Changes in Intracellular Free Ca^{2+} Concentration by Activation of α -Adrenoceptors in Rat Tail Artery

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Abstract—Changes in intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) by activation of α_1 - and α_2 -adrenoceptors were microfluorometrically measured in rat tail arteries loaded with the Ca^{2+} -sensitive fluorescent dye fura-2. Under the normal condition (1.8 mM Ca^{2+}), stimulation with the selective α_1 -adrenoceptor agonist phenylephrine very rapidly raised $[\text{Ca}^{2+}]_i$ to the peak concentration, plateauing at this level, whereas the stimulation with the selective α_2 -adrenoceptor agonist UK-14,304 gradually increased $[\text{Ca}^{2+}]_i$. In Ca^{2+} -free solution, the stimulation of α_1 -adrenoceptors caused a transient increase in $[\text{Ca}^{2+}]_i$, whereas the stimulation of α_2 -adrenoceptors had no effect. These results directly prove that α_1 -adrenoceptor activation induces both the release of intracellular Ca^{2+} and the influx of extracellular Ca^{2+} , whereas activation of α_2 -adrenoceptors evokes only the influx of extracellular Ca^{2+} . Plotting the increase in $[\text{Ca}^{2+}]_i$ against the developed contraction revealed that it was always linearly related regardless of the type of α -adrenoceptor stimulated. Therefore, the intracellular mechanisms after the increase in $[\text{Ca}^{2+}]_i$ are probably common to the vasocontractile responses mediated by α_1 - and α_2 -adrenoceptors.

It is generally accepted that both α_1 - and α_2 -adrenoceptors are present on vascular smooth muscle (1, 2). Furthermore, based on the observed effects of Ca^{2+} antagonists and Ca^{2+} agonists on the contractions or $^{45}\text{Ca}^{2+}$ uptake induced by selective α_1 - and α_2 -adrenoceptor agonists, it is hypothesized that the activation of α_2 -adrenoceptors is linked to the influx of extracellular Ca^{2+} , whereas activation of α_1 -adrenoceptors is coupled to the release of intracellular Ca^{2+} as well as the influx of extracellular Ca^{2+} (3–7). However, it remains unsolved whether the intracellular mechanisms after the increase in the concentration of intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$) are really the same in vasocontractile responses mediated by α_1 - and α_2 -adrenoceptors. Although it is generally accepted that smooth muscle contraction is primarily regulated by $[\text{Ca}^{2+}]_i$, possible modulation by factor(s) other than Ca^{2+} has also been postulated (8, 9). We have shown that contraction induced

by α_1 -adrenoceptor agonists is more sensitive to the calmodulin antagonist W-7 than that by α_2 -adrenoceptor agonists in the rat tail artery (6). It is possible that the intracellular regulation mechanism is different between the responses mediated by α_1 - and α_2 -adrenoceptors. To examine this possibility, it is necessary to measure direct changes in $[\text{Ca}^{2+}]_i$ by activation of α_1 - and α_2 -adrenoceptors in living vascular smooth muscle.

Recently, fluorescent Ca^{2+} indicators such as fura-2 and indo-1 have been developed for the measurement of $[\text{Ca}^{2+}]_i$ in living cells (10, 11). These dyes will make it possible to directly measure $[\text{Ca}^{2+}]_i$ changes in smooth muscle tissue because this tissue can not be readily studied by conventional methods using the Ca^{2+} -sensitive photoprotein aequorin, the Ca^{2+} -sensitive metallochromic dye arsenazo III or the Ca^{2+} -selective microelectrode (12–14). In the present study, we investigated the roles of $[\text{Ca}^{2+}]_i$ in α_1 - and α_2 -adrenoceptors-mediated vasoconstriction by applying this technique to rat tail arteries in which the

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existence of postsynaptic α_1 - and α_2 -adrenoceptors has been previously verified (6, 15, 16).

Materials and Methods

Male Wistar rats weighing 250 to 350 g were killed by a blow on the head and subsequently exsanguinated. Proximal segments of the tail arteries were quickly removed and placed in a dish filled with physiological salt solution (PSS) of the following composition: 118.0 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl_2 , 1.2 mM MgCl_2 , 1.2 mM NaH_2PO_4 , 25.0 mM NaHCO_3 , 11.1 mM glucose; the pH was 7.4. Under a dissecting microscope, adipose and connective tissues were removed as thoroughly as possible. The endothelial cells were removed by inserting the smoothed tip of a needle into the lumen.

In preliminary experiments, we found that changes in fluorescent intensity during contraction were detected more steadily from a transverse section than from intimal or adventitial surfaces. Therefore, ring preparations (about 0.7 mm in outside diameter and 1 mm in length) in which the smooth muscle layers in transverse section could be directly observed were used for the measurement of $[\text{Ca}^{2+}]_i$. The solution for loading the smooth muscle cells with fura-2 was prepared by adding 5 μM fura-2 acetoxymethylester (fura-2/AM), 0.02% Cremophor EL and 2 mg/ml bovine serum albumin to normal PSS. Cremophor EL is a non-cytotoxic detergent that helps to solubilize fura-2/AM in physiological media (13). The ring preparations were transferred to a plastic test tube containing 500 μl of the loading solution and incubated at 25°C for 4 hr with gentle shaking.

After the loading, the ring preparation was transferred to a 1 ml tissue chamber in which warmed (37°C) and oxygenated (95% O_2 -5% CO_2) PSS was continuously perfused at a speed of about 7 ml/min. The ring was hooked by a pair of stainless-steel wires attached to the chamber and fixed with sufficient stretching to minimize movement artifacts. Before the effects of drugs were tested, the tissue was allowed to equilibrate for 30 min to ensure stable responsiveness of the smooth muscle, to thoroughly wash out the remaining extracellular dye and to promote hydrolysis of

fura-2/AM by intracellular esterases. The drugs were applied by replacing the perfusing solution with the solution containing the desired concentrations of drugs.

We used an inverted fluorescence microscope (Olympus, IMT2-QRFC) to measure the fluorescence of fura-2. The light from a 100 W mercury lamp was filtered by either of two different interference filters (340 ± 5 nm and 380 ± 5 nm) in the excitation path and conducted to the specimen through a dichroic mirror and an epifluorescence objective (Olympus, DPlanApo 10 $\times$ UV). The fluorescence from the tissue was filtered by an interference filter (510 ± 15 nm) in the emission path. A circular area (0.045 mm^2) of the smooth muscle layer in the transverse section of the ring preparation was selected with a circular diaphragm, and the intensity of the fluorescence was measured with a photomultiplier (Hamamatsu Photonics, R1463) and recorded on an ink-writing oscillograph. The fluorescence at 340 nm or 380 nm excitation was alternately measured at intervals of 1 to 1.5 sec, and the ratio of 340 nm to 380 nm fluorescence was calculated. Unless necessary, excitation light was interrupted by a shutter in the excitation path to avoid photobleaching of fura-2. We did not determine absolute values of $[\text{Ca}^{2+}]_i$ for the following reasons: 1) the autofluorescence and the possible Ca^{2+} -insensitive fluorescence due to extracellularly remaining or incompletely hydrolyzed dyes (17) cannot be accurately subtracted from the total fluorescence signal in each measurement; 2) The dissociation constant (K_d) of fura-2 for Ca^{2+} which is required for the calculation of $[\text{Ca}^{2+}]_i$ (10) is unknown under the intracellular condition of the smooth muscle cells of rat tail arteries.

Helical strips (about 1 mm in width and 15 mm in length) were used for the measurement of the contractile force, because ring preparations used for the measurement of $[\text{Ca}^{2+}]_i$ developed only a very small tension that was technically difficult to measure as a mechanical change. The helical strip was vertically mounted in a 5-ml organ bath containing PSS, which was continuously oxygenated (95% O_2 - CO_2) and maintained at 37°C. The isometric tension was measured with a force-displacement transducer (Nihon Kohden, TB-

612T) and recorded on an ink-writing oscillograph (Nihon Kohden, WI 621-G). The details were the same as described previously (6).

The following drugs were used: Cremophor EL (Nacalai Tesque), fura-2/AM (1 mM in DMSO, Dojin), L-norepinephrine (NE) bitartrate (Wako), L-phenylephrine hydrochloride (Sigma), UK-14,304 tartrate (Pfizer). NE, phenylephrine and UK-14,304 did not show any additional fluorescence in the concentration ranges used in the present study.

Data were presented as the mean $\pm$ S.E.M. of n independent observations.

Results

The contractility of the rat tail artery was not significantly affected by the loading with fura-2. The maximal contractile forces induced by NE in the helical strips not loaded and loaded with fura-2 were 14.4 ± 1.3 mN ($n=4$) and 13.5 ± 1.2 mN ($n=4$), respectively. The pD_2 values for NE were 7.00 ± 0.16 ($n=4$) in the

arteries not loaded with fura-2 and 6.92 ± 0.12 ($n=4$) in the loaded arteries. The time-courses of the contractions induced by NE were also virtually the same in the arteries not loaded and loaded with fura-2.

Figure 1 shows examples of the fluorescence measurement with our present system. As shown in Fig. 1A the fluorescence at 340 nm and 380 nm excitation were detected in the rat tail arteries not loaded with fura-2, but neither changed by the application of 10^{-4} M NE, which produced the maximal contractions of the arteries (6). The intensity of the fluorescence at 340 nm and 380 nm excitation in the resting arteries loaded with fura-2 were about 4- to 5-fold and 6- to 7-fold stronger than those in the arteries not loaded with fura-2, respectively. Although the basal fluorescence slightly but continuously decreased the ratio of the fluorescence at 340 nm and 380 nm excitation remained constant unless the arteries were stimulated. Application of 10^{-5} M NE to the arteries loaded with fura-2

Fig. 1. Examples of fluorescence measurement in the rat tail arteries not loaded (A) or loaded with fura-2 (B). The fluorescence at 340 nm or 380 nm excitation was alternately recorded (upper panel), and the ratio of 340 nm to 380 nm fluorescence (F_{340}/F_{380} ratio) was calculated (lower panel). Ordinates of the upper panels are in arbitrary units. 10^{-4} M or 10^{-5} M NE was applied by perfusion during the time indicated by the bar.

increased the fluorescence at 340 nm excitation, decreased the fluorescence at 380 nm excitation and consequently increased the ratio of 340 nm to 380 nm fluorescence (Fig. 1B). After washing out the drug, the 340/380 nm fluorescence ratio recovered to the basal value. Basically the same results as Fig. 1 were obtained when 10^{-7} to 10^{-5} M phenylephrine or UK-14,304 was applied. The 340/380 nm fluorescence ratio in the arteries loaded with fura-2 increased from the basal value of 1.40 ± 0.04 ($n=15$) to the peak value of 2.17 ± 0.09 ($n=15$) after stimulation with 10^{-4} M NE.

Stimulation with 10^{-7} to 10^{-5} M phenylephrine, a selective α_1 -adrenoceptor agonist, greatly increased the 340/380 nm fluorescence ratio of the rat tail arteries loaded with fura-2 in a concentration-dependent manner. The 340/380 nm fluorescence ratio increased very rapidly, reached the peak within about 10–15 sec and remained at the same or a slightly lower level (Fig. 2A). Stimulation with 10^{-7} to 10^{-5} M UK-14,404, a selective α_2 -adrenoceptor agonist, also increased the 340/380 nm fluorescence ratio in a concentration dependent-manner. However, the increase in the 340/380 nm fluorescence ratio was gradual and required nearly 1 min to reach the peak (Fig. 2B). The time to reach the half maximal increase in 440/480 nm fluorescence ratio by stimulation with 10^{-5} M phenylephrine and 10^{-5} M UK-14,304 was 3.6 ± 0.4 sec ($n=5$) and 15.8 ± 3.9 sec ($n=5$), respectively.

The rate of the contraction induced by phenylephrine was much faster than that by UK-14,304 as observed in our previous paper (6). Time to reach the half maximal contraction by stimulation with 10^{-5} M phenylephrine and 10^{-5} M UK-14,304 was 4.4 ± 0.4 sec ($n=8$) and 11.5 ± 0.8 sec ($n=7$), respectively.

The rat tail arteries loaded with fura-2 were exposed to Ca^{2+} -free PSS without a calcium chelating agent for 10 min to remove the extracellular source of Ca^{2+} . The 340/380 nm fluorescence ratio decreased from 1.45 ± 0.05 ($n=10$) in normal PSS ($1.8 \text{ mM } \text{Ca}^{2+}$) to 1.18 ± 0.02 ($n=10$) after the exposure to Ca^{2+} -free PSS for 10 min. Stimulation with phenylephrine produced transient increases in the 340/380 nm fluorescence ratio under this

Fig. 2. Changes in the 340/380 nm fluorescence ratio in the rat tail arteries loaded with fura-2 during stimulation with various concentrations of phenylephrine (A) or UK-14,304 (B) in normal solution ($1.8 \text{ mM } \text{Ca}^{2+}$). The increase in 340/380 nm fluorescence ratio (F_{340}/F_{380} ratio) was expressed as a percentage of the maximal increase in fluorescence ratio produced by 10^{-4} M NE in each preparation. Abscissas indicate the time after exposure to the drug. The concentrations of the drugs were represented as the negative logarithm of the molar concentration in the right-hand side of each line. The data were the mean values of 5 observations.

condition, but stimulation with UK-14,304 never changed the 340/380 nm fluorescence ratio (Fig. 3).

The relation between the peak increase in 340/380 nm fluorescence ratio and the peak contraction produced by stimulation with various concentrations of phenylephrine and UK-14,304 are shown in Fig. 4. The development of the contraction correlated well with the increase in the 340/380 nm fluorescence ratio. The contractions of the same amplitude were induced by stimulation with phenyl-

Fig. 3. Changes in the 340/380 nm fluorescence ratio in the rat tail arteries loaded with fura-2 during stimulation with phenylephrine (A) or UK-14,304 (B) in the absence of extracellular Ca^{2+} . The arteries had been preincubated in Ca^{2+} -free solution for 10 min to remove the extracellular source of Ca^{2+} . The increase in 340/380 nm fluorescence ratio (F340/F380 ratio) was expressed as a percentage of the difference between the maximal fluorescence ratio during stimulation with 10^{-4} M NE in normal solution and the basal fluorescence ratio in the absence of extracellular Ca^{2+} in each preparation. The data were the mean values of 5 observations.

ephine and UK-14,304, which produced the same increase in the fluorescence ratio.

Discussion

There is a large amount of data describing the contractile responses of the smooth muscle tissues. Therefore, we attempted to measure changes in $[\text{Ca}^{2+}]_i$ in the contracting smooth muscle tissue to clarify the relationship between the $[\text{Ca}^{2+}]_i$ and the developed force. We were able to load the rat arteries with the fluorescent Ca^{2+} indicator fura-2

Fig. 4. The relation between the increases in $[\text{Ca}^{2+}]_i$ and the contractions of the rat tail arteries produced by various concentrations of phenylephrine ($\circ$) or UK-14,304 ($\bullet$) in normal solution (1.8 mM Ca^{2+}). The peak amplitude of the contraction induced by each concentration of agonist was expressed as a percentage of the maximal contraction induced by 10^{-4} M NE (ordinate). The peak increase in the 340/380 nm fluorescence ratio (F340/F380 ratio) induced by each concentration of agonist was expressed as a percentage of the maximal increase in fluorescence ratio induced by 10^{-4} M NE in each preparation (abscissa). As described in Materials and Methods, these two parameters were measured in separate preparations. The concentration of agonist was indicated as the negative logarithm of molar concentration near the symbol. The data were shown as the mean $\pm$ S.E.M. of 5 to 8 experiments.

which enabled us to detect efficiently changes of the fluorescence signal. However, it is necessary to ascertain that the loading with fura-2 does not affect the cellular functions (18, 19) and that the changes in the fluorescence signal are not interfered with by any factor not directly related to the changes in $[\text{Ca}^{2+}]_i$, e.g., fluorescence from reduced nicotinamide-adenine dinucleotide (NADH; 13), movement artifacts, etc.

In the present study, NE-induced contractile responses of the rat tail arteries loaded with fura-2 were almost the same as those of the arteries not loaded with fura-2 in terms of the maximal response, the sensitivity and the time-course. This result indicates that the extent of fura-2 loading in the present study does not significantly affect the contractile

responses of the rat tail arteries, although the evidence is circumstantial.

The results with the arteries not loaded with fura-2 suggest that the change in the autofluorescence including that from NADH is not involved in the change of fluorescence detected in the arteries loaded with fura-2 in the present study. The signal due to the changes in $[Ca^{2+}]_i$ and that due to the movement artifacts can be discriminated by monitoring the changes of fluorescence at two different excitation wavelengths. With the increase in $[Ca^{2+}]_i$, the fluorescence of fura-2 at 340 nm and 380 nm excitation increases and decreases, respectively (10), while the movement artifacts should increase the fluorescence at 340 nm and 380 nm excitation in parallel. In the present study, the fluorescence at 340 nm and 380 nm excitation in the rat tail arteries loaded with fura-2 oppositely increased and decreased by the stimulation with α -adrenoceptor agonists. Therefore, the changes in the fluorescence signal observed in the present study undoubtedly correspond to the changes in $[Ca^{2+}]_i$.

The use of 340/380 nm fluorescence ratio makes it possible to cancel out a slight but continuous decrease in the basal fluorescence probably due to photobleaching or leakage of the dye and to consequently distinguish the signal correspond to the changes in $[Ca^{2+}]_i$ with the changes in the 340/380 nm fluorescence ratio.

Using these techniques, we investigated how $[Ca^{2+}]_i$ changes by stimulation of α_1 - and α_2 -adrenoceptors in the rat tail artery. Both phenylephrine, a selective α_1 -adrenoceptor agonist, and UK-14,304, a selective α_2 -adrenoceptor agonist, increased $[Ca^{2+}]_i$ of the rat tail artery in a concentration-dependent manner. However, the time-courses of the changes in $[Ca^{2+}]_i$ induced by the stimulation of α_1 - and α_2 -adrenoceptors were markedly different. The activation of α_1 -adrenoceptors raised the $[Ca^{2+}]_i$ to the peak level very rapidly and maintained it at the same or a slightly lower level, whereas the activation of α_2 -adrenoceptors gradually increased the $[Ca^{2+}]_i$.

The exposure of the rat tail artery to Ca^{2+} -free PSS for 10 min significantly decreased the resting $[Ca^{2+}]_i$. This change is probably

due to the efflux of intracellular Ca^{2+} according to the changes in concentration gradients. The activation of α_1 -adrenoceptors produced a transient increase in $[Ca^{2+}]_i$ after exposure to Ca^{2+} -free PSS, whereas the increase in $[Ca^{2+}]_i$ induced by the activation of α_2 -adrenoceptors in the presence of extracellular Ca^{2+} completely disappeared by the removal of extracellular Ca^{2+} . These results directly prove that the activation of α_1 -adrenoceptors induces release of intracellular Ca^{2+} as well as influx of extracellular Ca^{2+} , whereas the activation of α_2 -adrenoceptors provokes only an influx of extracellular Ca^{2+} . The result that $[Ca^{2+}]_i$ was maintained at a slightly higher level than the basal level following a transient increase by stimulation with a higher concentration of phenylephrine after exposure to Ca^{2+} -free PSS may be due to influx of possibly remaining extracellular Ca^{2+} .

We measured $[Ca^{2+}]_i$ and contraction in separate preparations to measure more efficiently two parameters. Therefore, the individual data may involve preparation-dependent differences. However, the averaged data should provide substantial information about the relationship between $[Ca^{2+}]_i$ and contraction. The development of the contraction induced by α_1 -adrenoceptor agonists was much faster than that by α_2 -adrenoceptor agonists, corresponding well to the time-course of increase in $[Ca^{2+}]_i$. Furthermore, a plot of the relation between the increase in $[Ca^{2+}]_i$ and the developed contraction revealed that the same increase in $[Ca^{2+}]_i$ caused by the stimulation of α_1 - and α_2 -adrenoceptors produced the same amplitude of contraction. Therefore, the intracellular mechanisms after the increase in $[Ca^{2+}]_i$ are probably common to the vasocontractile responses mediated by α_1 - and α_2 -adrenoceptors. Differences in inhibitory effects of W-7 reported in our previous paper (6) may reflect differences in mechanism before the increase in $[Ca^{2+}]_i$. Simultaneous recording of $[Ca^{2+}]_i$ and contraction in the same cells is necessary to investigate in more detail the time-course of changes in $[Ca^{2+}]_i$ and contraction.

Morgan and Morgan (8) directly measured the changes in the intracellular Ca^{2+} level of the ferret portal vein with aequorin and observed that during the stimulation with

phenylephrine, the intracellular Ca^{2+} level rises rapidly but then declines rapidly to a very low level, while the contractile force is still maintained. DeFeo and Morgan (20) observed similar changes in $[\text{Ca}^{2+}]_i$ during stimulation with phenylephrine in enzymatically isolated single cells from the same tissue with the fluorescent Ca^{2+} indicator quin 2. Himpens and Casteels (21) reported similar results about the relation between $[\text{Ca}^{2+}]_i$ measured with quin 2 and tension development during stimulation with NE in strips of rabbit ear artery. However, the increases in $[\text{Ca}^{2+}]_i$ caused by the activation of α -adrenoceptors of the rat tail arteries observed in the present study were not transient but relatively sustained, consistent with the report by Ozaki et al. (13) in strips of rat aorta stimulated with NE. Although the differences may be partly due to the different properties of the indicators used, it is probable that the dependence on Ca^{2+} for maintaining the tension considerably differs with various tissues.

In conclusion, we measured the changes in $[\text{Ca}^{2+}]_i$ in the rat tail arteries using the fluorescent Ca^{2+} indicator fura-2 and, for the first time, directly proved that the vasoconstrictile responses mediated by α_1 - and α_2 -adrenoceptors are different in the mobilization of Ca^{2+} , but common in the intracellular mechanisms after the increase in $[\text{Ca}^{2+}]_i$.

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