Vol. 135, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 26, 1986 Pages 9-15

INTRACELLULAR FREE CALCIUM TRANSIENTS INDUCED BY NOREPINEPHRINE IN RAT AORTIC SMOOTH MUSCLE CELLS IN PRIMARY CULTURE

Yoshito Shogakiuchi, Hideo Kanaide, Sei Kobayashi, Junji Nishimura and Motoomi Nakamura

Research Institute of Angiocardiology and Cardiovascular Clinic, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan

Received January 6, 1986

SUMMARY: In cultured rat arterial smooth muscle cells treated with quin 2, cytosolic Ca^{2+} transients induced by norepinephrine were recorded microfluorometrically. In the presence or absence of extracellular Ca^{2+}, norepinephrine induced transient and dose-dependent elevations in cytosolic Ca^{2+}, with a similar time course, the peak levels being observed at 2 min. These transient elevations in cytosolic Ca^{2+} were dose-dependently inhibited by alpha-adrenergic antagonists, the order of potency being prazosin > phentolamine > yohimbine, irrespective of the presence of extracellular Ca^{2+}. We propose that with or without extracellular Ca^{2+}, norepinephrine activates mainly alpha-1 adrenoceptors leading to a release of Ca^{2+} from intracellular stores. This would explain the transient elevation in cytosolic Ca^{2+} in rat aortic vascular smooth muscle cells in primary culture.

Post-junctional alpha-adrenergic receptors in vascular smooth muscle cells (VSMCs) play an important role in physiological regulation of vascular tone and also in the development or maintenance of hypertension (1-3). It has been well established that alpha-adrenergic activation of VSMCs involves both the influx of Ca^{2+} and the release of intracellular Ca^{2+}. However, the relative dependence of the alpha-receptor-mediated contraction on these two Ca^{2+} sources is debatable (4-7). There is evidence of both alpha 1- and alpha 2- adrenoceptor subtypes in the plasma membrane of VSMCs (8,9), but the relative significance of these two subtypes in Ca^{2+} homeostasis in VSMCs is not well understood (7,10), probably due to the difficulty in directly measuring the cytosolic Ca^{2+} transient in VSMCs.

ABBREVIATIONS : $[Ca^{2+}]i$: intracellular Ca^{2+} concentration , EGTA: ethylene glycol-bis (β -aminoethylether) N, N'-tetraacetic acid , HEPES: N-2-Hydroxyethyl-piperazine-N'-2-ethane sulfonic acid , PSS: physiological saline solution , VSMCs: vascular smooth muscle cells , NE: norepinephrine .

0006-291X/86 $1.50

Intracellular Ca^{2+} concentrations ($[Ca^{2+}]i$) have apparently never been directly monitored during alpha-adrenergic activation in VSMCs, except for one study, in which the Ca^{2+} indicator aequorin was administered intracellularly by making the cells temporarily hyperpermeable, and the effects of phenylephrine on cytosolic Ca^{2+} levels in the ferret portal vein were investigated (11). In the present study, using the quin 2-microfluorometry we developed (12,13), we characterized norepinephrine(NE)-induced $[Ca^{2+}]i$ transients of VSMCs in primary culture from rat aorta and did studies to determine whether the NE activation of alpha-1 or alpha-2 adrenoceptors is responsible for the $[Ca^{2+}]i$ transients.

MATERIALS AND METHODS

Cell culture and loading cells with quin 2: Rat aortic medial smooth muscle cells were cultured as described (14). On days 5 to 6, just before reaching confluence, the cultured cells on Lux chamber slides were loaded with quin 2 as the acetoxymethyl ester (quin 2/AM), physiologically, as described (12,13). Unless otherwise indicated, the experiments were performed in physiological saline solution (PSS) at 25°C. The millimolar composition of the "normal" PSS (pH 7.4 at 25°C) was : NaCl 135; KCl 5; $CaCl_2$ 1; $MgCl_2$ 1; glucose 5.5; HEPES 10. The composition of Ca^{2+} free PSS was similar to "normal PSS", except that it contained 2mM EGTA instead of 1mM $CaCl_2$. We used primary cultured cells for all experiments. High cell viability (>95%) was maintained during the course of each experimental procedure, as assessed by the trypan blue exclusion test and our procedures (14).

Microfluorometry of quin 2: We recorded the fluorescence intensity of VSMCs using the microfluorometry, as described (12,13). Briefly, we used a fluorescence microscope (model Standard 18,Zeiss) equipped with a water immersion objective system (Plan-Neofluor 63, Zeiss) and an appropriate combination of filters, in which the cells were excited at wavelengths between 350 and 360 nm and analysed at fluorescent wavelengths between 470 and 560 nm. Using a pinhole diaphragm (Zeiss) in the light axis, the fluorescence intensity in a spot ($<1\mu m^2$) of the cytosol 3μm apart from the nucleus was measured. Each cell was exposed to the excitation light, only once, for not longer than 2 sec in order to avoid the photobleaching effect on the dye. To read the fluorescence intensity, input-output calculator (model 97S, Hewlett-Packard) was used.

Materials: Quin 2/AM was purchased from DOTITE(Japan). Yohimbine and l-norepinephrine were purchased from the Sigma Chemical Co. The following drugs were kindly donated from the respective manufacturers: prazosin(Pfeizer) and phentolamine(Ciba-Geigy(Japan)). All other reagents were of the highest grade available.

RESULTS AND DISCUSSION

Fluorescence photographs of quin 2-loaded VSMCs in normal PSS and in normal PSS containing 10^{-5}M NE for 2 min are shown in Figure 1 (A) and Figure

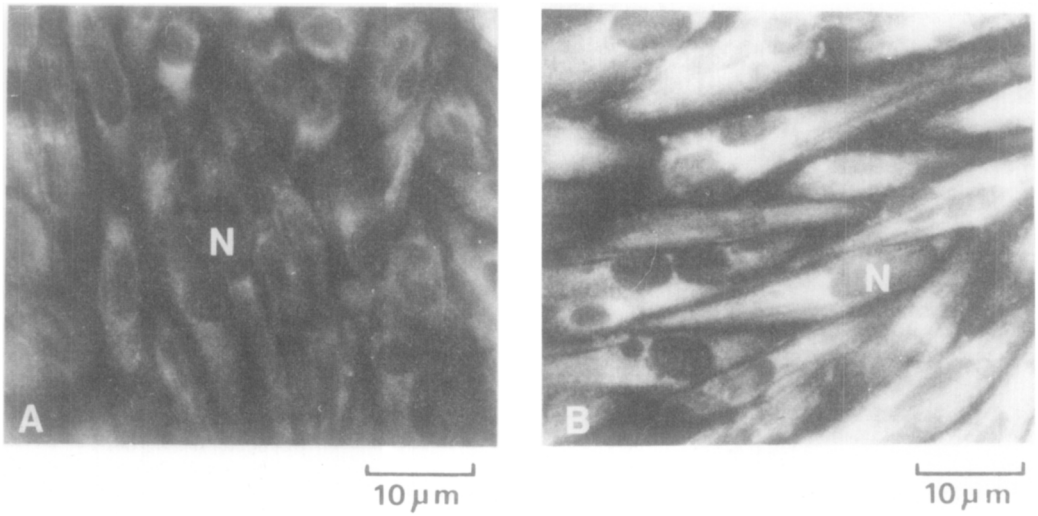

Figure 1. (A), fluorescence photomicrograph of VSMCs loaded with quin 2 in normal PSS. Marked fluorescence was observed in the cytosol, but not in the nucleus (N), myofilaments or in the extracellular space. The fluorescence intensity was measured in a small circle area (<1μm^2) 3μm apart from the nucleus in the cytosol. (B), fluorescence photomicrograph of quin 2-loaded cells 2 min after the addition of 10^{-5}M NE. Note that fluorescence increased in response to the application of 10^{-5}M NE. In order to avoid the photobleaching effect, which differed in individual cells, the microfluorometry was carried out within 2 seconds after exposure of the cells to the excitation light. To obtain a photograph with a sharp contrast, the time of exposure to the excitation light was 15 seconds in these two photographs, (A) and (B). Kodak Tri-X film (ASA 400, monochrome) was used to take the quin 2 fluorescence photographs.

1 (B), respectively. Marked fluorescence was observed almost exclusively in the cytosol, and the extracellular space, nuclei, and myofilaments stained negatively. During the course of exposure to NE with a concentration range between 10^{-9} and 10^{-4} M, neither contraction nor swelling of cells was noted, as determined by a phase contrast microscopy at x400.

Figure 2(A) shows one of the typical time courses of the response of fluorescence change of the cytosolic spot observed when VSMCs were exposed to 10^{-5}M NE, either in normal PSS or in Ca^{2+} free PSS containing 2mM EGTA. When VSMCs were exposed to Ca^{2+} free PSS containing 2mM EGTA prior to the application of NE, $[Ca^{2+}]i$ decreased gradually and reached a steady state level in 5 min. This level remained unchanged for at least 60 minutes, as previously noted (12,13). Both in the presence (1.0mM Ca^{2+}) and the absence of extracellular Ca^{2+}, NE caused a transient elevation in the fluorescence, with

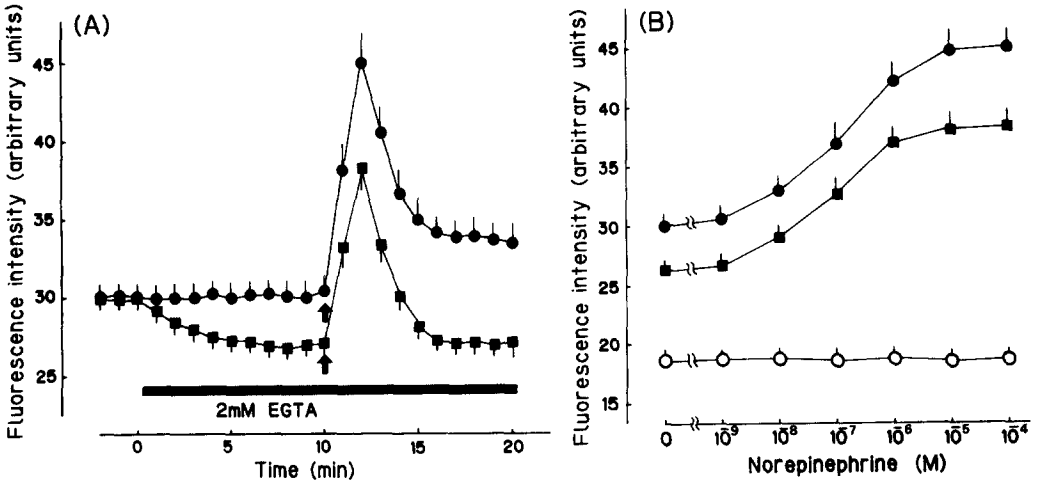

Figure 2. (A), a typical example of the effect of 10^{-5}M NE on fluorescence signal in VSMCs in normal PSS(●) and in Ca^{2+} free PSS containing 2 mM EGTA (■). NE was added at the time indicated by the arrow. Data are mean±SD of 8 cells. (B), dose-dependent effect of the peak levels of transient $[Ca^{2+}]i$ elevations in quin 2-loaded cells induced by NE in normal PSS(●) and in Ca^{2+} free PSS containing 2mM EGTA(■). Effect of various concentrations of NE on $[Ca^{2+}]i$ of quin 2-unloaded cells in normal PSS(○). Data are mean±SD of 5 experiments, and the number of cells counted in each experiment was 8. There was no change in the fluorescence levels of quin 2-unloaded cells(○), indicating that optical artifacts, if any, produced by possible contraction or swelling of cells by NE were negligible.

a similar time course. The fluorescence reached a maximal level at 2 min, and then declined to a fairly steady-state level within 6 min, despite the continuous application of NE. The peak values of the transient elevation (at 2 min) induced by NE were dose-dependent in the range between 10^{-8} and 10^{-5}M ($p < 0.05$ by an analysis of variance), both in the presence and absence of extracellular Ca^{2+} (Fig. 2B). The finding that NE induces a transient and dose-dependent rise in cytosolic Ca^{2+} concentrations in the absence of extracellular Ca^{2+} indicates that NE probably mediates a release of Ca^{2+} from intracellular stores. This proposal is compatible with recent observations that the contractile responses of various vascular tissues to NE are transient in the Ca^{2+} free solution and that the transient contraction is induced by a release of cellular Ca^{2+} (15-17).

As shown in Fig. 2(A), fluorescence levels declined to steady state pre-exposure levels in 6 min in the absence of extracellular Ca^{2+}. However, in the presence of extracellular Ca^{2+}, although the fluorescence declined to

steady state levels with 6 min of NE application, these levels were significantly higher than the pre-exposure ones. These findings have to be given due attention, because a similar phenomenon was noted in smooth muscle preparations of the ferret portal vein (11). These authors recorded intracellular Ca^{2+} levels using a bioluminescent protein, aequorin, during smooth muscle contraction, and found that the elevation of $[Ca^{2+}]i$-induced by phenylephrine was separated into two components; $[Ca^{2+}]i$ had risen rapidly to a peak and then fell rapidly to a lower level (component I) from which it declined more slowly, remaining above the base-line levels (component II), while contraction of smooth muscle preparations was maintained in the presence of phenylephrine. Also in the rat aorta, the contraction induced by NE could be separated into phasic (or fast) and tonic (or slow) components of the response, which reflect a release of intracellularly stored Ca^{2+} and an influx of extracellular Ca^{2+}, respectively (5,18,19). This conclusion is based on the following observations; 1) the tonic component of the contraction induced by NE did not occur in the absence of extracellular Ca^{2+}, while the phasic component was not affected, and 2) the tonic component was effectively inhibited by Ca^{2+} antagonists, while the phasic component was resistant. A similar conclusion may be drawn for our data, because the first transient component of $[Ca^{2+}]i$ change induced by NE was observed both in the presence and absence of extracellular Ca^{2+}, with a similar time course, while the second steady component was not detected in the absence of extracellular Ca^{2+}. Thus, the first transient component may mainly reflect a release of intracellular Ca^{2+} and the second one may be due to an influx of extracellular Ca^{2+}, in the presence of extracellular Ca^{2+}.

In order to identify the contributions of alpha-1 or alpha-2 adrenoceptor subtypes to a transient $[Ca^{2+}]i$ elevation by NE in cultured vascular smooth muscle cells of rat aorta, the effect of the three alpha-adrenergic antagonists on peak levels of $[Ca^{2+}]i$ elevation induced by application of 10^{-5}M NE for 2 min was studied in normal PSS and in Ca^{2+} free PSS containing 2mM EGTA. Prazosin, yohimbine and phentolamine were the selective alpha-1,

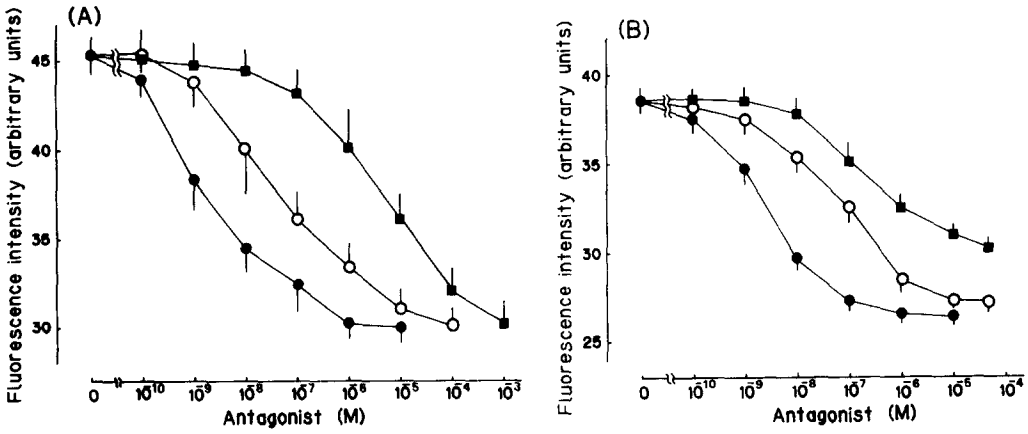

Figure 3. The effect of various concentrations of alpha-adrenergic antagonists on peak levels of cytosolic Ca^{2+} elevation induced by 10^{-5}M NE; prazosin (●), phentolamine (○) and yohimbine (■). The experiments were performed either in normal PSS (A) or in Ca^{2+} free PSS containing 2mM EGTA (B). Microfluorometry was carried out 2 minutes after NE and antagonists were simultaneously applied to the cells. Data are mean±SD of 5 experiments, and the number of cells counted in each experiment was 8.

selective alpha-2 and the non-selective alpha-antagonists, respectively (3,20). These antagonists, dose-dependently reduced the peak values of transient fluorescence elevations by 10^{-5}M NE, and the order of potency of these antagonists was prazosin > phentolamine > yohimbine, both in normal PSS (Fig. 3(A)) and Ca^{2+} free PSS containing 2mM EGTA (Fig. 3(B)). This order of antagonist effect is the same one for antagonists to an alpha-1 subtype of adrenoceptor. Thus, it is suggested that a transient $[Ca^{2+}]i$ elevation by application of NE for 2 min, both in the presence and absence of extracellular Ca^{2+}, correlates to an alpha-1 receptor subtype, in rat aorta cultured VSMCs. In the rat aorta, alpha-1 activation leads to a release of Ca^{2+} from intracellular stores, while alpha-2 activation induces an influx of extracellular Ca^{2+} (4,5). Thus, we suggest that, irrespective of the presence or absence of extracellular Ca^{2+}, NE mainly activates the alpha-1 adrenoceptor to induce a release of Ca^{2+} from the intracellular stores. These events lead to a transient and dose-dependent elevation of cytosolic Ca^{2+} in rat aortic VSMCs in primary culture.

ACKNOWLEDGMENTS

We thank M. Hasegawa for help with the experiments, N. Hayashi and N. Misaka for secretarial services, and M. Ohara for comments on the manuscript. This work was supported in part by grants-in-Aid for Scientific Research

(No.58570395 and 59440044) from the Ministry of the Education, Science and Culture, Japan.

REFERENCES

1. Hicks P.E., Nahorski S.R. and Cook N. (1983) Clin. Exp. Hypertension A5(3), 401-427.
2. Rutledge A., Swamy V.C. and Triggle D.J. (1984) Br. J. Pharmacol. 83, 103-111.
3. Langer S.Z. and Hicks P.E. (1984) J. Cardiovasc. Pharmacol. 6(suppl.4) S547-558.
4. Van Meel J.C.A., De Jonge A., Kalkman H.O., Wilffert B., Timmermans P. B.M.W.M. and Van Zwieten P.A. (1981) Eur. J. Pharmacol. 69, 205-208.
5. Godfraind T., Miller R.C. and Socrates Lima J. (1982) Br. J. Pharmacol. 77, 597-604.
6. De Mey J. and Vanhoutte P.M. (1981) Circ. Res. 48, 875-884.
7. Vanhoutte P.M. (1982) J. Cardiovasc. Pharmacol. 4(suppl 1), S91-96.
8. Bobik A. (1982) Life Sci. 30, 219-228.
9. Descombes J.J. and Stoclet J.C. (1985) Naunyn-Schmiedeberg's Arch Pharmacol. 329, 282-288.
10. Vanhoutte P.M. and Rimele T.J. (1982) J. Cardiovasc. Pharmacol. 4(suppl 3), S280-286.
11. Morgan J.P. and Morgan K.G. (1984) J. Physiol. 351, 155-167.
12. Kobayashi S., Kanaide H. and Nakamura M. (1985) Science 229, 553-556.
13. Kobayashi S., Kanaide H. and Nakamura M. (1985) Biochem. Biophys. Res. Comm. 129, 877-884.
14. Yamamoto H., Kanaide H. and Nakamura M. (1983) Br. J. Exp. Pathol. 64, 156-165.
15. Janssens W. and Verhaeghe R. (1984) J. Physiol. 347, 525-532.
16. Rooke T.W., Rimele T.J. and Vanhoutte P.M. (1984) Am. J. Physiol. 247, H259-H263.
17. Casteels R., Raeymaekers L., Suzuki H. and Van Eldere J. (1981) Pflugers Arch. 392, 139-145.
18. Downig O.A., Wilson K.A. and Wilson V.G. (1983) Br. J. Pharmacol. 80, 315-322.
19. Scarborough N.L. and Carrier G.O. (1984) J. Pharmacol. Exp. Ther. 231, 597-602.
20. Kobinger W. and Pichler L. (1981) Eur. J. Pharmacol. 73, 313-321.