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EFFECTS OF VALINOMYCIN ON CALCIUM MOBILIZATION IN VASCULAR SMOOTH MUSCLE CELLS INDUCED BY ANGIOTENSIN II

E. KOH, S. MORIMOTO, S. TAKAMOTO, R. MORITA, S. KIM, T. HIRONAKA, T. NABATA, T. ONISHI and T. OGIHARA

Department of Geriatric Medicine,
Osaka University Medical School, Osaka 553, Japan

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The effect of the specific potassium (K^+) ionophore valinomycin on increase in intracellular calcium concentration ($[Ca^{2+}]i$) was studied in vascular smooth muscle cells (VSMC). Valinomycin at more than 10^{-9} M dose-dependently suppressed phasic increase in $[Ca^{2+}]i$ in VSMC induced by angiotensin II (AII) in both control and Ca^{2+}-free solution, indicating that it suppressed the release of Ca^{2+} from intracellular Ca^{2+} stores. Nicorandil and cromakalim, which are both K^+ channel openers, also suppressed the increases in $[Ca^{2+}]i$ induced by AII in the Ca^{2+} free solution. However, valinomycin did not suppress AII-induced production of inositol 1,4,5-trisphosphate (IP_3), which is known to mediate the release of Ca^{2+}. These results indicate that decrease of intracellular K^+ induced by valinomycin suppressed the release of Ca^{2+} from intracellular Ca^{2+} stores induced by IP_3.

The concentration of intracellular calcium ($[Ca^{2+}]i$) is known to be about 10,000 times less than that of extracellular Ca^{2+} and to play a key role in contraction of vascular smooth muscle (1). On the other hand, the concentration of intracellular potassium ($[K^+]i$) is know to be about 20 times more than that in the extracellular space (3). Increase of extracellular potassium (K^+) increases the influx of calcium and causes contraction of vascular smooth muscle (4), but the effect of $[K^+]i$ on intracellular Ca^{2+} mobilization has not been clarified. In this work, we studied the effect of a K^+ specific ionophore, valinomycin (5) on

Abbreviations used: K^+, potassium; VSMC, vascular smooth muscle cells; $[Ca^{2+}]i$, intracellular calcium concentration; AII, angiotensin II; IP_3, inositol, 1,4,5-trisphosphate; $[K^+]i$, intracellular potassium concentration.

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intracellular Ca^{2+} mobilization in VSMC induced by a strong vasoconstrictor, angiotensin II (AII), which is known to induce opening of Ca^{2+} channels causing influx of Ca^{2+} and the production of inositol 1,4,5-trisphosphate (IP_3), which causes release of Ca^{2+} from intracellular Ca^{2+} stores (2).

MATERIALS AND METHODS

Materials

AII, valinomycin and monencin were purchased from Sigma Chemical Co. (St. Louis. MO). Fura 2 acetoxymethyl ester (fura 2 AM) was purchased from Dojin Co. (Kumamoto, Japan) and ionomycin from Calbiochem Behring (La. Jolla, CA). Nicorandil was a gift from Chugai Pharmaceutical Co. (Tokyo, Japan), and cromakalim was from Beecham Pharmaceutical Co. (Harlow, UK). An IP_3 assay kit (TRK 1000) was purchased from Amersham Int. Ltd. (Bucks, UK).

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

$[Ca^{2+}]i$ was measured as described before (6). Briefly, VSMC were prepared from the aorta of female Wistar rats by the explant method and cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. They were then preincubated in medium without serum for 24h, incubated with 2 μM fura 2 AM for 40 min and harvested with 500 IU/ml of disperse. The VSMC were then washed twice and suspended in ice cold Earle's balanced salt solution [116 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 5.5 mM glucose, 26 mM $NaHCO_3$ and 10 mM HEPES (pH 7.3)] containing 1mg/ml bovine serum albumin (BSA). The cells (1 X 10^6 /ml) were then placed in a 1 cm^2 quartz cuvette and warmed at 37°C for 10 min. Then their intensity of fluorescence was measured in a Hitachi Fluorescence Spectrophotometer F-3000 (Tokyo, Japan) with excitation and emission wavelengths of 340 and 495 nm, respectively. The composition of Ca^{2+}-free solution was the same except that $CaCl_2$ was omitted and 2 mM ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) was added. $[Ca^{2+}]i$ was calculated by the method of Grynklewicz et al. (7).

Measurement of IP_3 formation in VSMC

A suspension of VSMC in cold Earle's balanced salt solution containing 1 mg/ml BSA were warmed at 37°C for 10 min. Then 0.01 volume of 10^{-6} M AII was added and the mixture was promptly vortexed. After 10 seconds 0.2 volume of ice-cold 20% perchloric acid was added and the mixture was kept on ice for 20 min. Protein was removed by centrifugation at 2000 x g for 15 min at 4°C, and the supernatant was titrated to pH 7.5 with 10 N KOH and kept on ice for 60 min. After removal of insoluble $KClO_4$ by centrifugation, IP_3 was measured with a radioimmunoassay kit (TRK. 1000).

RESULTS

Effects of valinomycin on increase in $[Ca^{2+}]i$ of VSMC induced by AII

Figure 1 illustrates the effect of pretreatment of VSMC with valinomycin for 1 min on the increase in $[Ca^{2+}]i$ evoked by 10^{-8} M AII. In the presence

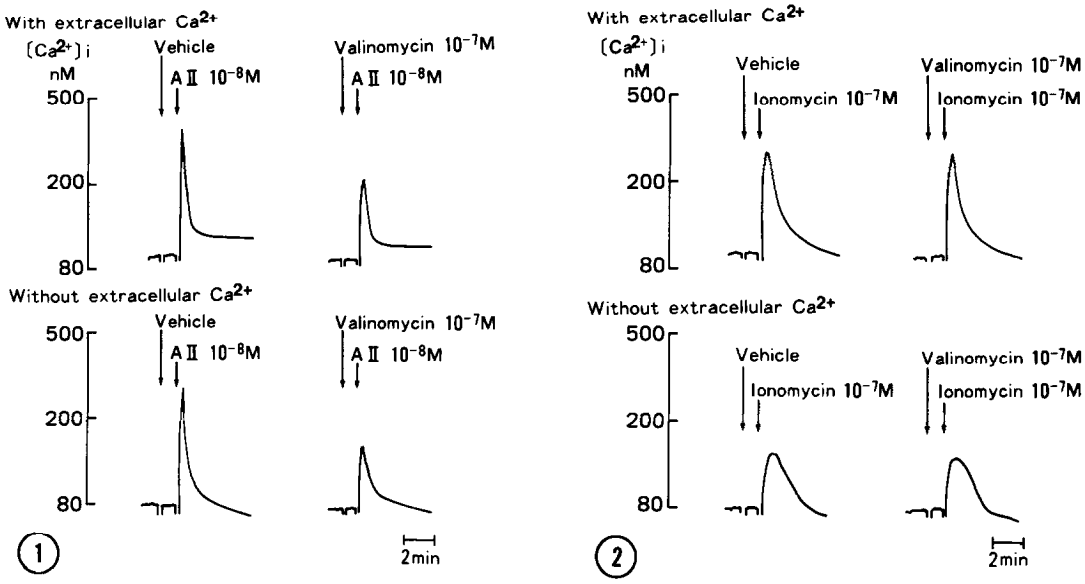


Fig. 1. Effects of pretreatment of VSMC with valinomycin on the increases in $[Ca^{2+}]i$ induced by angiotensin II (AII) in control solution and Ca^{2+}-free solution. Arrows indicate the times of addition of valinomycin and AII. Traces were obtained with portions of the same cell suspension in a single experiment.

Fig. 2. Effects of pretreatment of VSMC with valinomycin on the increases in $[Ca^{2+}]i$ induced by ionomycin in control solution and Ca^{2+}-free solution. Arrows indicate the times of addition of valinomycin and ionomycin.

of 1.8 mM extracellular Ca^{2+} (control solution), addition of AII caused phasic and then tonic increase in $[Ca^{2+}]i$, whereas in the absence of extracellular Ca^{2+} (Ca^{2+}-free solution) it evoked only phasic increase in $[Ca^{2+}]i$. Valinomycin had no effect on the basal level of $[Ca^{2+}]i$ in VSMC, but at concentrations of more than 10^{-9} M it suppressed the phasic increase in $[Ca^{2+}]i$ induced by AII dose-dependently in both control and Ca^{2+}-free solution (Fig. 1 and 3).

Effects of valinomycin on increase in $[Ca^{2+}]i$ of VSMC induced by ionomycin

Ionomycin, a calcium ionophore, at a concentration of 10^{-7} M also evoked phasic increase in $[Ca^{2+}]i$ of VSMC in both control and Ca^{2+}-free solution. Valinomycin at concentrations of up to 10^{-5} M did not suppress the phasic increase in $[Ca^{2+}]i$ induced by ionomycin either in control or Ca^{2+}-free solution (Fig. 2 and 3).

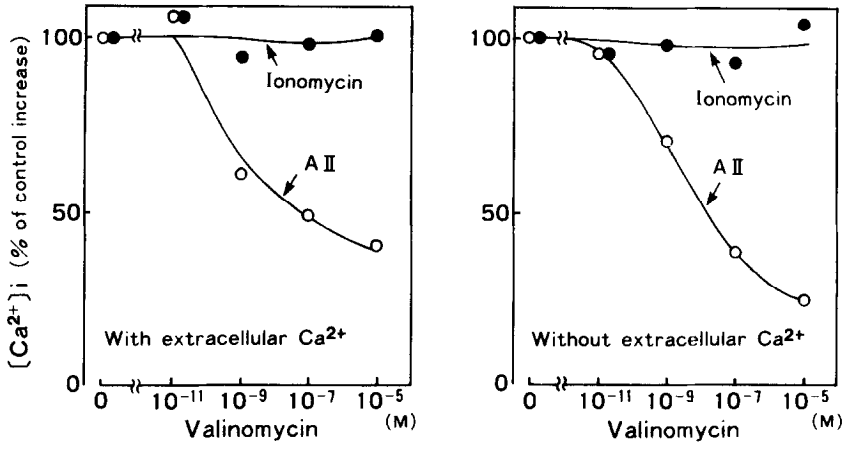


Fig. 3. Dose-dependent effects of valinomycin on the phasic increases in $[Ca^{2+}]i$ induced by 10^{-8} M AII (○) and 10^{-7} M ionomycin (●) in control solution (left panel) and Ca^{2+}-free solution (right panel). VSMC were pretreated with the indicated concentrations of valinomycin for 1 min. The basal $[Ca^{2+}]i$ was subtracted from the maximal $[Ca^{2+}]i$ observed after stimulation with 10^{-8} M AII or 10^{-7} M ionomycin. The phasic increases in $[Ca^{2+}]i$ evoked by AII and ionomycin are expressed as percentages of those observed in VSMC without valinomycin treatment. Values are means for triplicate determinations.

Effects of nicorandil, cromakalim and monencin on $[Ca^{2+}]i$ of VSMC

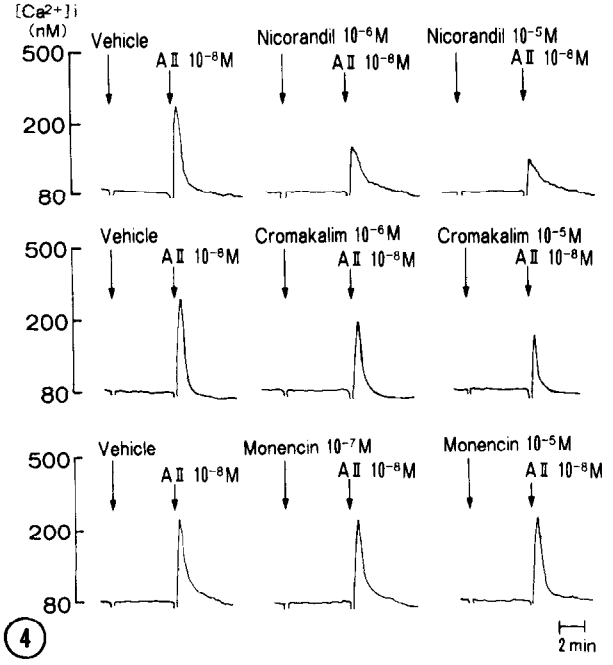
Pretreatment of VSMC with either nicorandil or cromakalim, which are both potassium channel openers (8,9), at concentrations of 10^{-6} M or more dose-dependently suppressed the phasic increase in $[Ca^{2+}]i$ induced by 10^{-8} M AII in Ca^{2+}-free solution (Fig. 4). Neither nicorandil nor cromakalim suppressed the phasic increase in $[Ca^{2+}]i$ induced by 10^{-7} M ionomycin (data not shown). Monencin, a sodium ionophore (5) also had not effect on the increase in $[Ca^{2+}]i$ induced by 10^{-8} M AII (Fig. 4).

Effects of valinomycin on the production of IP_3 induced by AII in VSMC

Figure 5 illustrates the effects of valinomycin on the production of IP_3 in VSMC induced by AII. AII at 10^{-8} M increased the concentration of IP_3 in VSMC to about 3 times the basal level, and this increase was not affected by valinomycin at concentrations of 10^{-7} M and 10^{-9} M.

DISCUSSION

Valinomycin, an ionophore that shows very much greater selectivity for K^+ than for Na^+ or Ca^{2+}, is known to increase the permeability of the



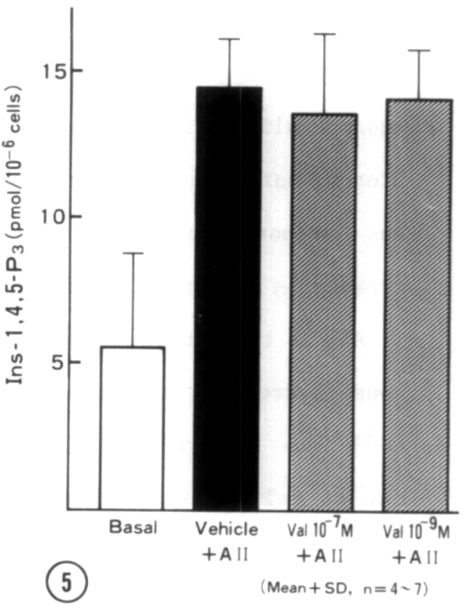


Fig. 4. Effects of pretreatment of VSMC with nicorandil, cromakalim and monencin on increase in $[Ca^{2+}]i$ induced by 10^{-8} M AII in the Ca^{2+}-free solution. Arrows indicate the times of addition of nicorandil, cromakalim, monencin and AII.

Fig. 5. Effects of pretreatment of VSMC with valinomycin for 1 min on the production of inositol 1,4,5-trisphosphate (IP_3) induced by 10^{-8} M AII in control solution. Values were obtained with portions of the same cell suspension in each experiment. The reactions were stopped 10 seconds after addition of AII.

plasma membrane to K^+ (5). Nicorandil and cromakalim are also reported to increase the K^+ conductance of the plasma membrane and dilate vascular smooth muscle (8,9). Because, the $[K^+]i$ is about 20 times the extracellular K^+ concentration (3), all three compounds are supposed to decrease $[K^+]i$ resulting in hyperpolarization of the plasma membrane in living cells in culture (5,8,9,11). In studies on fragmented sarcoplasmic reticulum from skeletal muscle, intracellular K^+ as well as Na^{2+} at their physiological concentrations were found to stimulate Ca^{2+}-ATPase dependent Ca^{2+} uptake (11), and valinomycin itself decreased ATPase activity by a mechanism independently of its effect on K^+ conductance (10). If K^+-ion and valinomycin itself have similar effects on the sarcoplasmic reticulum of living VSMC, valinomycin should induce increase in $[Ca^{2+}]i$. However, in our

conditions, valinomycin at concentrations of more than 10^{-9} M suppressed the phasic increase of $[Ca^{2+}]i$ in VSMC induced by AII in Ca^{2+}-free solution, suggesting that it that decrease in $[K^+]i$ induced by valinomycin and/or valinomycin itself suppressed AII-induced release of Ca^{2+} from Ca^{2+} stores, mainly in the sarcoplasmic reticulum of the VSMC. This supeculation was supported by the finding that the K^+ channel openers nicorandil and cromakalim also suppressed the increase in $[Ca^{2+}]i$ induced by AII.

AII is thought to bind to a specific receptor on the plasma membrane and cause hydrolysis of phosphatidyl inositol to produce IP_3, which releases Ca^{2+} from intracellular Ca^{2+} stores (2). On the other hand, ionomycin causes release of Ca^{2+} directly, independently of IP_3 (12). In our experiment, valinomycin did suppressed the increase of $[Ca^{2+}]i$ induced by AII but not that induced by ionomycin. However, valinomycin did not have any effect on the production of IP_3 induced by AII. These data indicate that the decrease of $[K^+]i$, and/or valinomycin itself suppressed the effects of IP_3 on the release of Ca^{2+} from intracellular Ca^{2+} stores, not the production of IP_3. The intracellular sodium concentration may not be involved appreciably in Ca^{2+} mobilization induced by AII, as monencin did not affect the increase of $[Ca^{2+}]i$ induced by AII. Muallem *et al.* (13) reported that the IP_3-induced release of ^{45}Ca from rat liver microsomes depended on the external K^+ concentration, suggesting that IP_3 causes efflux of Ca^{2+} from intracellular Ca^{2+} stores in exchange for K^+ influx. Our data are partially consistent with these results of Muallem *et al.* (13). Further investigations are needed on changes in the intracellular electrical potential and their effects on intracellular Ca^{2+} mobilization to elucidate the precise mechanism of the effect valinomycin in suppressing mobilization of intracellular Ca^{2+}.

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