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THE ELEVATION OF THE CYTOPLASMIC CALCIUM IONS IN VASCULAR SMOOTH MUSCLE CELLS IN SHR --MEASUREMENT OF THE FREE CALCIUM IONS IN SINGLE LIVING CELLS BY LASERMICROFLUOROSPECTROMETRY

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SUMMARY We have developed an accurate and sensitive system for the measurement of cytoplasmic free calcium concentrations ($[Ca^{++}]_i$) of a single cell by using UV-laser and Indo-1. By this method, we made the first successful measurement of $[Ca^{++}]_i$ of single living vascular smooth muscle cells. $[Ca^{++}]_i$ in spontaneously hypertensive rats was elevated and maintained after the 6th passage culture. However, $[Ca^{++}]_i$ in Goldblatt hypertensive rats was not elevated. Thus, these results suggest that the maintenance of high $[Ca^{++}]_i$ levels of vascular smooth muscle cells in spontaneously hypertensive rats is genetically regulated and that it is one of the mechanisms for hypertension.

To evaluate the role of $[Ca^{++}]_i$ in the elevation of blood pressure in SHR, it is essential to develop a method for the measurement of $[Ca^{++}]_i$ of VSMC, since $[Ca^{++}]_i$ of VSMC is thought to play an important role in the regulation of vascular tone and blood pressure. There have been a few reports about $[Ca^{++}]_i$ of suspended VSMC from SHR (1), but there has been no report which has measured $[Ca^{++}]_i$ in the physiological condition. We report here the first successful measurement of the absolute value of $[Ca^{++}]_i$ of a single living VSMC in the physiological condition by using the UV-laser (2) and Ca^{++}-sensitive dye Indo-1 (3). The assay system (shown schematically in Fig.1A) was developed to detect light with extremely high sensitivity

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ABBREVIATIONS: $[Ca^{++}]_i$, cytoplasmic free calcium concentrations; VSMC, vascular smooth muscle cells; UV, ultra-violet; SHR, spontaneously hypertensive rats; WKY, Wistar Kyoto rats; GBH, Goldblatt hypertensive rats; Hepes, N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Mops, 3-[N-Morpholino]propanesulfonic acid; EGTA, Ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N',-tetraacetic acid; DARSS, diode array rapid scanning system.

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within a small area (less than 2μm in diameter) inside a single cell by DARSS to analyze the spectra of the fluorescence using a multichannel analyzer and microcomputer and to calculate $[Ca^{++}]_i$ instantaneously.

MATERIALS AND METHODS

Rat aortic medial VSMC were cultured by modified Chamley method (4). Between days 6 to 8, just before reaching confluence, the cultured cells on quartz slides were washed three times with physiological saline solution and observed with a microscope, as described below, to record the autofluorescence spectra. After these procedure, the cells were incubated with Hepes buffered Dulbecco's modified Eagle medium containing 10μM Indo-1 AM (Molecular Probe) for 15-25 minutes at 37°C, and additionally incubated without Indo-1 AM for 15 minutes. Then the cells were washed three times with PSS at 37°C to remove the dye in the extracellular space. The physiological saline solution consisted of 135mM NaCl, 5mM KCl, 1mM $CaCl_2$, 1mM $MgCl_2$, 5.5mM glucose, and 10mM Hepes.(pH 7.4 at 37°C.) Cultured cells were characterized as VSMC by their typical hill-and-valley appearance and by the immunohistochemical confirmation of actin and myosin fibers (5). In each experiment high cellular viability (>98%) was maintained as assessed by the trypan blue exclusion test performed at the end of the experiment. Indo-1 loaded cells were observed with a fluorescence microscope equipped with a water-immersion objective system without a cover glass. The cells were excited with the UV-laser at 325nm and the fluorescence spectra at wavelength between 345-515nm were obtained by DARSS. In each cell, a spot (less than 2μm in diameter) 2μm from the nucleus was chosen for the measurement of the cytoplasmic fluorescence to avoid possible fluctuation caused by intracellular calcium gradients (6). Since Indo-1 has the special feature that the fluorescence spectra change in response to the calcium concentration, $[Ca^{++}]_i$ were calculated from the Indo-1 fluorescence spectra using a multichannel analyzer and microcomputer as described in the legend of Figure 1B. Although the signals of Indo-1 loaded cells are larger than those of autofluorescence (Fig.2), the autofluorescence spectra were subtracted from those of Indo-1 loaded cells in order to increase the accuracy of the calculated value of $[Ca^{++}]_i$, and $[Ca^{++}]_i$ was calculated instantaneously.

RESULTS AND DISCUSSION

Figure 1B shows the standard curve obtained by this assay system and you can see the good relationship between Indo-1 fluorescence ratio and $[Ca^{++}]$. The curve was so steep that the observational errors were within 10% in the physiological $[Ca^{++}]$ range (50-1000nM). Since $[Ca^{++}]_i$ were calculated not from the fluorescence intensity but from the fluorescence ratio in this assay system, the photobleaching of Indo-1 and intracellular Indo-1 concentration had no effect on the calculation of $[Ca^{++}]_i$. And by using the laser to excite the dye, the low energy excitation could produce the high fluorescence intensity from the low concentration of intracellular Indo-1 and there was little heat and dye influence to cells. These points are the great advantage of this system. Indeed the cardiac myocytes from the mice

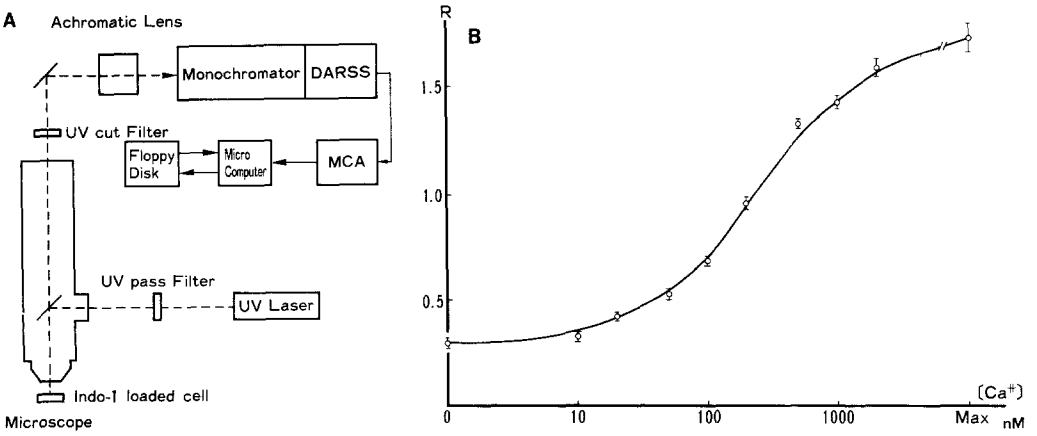

Fig.1. Acquisition of the fluorescence spectra of Indo-1 loaded cells and determination of $[Ca^{++}]_i$.

A. Schematic illustration of the fluorescence microscope (Zeiss, Photomicroscope III) with laser excitation. The UV-laser (Omnichrome, He-Cd laser, 339-2MS) generated a laser beam at wavelength 325nm and the laser power was less than 2mW. This laser beam was focused on cells less than 2μm in diameter through the objective lens (Zeiss, Ultra-Fluor x32) and the fluorescence was focused through the achromatic lens (Asahi Pentax, Ultra-achromatic Takumar) to the spectroscope (JASCO, CT25). The fluorescence spectra were obtained by DARSS (Tracor Northern, TN-6132) within 0.5 second and transferred to a multichannel analyzer (MCA) (Tracor Northern, TN-1710C) and microcomputer (NEC, PC-9801VM2). To reduce the noise, the UV pass filter (HOYA, UV34) and UV cut solution filter (1,4-Diphenylbutadiene 42mg/L Ether, optical length=10mm) (7) were used.

B. Relationship between Indo-1 fluorescence ratio and $[Ca^{++}]$. Indo-1 (Molecular Probe) was added to small volumes of buffer (115mM KCl, 20mM NaCl, 10mM Mops, 1.115mM K_2H_2EGTA, KOH to pH7.05, 37°C) at final concentration 0.3μM. Each volume contained a different $[Ca^{++}]$ which was controlled by varying the ratio of Ca-EGTA/EGTA (8). Fluorescence ratio (R), obtained by dividing the fluorescence values at 422nm by those at 468nm, bandwidth 3.5nm. These wave length were decided by the preliminary experiment. $[Ca^{++}]$ can be calculated from the equation $[Ca^{++}]=K(R-R_0)/(R_s-R)$, where R_0 is the ratio at 0 calcium and R_s is the ratio at calcium saturation. K represents $K_d(F_0/F_s)$, where K_d is the effective dissociation constant for Indo-1 in the appropriate conditions, F_0 is the fluorescence at 468nm in 0 Ca and F_s is the fluorescence at 468 nm in saturated calcium solutions.

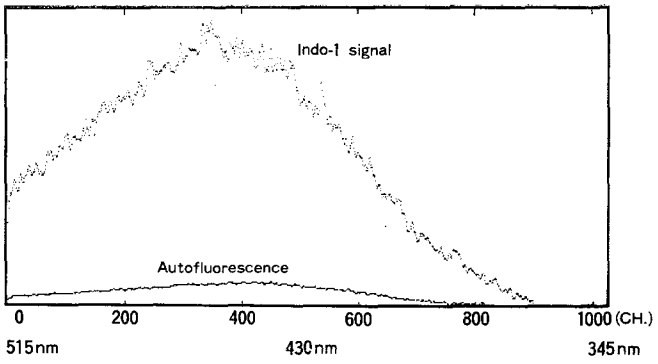

Fig.2. A typical example of the fluorescence spectrum from the Indo-1 loaded VSMC and the autofluorescence of the cell. The Indo-1 signal was obtained within 0.5 second from a single Indo-1 loaded VSMC and the autofluorescence was the average measurement obtained from 20 Indo-1 unloaded cells.

embryos could be observed beating during measurement of $[Ca^{++}]_i$ after incubation with Indo-1 AM as described above. (Data are not published.) Although there have been a few reports describing $[Ca^{++}]_i$ of suspended VSMC from WKY and SHR (1), $[Ca^{++}]_i$ of a single VSMC have not been measured accurately, nor in the physiological condition. With their assay method it is necessary to trypsinize and suspend VSMC in the assay solution. Suspended VSMC received significant cellular damage and they were not physiological, neither in the cellular form nor in the cellular function. On the contrary, since with our new assay system it is not necessary to suspend cells. We can measure $[Ca^{++}]_i$ of single cells sticking on quartz slides without any damaging procedures. By this assay system we could obtain the first successful measurements of $[Ca^{++}]_i$ in a single living VSMC in the physiological condition without cellular damage.

To evaluate the relationship between blood pressure and $[Ca^{++}]_i$ of VSMC in SHR, we observed blood pressure and $[Ca^{++}]_i$ over time. Blood pressure was markedly elevated in 8 and 12 week old SHR compared with blood pressure in age matched control WKY. However, in 4 week old SHR, blood pressure was much the same as in WKY. (Fig.3A) The mean $[Ca^{++}]_i$ in 8 and 12 week old SHR were significantly higher than those in 8 and 12 week old WKY, but the mean $[Ca^{++}]_i$ in 4 week old SHR was about the same as in 4 week old WKY. (Fig.3B and Table 1A) In Table 1B, $[Ca^{++}]_i$ of the 6th passage cultured VSMC are listed in comparison with $[Ca^{++}]_i$ of their primary cultured VSMC, and $[Ca^{++}]_i$ of the 6th passage cells were much the same as those of primary cells. In the 6th passage cultured VSMC, since the cells were cultured for more than 4 weeks, the hemodynamic effects of the high blood pressure in SHR on $[Ca^{++}]_i$ of VSMC *in vivo* were supposed to disappear when $[Ca^{++}]_i$ were measured. In 4 week old rats, both blood pressure and $[Ca^{++}]_i$ in SHR were much the same as those in WKY. But in 8 and 12 week old, both blood pressure and $[Ca^{++}]_i$ in SHR were significantly higher than those in WKY. These results suggested that the maintenance of high $[Ca^{++}]_i$ levels of VSMC in SHR is genetically regulated and that the expression of high $[Ca^{++}]_i$ is

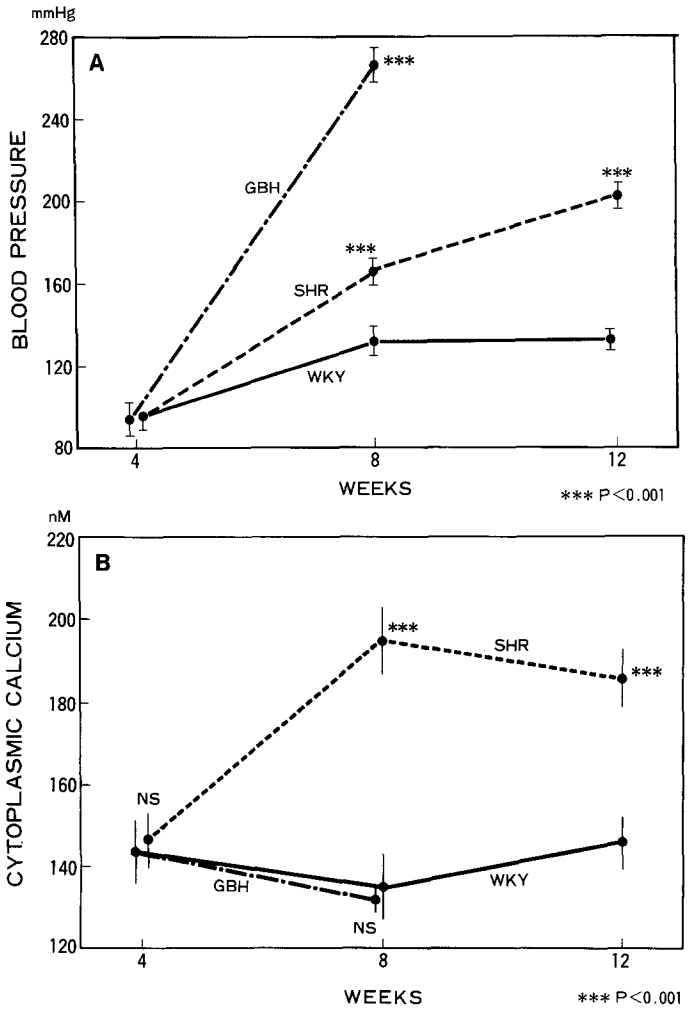

Fig.3A. Relationships between aging and blood pressure in WKY, SHR and GBH. Blood pressure was measured by the tail-cuff method using PE-300 (NARCO BIO-SYSTEMS). GBH were made from 4 week old WKY by 1 kidney 1 clip Goldblatt operation. mean±SE (n=8), ***;p<0.001 vs WKY

Fig.3B. Relationships between aging and $[Ca^{++}]_i$ in WKY, SHR and GBH. mean±SE, ***;p<0.001 vs WKY

Table 1A
Relationships between aging and $[Ca^{++}]_i$ in WKY, SHR and GBH
These data are the same in the Fig.3B.

Cytoplasmic Calcium (nM)

	4 W	8 W	12W
SHR	146±7(26)	195±8(44)	186±7(28)
WKY	144±8(22)	135±8(46)	146±7(22)
GBH		132±3(60)	

mean±SE (n=Cell number)

Table 1B
Effect of passage culture on $[Ca^{++}]_i$ in 8 week old WKY and 4,8 week old SHR

Cytoplasmic Calcium (nM)

	WKY	SHR	
	8 W	4 W	8 W
primary	135±8(46)	146±7(26)	195±8(44)
6th passage	139±4(30)	146±6(29)	187±7(30)

mean±SE (n=Cell number)

related to the expression of hypertension. To make clear these points, we made 1 kidney 1 clip GBH and measured $[Ca^{++}]_i$ and blood pressure. These results are also listed in Table 1A and shown in Figure 3B. Interestingly, in GBH, although blood pressure was significantly higher than in WKY, $[Ca^{++}]_i$ was much the same as in WKY. Since GBH are secondary hypertensive induced by activation of the renin-angiotensin system, the hormonal and hemodynamic effect on $[Ca^{++}]_i$ of VSMC should be eliminated during cell culture. Thus, the elevation of $[Ca^{++}]_i$ of VSMC was observed only in hereditary hypertensive rats, that is in SHR, and moreover in the overt hypertensive ones. These results strongly suggested that the maintenance of high $[Ca^{++}]_i$ levels of VSMC in SHR is genetically regulated and can be seen to serve as one of the important mechanisms for elevated blood pressure.

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REFERENCES

1. Nabika,T., Velletri,P.A., Beaven,M.A., Endo,J., and Lovenberg,W. (1985) Life Science. 37, 579-584
2. Urabe,H., Ishii,Y., Yamada,Y., Tsukakoshi,M., Ikegami,A., and Kasuya,T. (1985) Laser Science Progress Report of IPCR. 7, 16-18
3. Grynkiewicz,G., Poenie,M., and Tsien,R.Y. (1985) J. Biol. Chem. 260, 3440-3450
4. Chamley,J.H., Campbell,G.R., McConnell,J.D., and Stewart,U.G. (1977) Cell Tissue Res. 177, 503-522
5. Chamley-Campbell,J., Campbell,G.R., and Ross,R (1979) Physiol. Rev. 59, 1-61
6. Williams,D.A., Fogarty,K.E., Tsien,R.Y., and Fay,F.S. 1985 Nature 318, 558-561
7. Kasha,M. (1948) J. Opt. Soc. Am. 38, 929-934
8. Moisescu,D.G., and Pusch,H. (1975) Pflugers Archiv. 355, R122