Vol. 194, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1993 Pages 287-293

THAPSIGARGIN INCREASES CELLULAR FREE CALCIUM AND INTRACELLULAR SODIUM CONCENTRATIONS IN CULTURED RAT GLOMERULAR MESANGIAL CELLS

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Received May 25, 1993

SUMMARY: We examined whether thapsigargin increases cellular free calcium ($[Ca^{2+}]i$) and intracellular sodium concentration ($[Na^{+}]i$) in cultured rat glomerular mesangial cells. 1 x 10^{-6} M Thapsigargin increased $[Ca^{2+}]i$ to 244.4 from 86.6 nM, an increase sustained at least during the 15 min observation period. Such an increase in $[Ca^{2+}]i$ was transient in Ca^{2+}-free medium containing 1 x 10^{-4} M EGTA. An increase in $[Ca^{2+}]i$ by thapsigargin was not altered by 1 x 10^{-6} M nicardipine, a L-type Ca^{2+} channel blocker. Thapsigargin also produced a sustained rise in $[Na^{+}]i$ in a dose-dependent manner. However, preincubation of cells with Ca^{2+}-free medium completely blocked the increase in $[Na^{+}]i$ by thapsigargin. These results indicate that thapsigargin increases $[Ca^{2+}]i$ by blocking endoplasmic Ca^{2+}-ATPase and enhancing Ca^{2+} entry, and that the increased Ca^{2+} influx is triggering an increase in $[Na^{+}]i$ stimulated by thapsigargin per se in glomerular mesangial cells.

Agonist-induced increases in cellular free calcium ($[Ca^{2+}]i$) play an important role in triggering numerous cellular responses in glomerular mesangial cells (1 - 4). The initial rise in $[Ca^{2+}]i$ stimulated by agonists is dependent on the action of inositol 1, 4, 5-trisphosphate (IP_3) and IP_3 mobilizes $[Ca^{2+}]i$ from endoplasmic reticulum. The sustained phase of $[Ca^{2+}]i$ mobilization is derived from extracellular Ca^{2+}. We demonstrated that vasoconstrictor hormones including arginine vasopressin (AVP), angiotensin II and endothelin also increases intracellular sodium concentration ($[Na^{+}]i$), which is based on the mobilization of $[Ca^{2+}]i$, particularly on Na^{+}/Ca^{2+} and Na^{+}/H^{+} exchanges (5).

Thapsigargin has been recognized to inhibit endoplasmic Ca^{2+}-ATPase activity, resulting in increasing $[Ca^{2+}]i$ in a number of cells (6 - 10). Recent reports showed that such an increase in $[Ca^{2+}]i$ by thapsigargin is also due to the increase in Ca^{2+} entry in addition to the blunted activity of endoplasmic Ca^{2+}-ATPase (11 - 13).

The present study was undertaken to determine whether thapsigargin increases $[Ca^{2+}]i$ in cultured rat glomerular mesangial cells. Also, wheth-

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er $[Na^+]i$ is mobilized by thapsigargin was examined, because there is Na^+/Ca^{2+} exchange system across plasma membrane.

MATERIALS AND METHODS

Cell culture

The experimental procedure was similar to our previous report (5), modified from the method of Kreisberg and Karnovsky (14). Male Sprague-Dawley rats weighing 150 - 175 g were used. Kidneys were removed under sterile condition, and cortical tissues were cut away from the medulla. They were minced with physiological saline solution (PSS; 140 mM NaCl, 4.6 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES,pH 7.4) by a sharp razor blade, and then the minced renal cortical tissues were incubated with 3 ml collagenase (1 mg/ml; Worthington Biochemicals, Freehold, N. J.) for 60 min at 37°C. After centrifuging the tubes at 500 g for 4 min at room temperature, the pellets were resuspended with Dulbecco's Modified Eagle's Medium (Flow Laboratories, McLean, VA) supplemented with 20% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The dispersed glomeruli were harvested into 35 x 10-mm plastic dishes with the medium and kept in a humidified incubator at 37°C under 95% air and 5% CO_2.

After the culture cells were confluent, they were subcultured using Ca^{2+}- and Mg^{2+}-free Hanks' solution containing 0.025% trypsin and 0.01% EDTA. The dispersed cells were collected into culture tubes and centrifuged at 500 g for 5 min at room temperature. The pellets were resuspended in Dulbecco's Modified Eagle's Medium containing 20% fetal bovine serum, penicillin and streptomycin and cultured in a humidified incubator. The culture cells at 3 - 10th passages were subjected to the following studies on days 7 - 10 of the subculture. For measurement of $[Ca^{2+}]i$ and $[Na^+]i$ the cells were cultured on thin glass slides (13-mm in diameter; Matsunami Kogyo Co., Osaka, Japan).

Measurement of $[Ca^{2+}]i$

The experimental procedure was similar to that used in our previous studies (15, 16). The cells were rinsed twice with 1 ml PSS and loaded with 5 μM fura-2/AM (Dojin Biochemicals, Kumamoto, Japan) in a volume of 0.25 ml for 60 min at 37°C. After aspiration of the fura-2/AM solution, the glass slide was rinsed and then placed in a 1 x 1-cm quartz cuvette with the aid of a special holder in a fluorescence spectrophotometer (CAF-100, Japan Spectroscopic Co., Tokyo, Japan). The dual wavelength excitation method for measurement of fura-2 fluorescence was used. The fluorescence was monitored at 500 nm with excitation wavelengths of 340 and 380 nm in the ratio mode. The effectors were added after a stable fluorescence signal (R) was achieved. Thapsigargin (Sigma, St. Louis, MO), AVP (Sigma) and endothelin (Peptides Institute, Osaka, Japan) were used. Also, the cells were preincubated for 10-20 min with Ca^{2+}-free medium containing 1 x 10^{-4} M EGTA or 1 x 10^{-6} M nicardipine (Sigma), a L-type Ca^{2+} channel antagonist, and then studied in the same manner. From the ratio of fluorescence at 340 and 380 nm, the $[Ca^{2+}]i$ was determined, as described by Grynkiewicz et al (17), using the following expression: $[Ca^{2+}]i$ (nM) = Kd x (R - Rmin)/(Rmax - R) x β , where R is the ratio of fluorescence of the sample at 340 and 380 nm, and Rmax and Rmin were determined by treating the cells with 5 x 10^{-5} M digitonin and 1 x 10^{-2} M $MnCl_2$, respectively. The term β is the ratio of fluorescence of fura-2 at 380 nm in zero and saturating Ca^{2+} concentrations. Kd is the dissociation constant of fura-2 for Ca^{2+}, assumed to be 224 nM at 37°C (17).

Measurement of $[Na^+]i$

The experimental procedure was similar to that described in our previous studies (5, 18). The cells were rinsed twice with 1 ml PSS and

loaded with 10 μM SBFI/AM (Molecular Probes, Inc., Eugene, OR) in a volume of 0.25 ml for 3 h at 37°C. SBFI/AM was dissolved in PSS containing 0.02% pluronic F-127, a nonionic detergent. After aspirating the SBFI/AM solution, the glass slides were rinsed and then placed in a 1 x 1-cm quartz cuvette with the aid of a special holder in a fluorescence spectrophotometer (CAF-100). The dual wavelength excitation method for measurement of SBFI fluorescence was used. The fluorescence was monitored at 500 nm with excitation wavelengths of 340 and 380 nm in the ratio mode. After a stable fluorescence signal was achieved, the effectors were added. $[Na^+]i$ was calibrated by equilibrating $[Na^+]i$ with the extracellular Na^+ concentration using 1 x 10^{-6} M gramicidin. The reference standard solutions were made from appropriate mixtures of Na^+ and K^+ solutions, based on the solution of PSS. The total concentrations of Na^+ and K^+ were adjusted to 135 mM. The $[Na^+]i$ was determined by the relation between the ratio and the authentic $[Na^+]i$.

Measurement of cellular pH (pHi)

The experimental procedure was similar to that reported in our previous studies (5, 18). The cells were rinsed twice with 1 ml PSS and loaded with 2 μM BCECF/AM (Molecular Probes), for 60 min at 37°C. BCECF/AM was dissolved in PSS. The fluorescence was monitored at 530 nm, with excitation wavelengths of 450 and 500 nm in the ratio mode. After measurement of the basal pHi level, 1 x 10^{-6} M thapsigargin was added. The fluorescence signal was calibrated at several pH values (6.6, 7.0 and 7.4) in the KCl solution (140 mM KCl, 4.6 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES) containing the K^+/H^+ ionophore nigericin (10 μg/ml).

Statistics

All values of $[Ca^{2+}]i$ and $[Na^+]i$ were analyzed by Student's t-test. $P<0.05$ was considered significant.

RESULTS

Figure 1 shows $[Ca^{2+}]i$ induced by thapsigargin in cultured rat glomerular mesangial cells. 1 x 10^{-6} M Thapsigargin gradually increased $[Ca^{2+}]i$, which reached a peak level 5 min after the addition. An increase in $[Ca^{2+}]i$ produced by thapsigargin was obtained at least during the 15 min observation period. Statistically, 1 x 10^{-6} M thapsigargin increased $[Ca^{2+}]i$ to 244.4 ± 28.3 nM from 86.6 ± 4.1 nM ($P<0.01$, Fig. 2). In contrast, 1 x 10^{-6} M thapsigargin-induced increase in $[Ca^{2+}]i$ was transient when cells were preincubated in Ca^{2+}-free medium containing 1 x 10^{-4} M EGTA. Basal $[Ca^{2+}]i$ decreased to 54.3 ± 2.7 nM and 1 x 10^{-6} M thapsigargin increased $[Ca^{2+}]i$ to only 81.2 ± 8.3 nM (Fig. 2). Its increase returned to the basal level 5 min after the addition of thapsigargin. The study was also performed with 1 x 10^{-6} M nicardipine. 1 x 10^{-6} M Thapsigargin increased $[Ca^{2+}]i$ to 209.5 ± 18.1 nM from 61.4 ± 8.3 nM ($P<0.01$, $n = 4$). An increase in $[Ca^{2+}]i$ by thapsigargin was not altered by the nicardipine pretreatment.

Figures 3 and 4 show that thapsigargin also increased $[Na^+]i$ in cultured rat glomerular mesangial cells. As shown in Fig. 3, 1 x 10^{-6} M thapsigargin raised $[Na^+]i$ gradually. $[Na^+]i$ induced by 1 x 10^{-6} M thapsi-

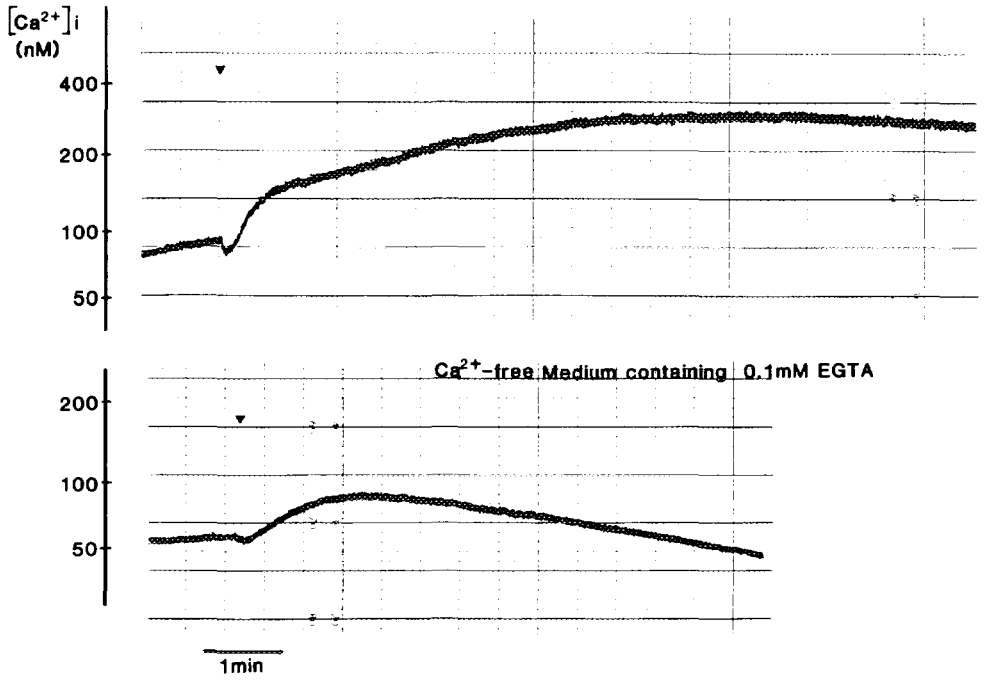


Fig. 1. Effect of 1 x 10^{-6} M thapsigargin on cellular free calcium concentration ($[Ca^{2+}]i$) in cultured rat glomerular mesangial cells. (Upper) the control, (Lower) Ca^{2+}-free medium containing 1 x 10^{-4} M EGTA.

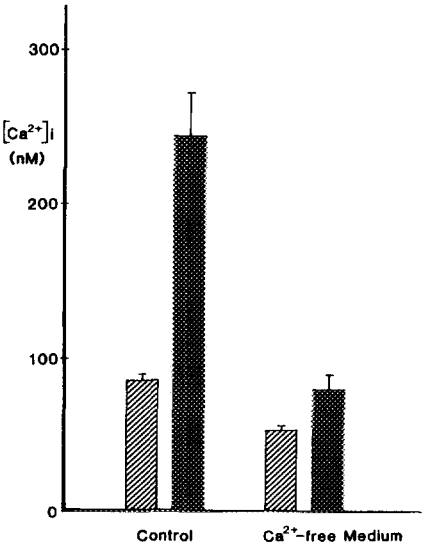
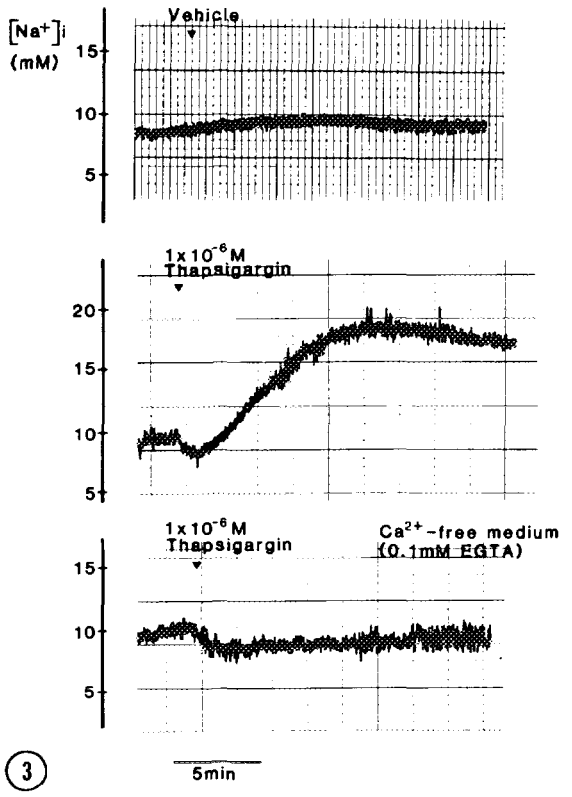


Fig. 2. Effect of Ca^{2+}-free condition on 1 x 10^{-6} M thapsigargin-induced increase in $[Ca^{2+}]i$ in cultured rat glomerular mesangial cells. Hatched bars show the basal level of $[Ca^{2+}]i$. Solid bars show 1 x 10^{-6} M thapsigargin-induced increase in $[Ca^{2+}]i$. Values are means $\pm$ SEM, n = 6.



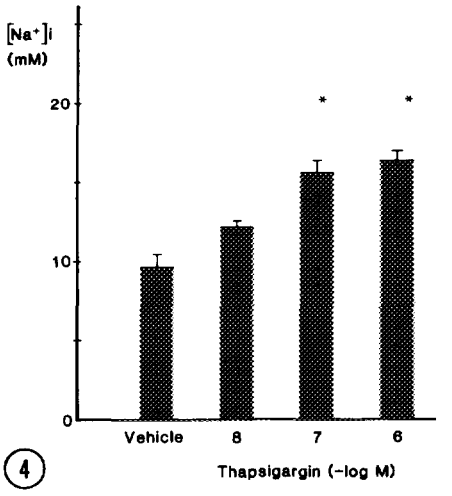


Fig. 3. Effect of 1 x 10^{-6} M thapsigargin on cellular sodium concentration ($[Na^+]i$) in cultured rat glomerular mesangial cells. (Top) the vehicle, (Middle) 1 x 10^{-6} M thapsigargin, (Bottom) 1 x 10^{-6} M thapsigargin in Ca^{2+}-free medium containing 1 x 10^{-4} M EGTA.

Fig. 4. Dose-dependent increase in $[Na^+]i$ by thapsigargin in cultured rat glomerular mesangial cells. *P<0.01 vs. the vehicle. Values are means ± SEM, n = 6.

gargin reached a peak level 8 min later. $[Na^+]i$ elevated to 16.4 ± 0.7 mM from 9.5 ± 0.9 mM (P<0.01). The time course of thapsigargin-induced increase in $[Na^+]i$ was slower than that of $[Ca^{2+}]i$. Thapsigargin increased $[Na^+]i$ in a dose-dependent manner (Fig. 4). However, when cells were preincubated with Ca^{2+}-free medium containing 1 x 10^{-4} M EGTA, there was no increase in $[Na^+]i$ in response to 1 x 10^{-6} M thapsigargin (10.4 ± 0.4 mM to 10.1 ± 0.2 mM).

AVP and endothelin increase both $[Ca^{2+}]i$ and $[Na^+]i$ in a dose-dependent manner in cultured rat glomerular mesangial cells (5). However, there was no response of $[Ca^{2+}]i$ and $[Na^+]i$ to AVP or endothelin in cells pretreated with thapsigargin (data not shown).

Lastly, we measured the pHi induced by 1 x 10^{-6} M thapsigargin. Thapsigargin caused a sustained decrease in pHi by 0.05 ± 0.02 (P<0.01, n = 6).

DISCUSSION

In the present study we clearly demonstrated that thapsigargin increased $[Ca^{2+}]i$ mediated by two pathways in cultured rat glomerular mesangial cells. Namely, thapsigargin mobilized $[Ca^{2+}]i$ from intracellular space which is probably dependent on the inhibition of endoplasmic Ca^{2+}-ATPase, as described earlier (6 - 10). Another factor activating Ca^{2+} entry is also involved because thapsigargin-mobilized $[Ca^{2+}]i$ was transient in Ca^{2+}-free condition and its peak value was significantly less in Ca^{2+}-free condition than that in the control condition. Our additional study demonstrated that 1×10^{-6} M thapsigargin also increased $[Ca^{2+}]i$ from 89.9 $\pm$ 6.8 to 226.2 $\pm$ 21.9 nM (n = 5, $P<0.01$) in the cultured rat renal papillary collecting tubule cells. Ca^{2+}-free condition markedly reduced an increase in $[Ca^{2+}]i$ by thapsigargin (data not shown). However, an increase in $[Ca^{2+}]i$ by thapsigargin was not affected by nicardripin, a L-type Ca^{2+} channel blocker, in rat glomerular mesangial cells. These findings further support that the mobilization of $[Ca^{2+}]i$ by thapsigargin is mediated through an increase in Ca^{2+} entry across plasma membrane, an effect independent of L-type Ca^{2+} channel activation, as well as an inhibition of Ca^{2+} uptake into the endoplasmic reticulum. Similar results were obtained in several recent studies, suggesting that thapsigargin enhances cellular Ca^{2+} influx from the extracellular space in PC 12 cells (11), adrenal glomerulosa cells (12) and vascular smooth muscle cells (13). In thapsigargin-treated cells vasoconstrictor hormones such as AVP and endothelin did not further increase $[Ca^{2+}]i$.

Thapsigargin also increased $[Na^{+}]i$ in cultured rat glomerular mesangial cells. To our knowledge, this is the first report to show thapsigargin-induced increase in $[Na^{+}]i$. The change in $[Na^{+}]i$ in response to thapsigargin was slower than that in $[Ca^{2+}]i$. The increase in $[Na^{+}]i$ disappeared in cells pretreated with Ca^{2+}-free medium. Since the mobilization of $[Ca^{2+}]i$ by thapsigargin is based on the inhibition of endoplasmic Ca^{2+}-ATPase under Ca^{2+}-free condition, the increment in $[Ca^{2+}]i$ derived from the intracellular space does not affect the mobilization of $[Na^{+}]i$. Rather, cellular Ca^{2+} influx is important for the $[Na^{+}]i$ mobilization. There is Na^{+}/Ca^{2+} exchange in glomerular mesangial cells (5). An increase in $[Ca^{2+}]i$ results in increasing Ca^{2+} efflux, which activates Na^{+}/Ca^{2+} exchange to enhance Na^{+} influx. This is in consistence with the finding of thapsigargin-induced reduction in pHi. As shown in our previous report (5), agonists including AVP, angiotensin II and endothelin cause an initial acidification, followed by the sustained alkalinization. Such an alkalinization is closely related to the activation of Na^{+}/H^{+} exchange.

Cellular alkalinization enhances cell contraction, cellular growth and others in glomerular mesangial cells (4). Thapsigargin is not suggested to activate Na^+/H^+ exchange. Therefore, the physiological significance of mobilization of $[Ca^{2+}]i$ and $[Na^+]i$ by thapsigargin remained to be determined.

ACKNOWLEDGMENT: The present study was supported by a grant from the Ministry of Education, Science and Culture of Japan.

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