *n* indicates the number of cells.

RESULTS

Nuclear Ca^{2+} Response by Laser Scanning Confocal Microscopy (LSCM)

The nucleus of rat kidney IMCD cells was recognized in a phase contrast microscopy (Fig. 1a), but was usually indistinct in the pseudo-color image with a confocal microscopy at rest (Fig. 1b). No significant difference was observed between nuclear (Fl_n) and cytoplasmic (Fl_c) fluorescence (they were 22.5 ± 1.3 and 21.6 ± 1.1, n=165), although fluorescence intensity was usually higher on the border than in the middle of the colony. This suggests that Ca^{2+}-sensitive dye (Fluo-3-AM) was equally distributed between the nuclear and cytoplasmic compartments, and that even in the same colony either uptake of Ca^{2+} indicator or its deesterification was different. On the contrary, when Fl_n and Fl_c were increased during stimulation with ATP (bath), nuclear regions were transiently distinct and were recognized, probably due to their higher intensity (Fig. 1c, d). 39 and 96% of the cells were responding to 2 and 100 μM ATP, respectively (n=114 and 77). Uniform Ca^{2+} distribution at rest and apparently higher nuclear Ca^{2+} levels after application of ATP were observed. Nuclear and cytosolic Ca^{2+}-rise was seemed to be synchronized in the same cell, but time-courses of cellular Ca^{2+} changes were different even in the same colony.

Time Course of Nuclear and Cytosolic Ca^{2+} Signals

After application of ATP (100 μM) to the bath, Fl_n and Fl_c began to rise simultaneously without delay (Fig. 2a). During stimulation, at least in the initial 20 s, Fl_n was significantly higher than Fl_c. Mean values of the maximum Fl_n and Fl_c were 76.2 ± 5.9 and 46.1 ± 3.1 (n=74). Increasing fluorescence intensity must correspond to increasing Ca^{2+}, but nuclear and cytosolic Ca^{2+} concentrations were not quantified further due to non-rational images of Fluo-3 (22). Alternatively, the ratio (Fl_n/Fl_c) was estimated to investigate the nuclear and cytosolic Ca^{2+} difference. It was increased from 1.06 ± 0.03 (resting) to 1.71 ± 0.02 (peak), suggesting that after application of ATP Ca^{2+} concentration in nucleus may be higher than in cytosol. Ca^{2+} rise was transient and was not oscillated during the recording time of 60 s. Caffeine (10-30 mM), known to release Ca^{2+} from the Ca^{2+} induced Ca^{2+}-release system in guinea pig taenia caeci (23), failed to increase either nuclear or cytosolic Ca^{2+} concentration (>150 the IMCD cells of 6 individual colonies, data not shown).

Ca^{2+} Signals in a Ca^{2+} Free Solution (Bath)

3 times replacement with a Ca^{2+} free solution plus the Ca^{2+} chelator EGTA (100 μM) slightly decreased a level of intensity of the pseudo-color images. No significant difference was observed between the two compartments (Fl_n and Fl_c were 19.1±1.5 and 18.1 ± 1.5, respectively, n=73). In response to ATP (100 μM), Fl_n and Fl_c of the IMCD cells were increased with a longer lag time (8 s). 58% of the cells were stimulated. The ratio increased from 1.09 ± 0.02 (resting) to 1.52 ± 0.03 (peak)(Fig.2b). These results indicate that a main source of the nuclear and cytosolic Ca^{2+} was an internal Ca^{2+} store, and that Ca^{2+} may be synchronously released in response to the same intracellular second messenger system.

Calcium Signals in Response to ATP Analogues and AVP

Cellular Ca^{2+} signals induced by ATP analogues and AVP were examined. UTP similarly increased the nuclear and cytosolic Ca2+, but ADP and AMP did not. Percent increase in the nuclear Ca^{2+} was 347 ± 24.3, 413 ± 30.8, 126 ± 14.6, and 110 ± 2.9% in response to UTP, ATP, ADP, and AMP of 100 μM, respectively (n=24-43). Their peak ratios (Fl_n/Fl_c) were 1.55 ± 0.04, 1.62 ± 0.06, 1.06 ± 0.06, and 1.14 ± 0.08 in the same sequence. When the agonists (ATP, UTP) were sequentially added, the IMCD cells were only responding to the first one. ATP and UTP may share the overlapping Ca^{2+} store in the cell and/or the common receptor, probably P_{2u} in the surface cell membrane (5,6). According to the relative potency of selective agonists for the P_2-purinergic receptor subtype (1), the P_{2y} receptor has been identified in the proximal tubule (4) and in A6 cells (8).

AVP is known to increase intracellular Ca^{2+} in the fresh (24) and cultured (25) IMCD cells. In the present study, AVP increased the nuclear and cytosolic Ca^{2+} level within 30 s, with or without Ca^{2+} in the bath. In a normal solution containing 1 mM Ca^{2+}, 19% (27/140

cells from 4 colonies) and 32% (36/113 cells from 5 colonies) of the IMCD cells were responding to 1 and 5 μM AVP, respectively. Their peak ratios (Fl_n/Fl_c) were 1.68 $\pm$ 0.04 and 1.79 $\pm$ 0.06, respectively. In the Ca^{2+} free condition 15% (4/26 cells) of the IMCD cells were responding to 1 μM AVP. Its peak ratio was 1.45 $\pm$ 0.12.

Faster Time Resolution by LSCM (Line Scanning)

In neuron (15) and in RBL cells (16) cytosolic Ca^{2+} increased faster by about 200 ms than nuclear Ca^{2+}. In hamster oocyte nuclear Ca^{2+} reached the cytosolic Ca^{2+} level within 2 s (18). In order to evaluate the temporal difference between the nuclear and cytoplasmic Ca^{2+} concentration more precisely, we made scans repeated every 100 ms over periods of 60 s across a line that intersected the nucleus (n=6). Figure 3a represents an example of such scanning of 570 traces. The short and long bars indicate the nuclear and cellular regions, respectively. At every moment Fl_n seemed to be higher than Fl_c during application of ATP. Further, intranuclear intensity was lower in the center than the inner circumference of the nucleus during the initial Ca^{2+} increasing phase.

Time-dependent changes of Fl_n and Fl_c were illustrated in Fig. 3b. Calculated pixels in the cytosolic area were limited in perinuclear sides and their number was equal to that of the nucleus (35 pixels). In response to ATP Fl_n and Fl_c increased abruptly after a short lag time and seemed to be synchronous. Rapid increase in Fl_n always exceeded a level of Fl_c during and especially in the middle of the stimulation. The ratio (Fl_n/Fl_c) transiently increased from 1.06 $\pm$ 0.04 (resting, n=60) to 2.1 $\pm$ 0.1 (peak, n=6) and returned toward the control level. This confirms the view that nuclear and cytosolic Ca^{2+} is released from the internal Ca^{2+} stores stimulated by the same cellular messenger, and disagrees with the view that nuclear Ca^{2+} was diffused from a cytosolic Ca^{2+} raised during stimulation (15-17).

Young Adult Rat Kidney

It is important to determine that nuclear Ca^{2+} signaling in the present study is not a unique property of

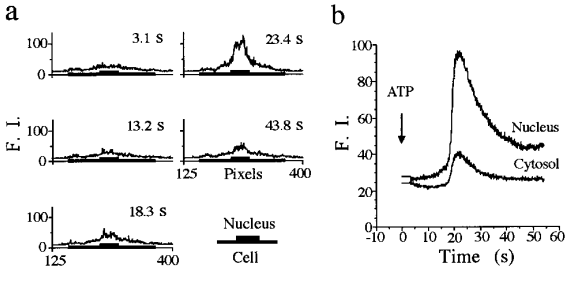

FIG. 3. Faster time resolution by line scanning. (**a**) 5 representative images of fluorescence intensity in a line scanning mode. Short and long bars indicate nuclear (Fl_n) and cellular (Fl_c) regions, respectively. Note that Fl_n was always larger than Fl_c during the stimulation. (**b**) Time courses of Fl_n and Fl_c after application of ATP.

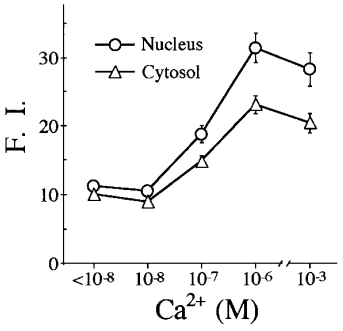

FIG. 4. Fl_n and Fl_c as a function of various concentrations of Ca^{2+} with ionomycin (10 μM). Note that Fl_n was significantly larger than Fl_c at the higher Ca^{2+} concentrations (n=11).

cells from neonatal kidneys which have the potential to differentiate. The IMCD cells from young adult rats (4 animals of 4-8 weeks) were similarly cultured and were used for confocal fluorescence measurement in the same procedure. Interestingly, the nuclear/cytosolic ratio of Fluo-3 fluorescence was significantly less than 1 (0.85 $\pm$ 0.02, n=174) at rest, but increased similarly to 1.64 $\pm$ 0.03 during stimulation by ATP. Nuclear Ca^{2+} response to ATP may play an important role for cellular function not only in the neonatal kidney, but also in the adult.

Nuclear Factor

Although nuclear fluorescence was higher than cytosolic one throughout the stimulation by ATP, UTP, and AVP, was nuclear Ca2+ concentration really higher than cytosolic Ca^{2+}? Nuclear and cytosolic Ca^{2+} fluorescence signals were calibrated in vivo with a Ca^{2+}-EGTA buffer system containing the Ca^{2+} ionophore, ionomycin. In the presence of ionomycin (10 μM), we can safely assume that nuclear and cytosolic Ca^{2+} is approximately equilibrated with the extracellular Ca^{2+} concentration. Figure 4 illustrates the nuclear and cytosolic fluorescence intensity as a function of various concentrations of Ca^{2+}. The ratio (Fl_n/Fl_c) was 1.11-1.16 at and below 10^{-8} M Ca^{2+}, but increased to 1.37-1.39 at and higher than 10^{-6} M Ca^{2+} (In a normal solution containing 1 mM Ca^{2+} without ionomycin, the ratio at rest was 1.10, n=11). This apparently disagrees with the hypothesis that in the presence of ionomycin Ca^{2+} ions are freely distributed in the cellular compartments. On the contrary, we postulate an alternative possibility that nuclear fluorescence intensity may be amplified in a different manner from the cytosol at least at the higher concentrations of Ca^{2+}. The amplifying mechanism (called nuclear factor in Brini et al. (26)) is unknown, but may be due to various factors in nuclei, such as chromatins (12), nucleic acids (26), pH and Mg^{2+} (27).

DISCUSSION

With a laser scanning confocal microscopy, temporal and spatial difference in nuclear (Fl_n) and cytosolic (Fl_c) fluorescence intensity was investigated, at rest and during stimulation with ATP and AVP. Three lines of evidence demonstrate that the nucleus (probably, nuclear envelope) is the source of nuclear Ca^{2+} signals in the rat kidney IMCD cells. 1) Fl_n and Fl_c were synchronously increased after application of ATP, with or without Ca^{2+} in the bath. 2) Even with faster time resolution (100 ms), Fl_n was not behind Fl_c in Ca^{2+} signals. 3) Nucleocytosolic gradient in Ca^{2+} signaling was maintained throughout the stimulation.

Synchronous increase in Ca^{2+} concentrations between nucleus and cytosol postulates two possibilities: First, nuclear Ca^{2+} was released from the nuclear envelope which may be regulated by the same second messenger system as the cytosolic Ca^{2+} store was. Second, a passive influx to the nucleus of calcium released from the cytosolic Ca^{2+} stores was too fast to be detected. Extracellular AVP (19,20) and ATP (1) are known to generate IP_3 and increase intracellular Ca^{2+} in various cell types including renal tubule cells. In the present study IP_3-generating agonists, such as ATP, UTP, and AVP similarly increased nuclear and cytosolic Ca^{2+}. However, caffeine (10-30 mM), known to release Ca^{2+} from the Ca^{2+}-induced Ca^{2+}-release system (CICR)(23), failed to increase an intracellular Ca^{2+}. IP_3 is probably the second messenger for both nuclear and cytosolic Ca^{2+} signals and CICR system may not be related with the nuclear and cytosolic Ca^{2+} distribution in the present study. Four lines of evidence support that IP_3 can work as a signal for Ca^{2+}-releasing at the inner membrane of nuclear envelopes: 1) Nuclear pore complexes are permeable to small molecules with a physical diameter of less than about 9 nm (28) or a molecular mass of 10-25 kDa (29), such as IP_3. 2) Spreading rate of IP_3 (283 $\mu m^2/s$) was faster than that of Ca^{2+} (13-65 $\mu m^2/s$) (30). It may be fast enough to increase nuclear and cytosolic Ca^{2+} without detectable delay. 3) nuclear envelopes of hepatocyte contained IP_3 production systems (31) and IP_3 receptors for Ca^{2+}-releasing (32-35). 4) Isolated nuclei (nuclear envelopes) could increase nuclear Ca^{2+} in response to IP_3 (14). By contrast, nuclear inner membrane of hamster oocyte was not responding to IP_3 injected through a glass micropipette (18).

Although time-resolution of 100 ms in the present study was not slow compared with 200 ms (15), we did not detect the difference in Ca^{2+} rise between nucleoplasm and cytoplasm. Further, the rise of Fl_n was always greater than that of Fl_c, when the IMCD cells were stimulated by extracellular ATP. Therefore, we concluded that nuclear Ca^{2+} may be directly released from the nuclear envelopes.

Nucleocytosolic Ca^{2+} gradient may occur, if Ca^{2+}-ATPase in the nuclear envelopes actively transports Ca^{2+} into the nucleus (32,36,37). Alternatively, smaller nuclear space may account for the higher nuclear Ca^{2+} concentrations during stimulation. With the aid of three-dimensional analysis of LSCM, nuclear volume was estimated as about 1/3 of cytoplasm (0.62 ± 0.13 pl) (n=4). Thus, it is reasonable that nuclear Ca^{2+} is higher than perinuclear cytosolic Ca^{2+} during stimulation. However, in the present study nuclear Ca^{2+} was higher than cytosolic Ca^{2+} even in the presence of ionomycin (Fig. 4): the nuclear/cytosolic fluorescence ratio was 1.1 at and below 10^{-8} M Ca^{2+}, 1.3 at 10^{-7} M Ca^{2+}, and 1.4 at and above 10^{-6} M Ca^{2+}. This postulates an alternative possibility that nuclear fluorescence intensity was more amplified than cytosolic one at the higher Ca^{2+} concentrations. Chromatins, nucleic acids, pH, and Mg^{2+} may affect an affinity of Ca^{2+} to Fluo-3 and change fluorescence intensity (12,26,27). It may be confusing, but Przywara et al. (12) showed that in sympathetic neuron, Ca^{2+} was higher in nucleus than in cytoplasm during high potassium depolarization, ionomycin or Ach treatment, but that in astrocytes and fibroblast, no differential increase was observed with even ionomycin treatment.

Finally, it is unlikely that artifacts due to cell thickness or uneven dye distribution (or sequestration) may account for $Fl_n / Fl_c > 1$. Because we used a laser scanning confocal microscopy with the lateral and Z resolution being 0.2 and 1.2 μm, respectively, with a pin hole of 25 μm in diameter. These values are more precise than those obtained with the previous system in which a vertical resolution was 3 μm (15). Further, we found that scratching the surface cell membrane by a patch-electrode made the cell dimmed (toward a level of background) without lysis under a microscopy. Therefore, we do not think that Fluo-3 was trapped in or bound to the intracellular compartments.

The kidney tubular transport is regulated by various hormones, including ATP (1) and AVP (19,20). Increase in cellular Ca^{2+} activates the renal ion channels (7,8) and modulates the urea and water transport stimulated by AVP via the cAMP signaling pathway (19-21). However, in the IMCD cells physiological significance of the nuclear Ca^{2+} signaling is, at present, unknown. In the present study the ratio of nuclear/cytosolic Ca^{2+} fluorescence at resting was significantly higher in neonates (1.03 ± 0.01, n=463) than in young adults (0.85 ± 0.02, n=174). This may account for a rapid growing and a high differentiation ability during the neonates.

In conclusion, nuclear and cytosolic Ca^{2+} signals during stimulation with ATP were synchronous and were not independently regulated in rat IMCD cells. The source of nuclear Ca^{2+} is probably the nuclear envelope. Nucleocytosolic gradient in fluorescence may be partly due to amplification of the nuclear factor and partly due to a smaller space in the nucleus. Nuclear envelope is not a major barrier for Ca^{2+} diffusion between the nuclear and cytosolic compartments.

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