Calcium-Induced Vasodilation Due to Increase in Nitric Oxide Formation in the Vascular Bed of Rabbit Ear Preparation

Naoyuki Takasugi

Department of Pharmacology, Hiroshima University School of Medicine, Kasumi, Minami-ku, Hiroshima 734, Japan

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ABSTRACT—Participation of calcium-induced vasodilation (due to an increase in synthesized nitric oxide (NO) content in endothelial cells) in the arterio-venous circulation, including the vascular bed was investigated by the vessel perfusion method in the isolated rabbit ear preparation. The perfusion medium used was a tris-buffered solution. When CaCl₂ (6.25, 12.5 and 25 mg) was injected in the perfused vessel of the rabbit ear preparation, dose-dependent vasocontraction was observed when vascular tone was kept at a normal level. However, CaCl₂ dose-dependently induced vasodilation of the vessel when it was continuously contracted by norepinephrine (1.2 × 10⁻⁷ M). This calcium-induced vasodilation was inhibited in the presence of NG-nitro-L-arginine (5 × 10⁻⁵ M), a selective inhibitor of NO synthesis, and methylene blue, a guanylate cyclase inhibitor, although it was rarely affected by indomethacin (10⁻⁵ M), a cyclooxygenase inhibitor. Calcium-induced vasodilation was also obtained in the in situ circulation containing vascular bed, and this suggests that the vasodilation was due to a Ca²⁺-induced increase in the synthesis of NO derived from endothelial cells.

Keywords: Vasodilation (Ca²⁺-induced), Nitric oxide, NG-Nitro-L-arginine, Vascular bed, Ear (rabbit)

It is well-documented that an elevation of intracellular Ca²⁺ concentration forms the calcium-calmodulin complex that activates myosin light chain kinase and induces vasoconstriction following the phosphorylation of myosin (1, 2). However, it has been reported that the increases in external Ca²⁺ produced vasorelaxation in KCl- and epinephrine-contracted helical strips of cerebral arteries of dog and rabbit aorta, respectively (3, 4). Recent work using isolated vessel preparations has suggested that the Ca²⁺-induced vasorelaxation is related to endothelium-derived relaxing factors (EDRFs) and nitric oxide (NO) derived from the endothelium as this effect was blocked by methylene blue, a guanylate cyclase inhibitor (5–7). However, the afore-mentioned findings (5–7) on calcium-induced vasorelaxation were obtained by using isolated arteries in vitro. The fate of high Ca²⁺ contents in the arterio-venous circulation (including vascular bed) seemed unclear. More recently, it was found that the release of EDRF was greater in veins than the corresponding arteries (8). In addition, effects of endothelin-1 on the venous vessels were found to be more potent than those on the arterial vessels of the vascular network in the isolated rabbit ear preparation (9). Thus, EDRF release from the endothelium in veins differs from that in arteries.

Therefore, the present study was performed to examine the effects of Ca²⁺ on the arterio-venous circulation including the vascular bed using vessel perfusion methods in isolated rabbit ear preparation and further to confirm that the possible vasodilation by Ca²⁺ was due to the increased NO.

MATERIALS AND METHODS

Adult healthy male rabbits (New Zealand White) weighing 2–3.5 kg, were used. The animals were anesthetized with pentobarbital-Na (30 mg/kg, i.p.), and then a polyethylene cannula was inserted into the central artery of the ear. The vessel was perfused at a constant pressure of 55 cmH₂O with the nutrition perfusate, which was aerated with 100% oxygen and kept at 37°C. The perfusate was composed of the following: 154 mM NaCl, 5.6 mM KCl, 2 mM CaCl₂, 12.7 mM tris (hydroxymethyl) aminomethane, and 5.6 mM glucose, adjusted to pH 7.4 with HCl. Since normal plasma contains 2 mM Ca²⁺ (10), 2 mM CaCl₂ was included in the present perfusate. When the ear lost color following administration of perfusion fluid, it
was removed. The isolated ear preparation was set on a plastic plate inclined at 45° to a horizontal plane and perfused at a constant pressure of 55 cmH₂O. The vasoconstricting and vasodilating drug responses were evaluated by the Krawkow-Pissemski method; that is, counting the number of drops of perfusion from the vessel through the cannula. The number of drops was continuously recorded on a recticorder (RJG4022, Nihon Kohden, Tokyo). Drugs and CaCl₂ were injected into the rubber tube connected to the upper portion of the cannula in amounts of 0.1 - 0.2 ml. A 2-l Mariotte bottle was prepared for continuous administration of drugs (norepinephrine, N²-nitro-L-arginine (L-NNA), methylene blue or indomethacin). The effects of isoproterenol and high CaCl₂ concentration in the presence and absence of drugs were examined when a constant perfusion drop rate was obtained, with the perfusate alone or containing norepinephrine (1.2 × 10⁻⁷ M), for over 30 min. Similarly, the perfusate containing L-NNA, methylene blue, or indomethacin was perfused through the vessel for over 30 min before injection of CaCl₂. The drugs used were as follows: l-isoproterenol hydrochloride (Nikken Chem. Co., Tokyo), phentolamine hydrochloride (Ciba-Geigy Co., Basel, Switzerland), pindolol (Sigma, St. Louis, MO, USA), d,l-norepinephrine hydrochloride (Sankyo, Tokyo), L-NNA (Sigma), methylene blue (Sigma), indomethacin (Sigma), and trizma base (Sigma). Indomethacin was dissolved in ethanol solution (10⁻² M). The stock solution was stored at 4°C and diluted to 10⁻⁷ M with norepinephrine (1.2 × 10⁻⁷ M). This perfusate contained 0.01% ethanol (final concentration), and this did not affect vascular tone in the present preparation.

All data are given as the mean±S.E. Statistical significance was determined by the paired Student’s t-test. P values of less than 0.05 were considered to indicate a statistically significant difference.

RESULTS

Effects of isoproterenol and Ca²⁺

Isoproterenol (0.5 - 2.0 μg) produced vasodilation and vasoconstriction in the presence of phentolamine (1.3 × 10⁻⁵ M) and pindolol (2 × 10⁻⁶ M), respectively. When CaCl₂ at doses of 6.25, 12.5, and 25 mg was injected into the vessel, the number of perfusion drops from the ear vessel decreased in a dose-dependent manner, indicating vasoconstriction was elicited. However, when CaCl₂ was injected to a vessel contracted by continuous perfusion of norepinephrine (1.2 × 10⁻⁷ M), a dose-dependent increase in perfusion drops of vasorelaxation was observed. This vasodilation became maximum at 2, 5 and 20 min post-administration and lasted for 30–50, 30–60 and 30–90 min with CaCl₂ at doses of 6.25, 12.5 and 25 mg, respectively. A typical time course of Ca²⁺-vasodilation is shown in Fig. 1.

Effects of L-NNA on calcium-induced vasodilation

The ear vessel was perfused with the tris-buffered nutrition perfusate containing L-NNA (5 × 10⁻⁵ M), a NO-synthesis inhibitor, and norepinephrine. The concentration of the perfusate was reduced to 5.9 × 10⁻⁸ M to produce a vasoconstriction effect equipotent to that induced by norepinephrine alone (1.2 × 10⁻⁷ M) since L-NNA (5 × 10⁻⁵ M) alone produced a vasoconstriction that was 10% of the one produced by norepinephrine (1.2 × 10⁻⁷ M). Then CaCl₂ at doses of 6.25, 12.5 and 25 mg was given successively at 20–50 min intervals 30 min later with continuous perfusion of L-NNA and norepinephrine. A transient decrease in the drop count was seen in 2 of 4 vessels 2–5 min after injection of CaCl₂ at 12.5 and 25 mg. Calcium-induced increases in the drop count were inhibited in all 4 vessels tested. The means of the accumulated drop count at 5, 10, 15 and 20 min post-injection of 12.5 mg CaCl₂ in 4 preparations were significantly suppressed in
the L-NNA-treated vessels compared with the non-treated samples (Fig. 2A). The means of the accumulated drop counts for 20 min following injection of 6.25, 12.5 and 25 mg of CaCl₂ were also significantly decreased in the presence of L-NNA (Fig. 2B).

**Effects of methylene blue on calcium-induced vasodilation**

When methylene blue (10⁻⁵ M) was perfused concomitantly with norepinephrine (5.9 x 10⁻⁸ M) at a rate similar to L-NNA perfusion, the CaCl₂-induced increases in drop counts were also inhibited in all 3 vessels examined.

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**Fig. 2.** Inhibitory effect of N°-nitro-L-arginine (L-NNA) on CaCl₂-induced vasodilation in the vascular bed of isolated rabbit ear. A: Effects of CaCl₂ (12.5 mg) injection on the vascular bed continuously contracted by norepinephrine in the presence and absence of L-NNA (5 x 10⁻⁵ M). Total numbers of drop counts at 5, 10, 15, and 20 min post-injection of CaCl₂ were plotted at the corresponding time intervals in the abscissas. B: Effects of L-NNA on the change of accumulated perfusion drop counts during the 20 min post-CaCl₂ injection period at the respective doses of 6.25, 12.5 and 25 mg. Filled symbols represent the presence of norepinephrine (1.2 x 10⁻⁷ M). Open symbols indicate the presence of norepinephrine (5.9 x 10⁻⁸ M) and L-NNA (5 x 10⁻⁵ M). Each value and vertical bar is a mean ± S.E. (n=4). Lack of vertical bars in the values indicates that S.E. was negligibly small. *P<0.05, **P<0.02 and ***P<0.01, statistically significant difference compared to the absence of L-NNA.

**Fig. 3.** Inhibitory effect of methylene blue on CaCl₂-induced vasodilation in the vascular bed of isolated rabbit ear. A: Effects of CaCl₂ (12.5 mg) injection on the vascular bed previously contracted by norepinephrine in the presence and absence of methylene blue (10⁻⁵ M) were examined. Methylene blue was continuously perfused for 30 min before CaCl₂ injection. Total numbers of drop counts at 5, 10, 15, and 20 min post-injection of CaCl₂ were plotted at 5, 10, 15, and 20 min in the abscissas. B: Effects of methylene blue on the accumulated perfusion drops during the 20 min post-CaCl₂ injection period at the respective doses of 6.25, 12.5 and 25 mg. Filled symbols represent the presence of norepinephrine (1.2 x 10⁻⁷ M). Open symbols indicate the presence of norepinephrine (5.9 x 10⁻⁸ M) and methylene blue (10⁻⁵ M). Each value and vertical bar is a mean ± S.E. (n=3). *P<0.05, **P<0.02 and ***P<0.01, statistically significant differences compared with CaCl₂ in the presence of norepinephrine.
A transient decrease in drop count was also seen 5-15 min after injection of CaCl\textsubscript{2} in 2 of 3 vessels (observed in the presence of L-NNA). The means of the drop counts accumulated during the 5, 10, 15 and 20 min after CaCl\textsubscript{2} injection at the dose of 12.5 mg in 3 preparations were significantly reduced in the methylene blue-treated vessels compared with the non-treated vessels (Fig. 3A). The means of the accumulated perfusion drop counts during the 20 min period by injection of CaCl\textsubscript{2} at 6.25, 12.5 and 25 mg were significantly inhibited in the presence of methylene blue (Fig. 3B).

**Effects of indomethacin on calcium-induced vasodilation**

The vascular bed was perfused for at least 30 min with a solution containing indomethacin (10\textsuperscript{-5} M) and norepinephrine (1.2 \times 10\textsuperscript{-7} M). The treatment with such a dose produced a vasoconstriction equipotent to that obtained with norepinephrine alone (1.2 \times 10\textsuperscript{-7} M). Under such conditions, CaCl\textsubscript{2} (6.25, 12.5 and 25 mg) was administered to the vessel. However, Ca\textsuperscript{2+}-induced vasodilation was not affected in the presence of indomethacin. Figure 4A shows the failure of the effects by indomethacin to influence Ca\textsuperscript{2+}-induced vasodilation. In the presence of indomethacin, the means of the accumulated perfusion drop counts during the 20-min period after administration with CaCl\textsubscript{2} at 6.25, 12.5 and 25 mg were not significantly altered compared with those without indomethacin (Fig. 4B).

**DISCUSSION**

The isolated rabbit ear preparation with a vascular network is a unique model developed by Krawkow and Pissenski (11). This method is suitable for examining the effects of Ca\textsuperscript{2+} on a circulation containing a vascular bed closely resembling that in vivo. When this preparation was continuously perfused with physiological solution, isoproterenol produced \(\alpha\) and \(\beta\)-adrenoceptors-mediated vasoconstriction and vasodilation, respectively (data not shown). This indicates that such a preparation may cause both contraction and dilation to occur.

Injection of CaCl\textsubscript{2} produced a dose-dependent contraction of the normal untreated vessel, as expected (data not shown). However, when CaCl\textsubscript{2} was applied to the vessel, which had been previously contracted by continuous perfusion of norepinephrine, vasodilation was obtained in a dose-dependent manner. This dual effect of CaCl\textsubscript{2} was first observed by Toda (4) using a rabbit aorta ring preparation. Calcium-induced vasodilation has been detected in the aorta ring preparation (12) and coronary artery (5), which had been previously contracted by a high concentration of KCl in the absence and presence of a Ca\textsuperscript{2+} antagonist, respectively. The vasodilation obtained in the present study using an in vivo preparation was inhibited by pretreatment with L-NNA, known to be an inhibitor of NO synthesis (13), and methylene blue, a guanylate cyclase inhibitor (14). Taken together with the findings by other investigators (5, 12) that calcium-induced vasodila-
tion was not detected in the endothelium-removed preparations, my results suggest that the calcium-induced vasodilation is, at least in part, due to synthesis and/or release of NO in the endothelium by calcium, since an increase in Ca$^{2+}$ influx reportedly induces an increase in synthesis and release of NO (15–19).

Transient vasocontraction, which was sometimes observed with injection of CaCl$_2$ (6.25, 12.5 and 25 mg) in the presence of L-NNA and methylene blue (Figs. 2A and 3A), is probably due to calcium-induced contraction following the inhibition of NO synthesis. Perfusion of L-NNA (5 x 10$^{-5}$ M) and methylene blue (10$^{-5}$ M), which are sufficient doses to induce the blockade of NO synthesis (13, 20) and guanylate cyclase in smooth muscle (14, 21), respectively, did not completely inhibit the calcium-induced vasodilation of the ear vessel. Therefore, the possibility can not completely be excluded that other EDRFs are simultaneously responsible for calcium-induced vasodilation. However, indomethacin (10$^{-5}$ M), which blocks formation of PGI$_2$ (22, 23), did not inhibit calcium-induced vasodilation, but rather enhanced it. Failure by indomethacin to inhibit the calcium-induced vasodilation has been also observed in coronary artery (12). Therefore, it seems unlikely that PGI$_2$ is attributed to the calcium-induced vasodilation at least in the vascular bed of rabbit ear.

In conclusion, the calcium-induced vasodilation in the vascular bed of rabbit ear is suggested to be due to synthesis and/or release of NO in the endothelium cells, although a simultaneous influence of other EDRFs can not completely be excluded at present.

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