Mechanisms of the Enhanced Contractile Response to a Low Concentration of Phorbol 12,13-Dibutyrate in Thoracic Aorta Isolated from Rats with Dietary Magnesium Deficiency

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ABSTRACT—The mechanisms underlying the enhanced contractile response to phorbol 12,13-dibutyrate (PDBu) were examined in de-endothelialized thoracic aortas isolated from rats with dietary magnesium (Mg) deficiency. PDBu (1.0 nM)-induced contractions were significantly larger in Mg-deficient rats than in the controls. The contraction was completely inhibited by nifedipine, removal of external Ca$^{2+}$ or by 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7). PDBu (1.0 nM) and phorbol 12-myristate 13-acetate (1.0 μM) significantly decreased the $K_D$ value and increased the $B_{max}$ for the binding of $[^3H]$PN200-110 to the aortas. The degree of the decrease in the $K_D$ value was significantly greater in Mg-deficient rats than in the controls. The PDBu-induced decrease in the $K_D$ value was abolished by H7. These results suggest that activation of protein kinase C by phorbol esters may participate in the activation of L-type Ca$^{2+}$ channels, which increases both the affinity of $[^3H]$PN200-110 binding and the magnitude of the external Ca$^{2+}$-dependent contraction. Dietary Mg-deficiency may enhance these processes.

Keywords: Mg-deficient rat, Phorbol 12,13-dibutyrate, Protein kinase C, $[^3H]$PN200-110 binding, Ca$^{2+}$ channel

It has been reported that the Ca$^{2+}$/phospholipid-dependent protein kinase, protein kinase C (PKC), plays an important role in the control of smooth muscle function (1, 2). In vascular smooth muscle, some phorbol esters directly activate PKC and produce vasoconstriction (3-6). Although the mechanisms of vasoconstriction through activation of PKC are complex (7-10), one of them is partially associated with the activation of L-type Ca$^{2+}$ channels (11-13).

Our previous study showed that the enhanced contractile response to phenylephrine (an $\alpha_1$-agonist, PE) and increased binding affinity of $[^3H]$PN200-110 (a Ca$^{2+}$ channel antagonist) (14) to aortas isolated from rats with dietary magnesium (Mg)-deficiency via the stimulation of $\alpha_1$-adrenoceptors were inhibited by pretreatment with 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) (15). These results suggest that the activation of PKC plays an important role in the enhanced vasoconstriction via the L-type Ca$^{2+}$ channel. To confirm the above suggestion, the present study was undertaken to clarify the mechanisms underlying the enhanced contractile response to an exogenous PKC activator, phorbol 12,13-dibutyrate (PDBu), in the thoracic aorta isolated from rats with dietary Mg deficiency, using functional and $[^3H]$PN200-110 binding studies.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (7- to 9-weeks-old) were fed a Mg-deficient diet (Mg$^{2+}$, 0.001%) for 30 days. A control group received a normal diet (Mg$^{2+}$, 0.07%). The composition of the purified experimental diet and general observations on Mg-deficient rats have been described in detail in our previous papers (15, 16). The rats were pair-fed and allowed free access to deionized water. Each rat was housed individually in a stainless steel cage at an ambient temperature of 22-24°C under a 12-hr light-dark cycle.

Functional study

After an experimental period of 30 days, each rat was decapitated, and the thoracic aorta was removed immedi-
ately. The aorta was cleaned of all fat and connective tissue and cut into a helical strip about 2-mm-wide and 15-mm-long. Each strip was mounted in an organ bath containing 10 ml physiological salt solution (PSS) with the following composition: 119 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM KH₂PO₄, 25 mM NaHCO₃ and 10 mM glucose. The organ bath solution was maintained at 37°C and gassed with 95% O₂ and 5% CO₂.

A resting tension of 10 mN was applied to the aortic strip, and an equilibration period of 2 hr was allowed before the strip was exposed to drugs. The isometric muscle tension was recorded with a force-transducer (TB-611T; Nihon Kohden Kogyo Co., Tokyo) connected to a polygraph (AP-621G, Nihon Kohden Kogyo Co.). To obtain the external Ca²⁺-independent contraction, aortic strips were exposed to Ca²⁺-free PSS containing 0.01 mM glycoletherdiaminetetraacetic acid (EGTA) for 20 min, followed by replacement with Ca²⁺-free PSS without EGTA for 20 min. When antagonists were used, each was added to the organ bath 30 min before the concentration-response curves of the agonists were obtained.

In both functional and binding studies, we used preparations denuded of endothelium to avoid the effects of this tissue (17). The endothelium was removed by gently rubbing the intimal surface with a swab wetted with PSS. The absence of endothelium was verified by lack of relaxation when acetylcholine was added.

**Binding study**

The binding of [³H]PN200-110 to de-endothelialized aortic strips was determined by the method of Morel and Godfraind (18). Strips of aorta (0.8-1.0 mg) were incubated in PSS with [³H]PN200-110 (0.02-0.75 nM) for 90 min at 37°C in the dark. When the effects of PDBu or PMA were examined, these were added to the PSS at the beginning of incubation. At the end of the incubation, each strip was washed 3 times with ice-cold PSS and then dried on filter paper, weighed and dissolved in 0.1 ml of a mixture of perchloric acid : hydrogen peroxide (1 : 1). Radioactivity was counted using a liquid scintillation counter (LSC-3050; Aloka Co., Tokyo). To determine the non-specific binding, 3 μM nifedipine was included in the incubation medium. The difference between the total binding and non-specific binding was taken as the specific binding.

**Analysis of parameters**

The dissociation constant (K_D), the maximal density of specific binding sites (B_max) and the Hill coefficient were determined by Scatchard analysis using an equilibrium binding data analysis computer program (19).

**Statistical analyses**

The data are expressed as means±S.E. and analyzed statistically by Student’s t-test and Sheffe’s test for comparisons involving three or more values. Unless stated otherwise, n refers to the number of animals. Differences at a probability of less than 0.05 were considered to be significant.

**Drugs**

Drugs used were: phorbol 12,13-dibutyrate, phorbol 12-myristate 13 acetate, 4-alpha-phorbol 12,13-didecanoate and EGTA (Wako Pure Chemical Industries, Ltd., Osaka); nifedipine (Sigma Chemical Co., St. Louis, MO, USA); l-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) (Research Biochemicals, Inc., Natick, MA, USA); and (+)-[³H]PN200-110 (83 Ci/mmol; Amersham, Tokyo).

**RESULTS**

**Contractile response to phorbol 12,13-dibutyrate**

Figure 1 shows the contractile response to 1.0 nM PDBu, an activator of PKC, in thoracic aortas isolated from the control and Mg-deficient rats. PDBu produced a slowly developing contraction. The time of the beginning of the contractile response did not differ significantly between the control and Mg-deficient rats. After about 80 min, the contractile responses to PDBu were significantly greater in Mg-deficient rats than in the controls.

Pretreatment with H7 (10 μM) or nifedipine (10 nM)
Table 1. Effects of H7, nifedipine or removal of external Ca\(^{2+}\) on 1.0 nM PDBu-induced contraction of thoracic aortas isolated from control and Mg-deficient rats

|            | Control (%) | Mg-deficient (%) |
|------------|-------------|------------------|
| Ca (+)     | 172.3±3.7   | 189.6±4.6\(^a\) |
| + H7 (10 \(\mu\)M) | 0          | 0                |
| + Nifedipine (10 nM) | 0          | 0                |
| Ca (−)     | 0           | 0                |

Contraction induced by 60 mM KCl were taken as 100\%; mean absolute values were 6.8±0.3 mN in the Mg-deficient rats and 6.8±0.3 mN in the control rats. There was no significant difference between the two values. Each value represents the mean±S.E.M. from 10 rats. \(^a\)Significantly different from the corresponding controls (P < 0.05).

completely abolished the contractile responses induced by 1.0 nM PDBu in both groups. In Ca\(^{2+}\)-free PSS, 1.0 nM PDBu produced no contraction in either group. These results are summarized in Table 1.

In aortas from both groups, 4-alpha-phorbol 12,13-didecanoate, a non-activator of PKC, produced no contractile responses even at 1.0 \(\mu\)M (data not shown).

Binding of \[^{3}\text{H}]\text{PN200-110} to aortas

The specific binding of \[^{3}\text{H}]\text{PN200-110} to aortas was determined in the control and Mg-deficient rats. In normal PSS (resting state), \[^{3}\text{H}]\text{PN200-110} bound with high affinity in a saturable manner, as reported previously (15). Scatchard plots of the specific binding were linear in both

![Scatchard plots](image-url)
Table 2. Characteristics of [3H]PN200-110 binding to thoracic aortas isolated from control and Mg-deficient rats

|                      | K_D (nM)       | B_max (fmol/mg wet tissue) |
|----------------------|----------------|---------------------------|
|                      | Control        | Mg-deficient              |
| Vehicle              | 0.234±0.016    | 0.258±0.011               |
| PMA (1.0 µM)         | 0.119±0.005⁵   | 0.077±0.011⁶             |
| PDBu (1.0 nM)        | 0.116±0.015⁵   | 0.066±0.011⁶             |
| PDBu (1.0 nM) + H7 (10 µM) | 0.227±0.049   | 0.235±0.027               |

Each value represents the mean±S.E.M. from 4–8 rats. *Significantly different from the corresponding control (P<0.05). **Significantly different from the vehicle-treated preparation (P<0.05).

groups (Fig. 2a), indicating a single class of binding site. The K_D value obtained from Mg-deficient rats was not significantly different from that of the controls, although B_max was significantly larger in Mg-deficient rats (Fig. 2a and Table 2).

In the presence of 1.0 nM PDBu or 1.0 µM PMA, the specific binding of [3H]PN200-110 increased without any change in the non-specific binding, and the Scatchard plots were linear in both cases (Fig. 2, b and c), indicating a single class of binding sites. Calculated K_D and B_max values are shown in Table 2. PDBu and PMA significantly decreased K_D in both groups and increased B_max in the control. In the presence of PDBu or PMA, the K_D value was significantly smaller in the Mg-deficient rats than in the controls.

The effects of H7 on [3H]PN200-110 specific binding were investigated in aortas stimulated with 1.0 nM PDBu. Scatchard plots of specific binding were linear in both the control and Mg-deficient rat aortas (Fig. 2d), indicating a single class of binding sites. The calculated K_D and B_max values are shown in Table 2. H7 restored the characteristics of PDBu-induced [3H]PN200-110 specific binding to the vehicle state in both groups.

DISCUSSION

The present results show that subchronic dietary Mg-deficiency in adult male rats can enhance the contractile response of the thoracic aorta to a low concentration (1.0 nM) of PDBu (an activator of PKC). However, 4-alpha-phorbol 12,13-didecanoate (1.0 µM), a non-activator of PKC, produced no response in either control or Mg-deficient rats. The PDBu-induced contraction was completely inhibited by H7 (an inhibitor of PKC) (20). These results indicate that the contractile response via activation of PKC is enhanced in thoracic aortas isolated from Mg-deficient rats.

Although the mechanisms of phorbol ester-induced vasoconstriction are complex (7–10), the contraction induced by a low concentration (1.0 nM) of PDBu was completely inhibited by removal of external Ca²⁺ or by nifedipine (10 nM). The results suggest that in rat aortas, the contraction induced by 1.0 nM PDBu is entirely dependent on extracellular Ca²⁺ influx via L-type Ca²⁺ channels. It is well known that L-type Ca²⁺ channels play an important role in Ca²⁺ influx into vascular smooth muscle cells (21). It has been reported that phorbol ester-induced vasoconstriction is partially inhibited by a dihydropyridine Ca²⁺ channel antagonist (22, 23) and that phorbol esters increase the Ba²⁺ current through L-type Ca²⁺ channels (12, 13). Our previous studies using ⁴⁰Ca and nifedipine suggested that in thoracic aorta isolated from Mg-deficient rats, enhanced Ca²⁺ influx via L-type Ca²⁺ channels resulted in enhanced noradrenaline-induced contraction (16, 24). The present results suggest the possibility that the opening of L-type Ca²⁺ channels via activation of PKC is involved in the external Ca²⁺-dependent contractile response to PDBu. These processes might be enhanced in the aorta isolated from Mg-deficient rats.

It is well known that L-type Ca²⁺ channels exist in different conformational states (closed, open or inactivated), and dihydropyridines have a high affinity for the inactivated state, which is a conformational state after the open state (25, 26). It has been shown that activation of PKC increases the binding affinity of dihydropyridines for vascular smooth muscle (27). As shown in Table 2, phorbol esters decreased the K_D of dihydropyridine binding, and H7 inhibited the decrease in the K_D. The decrease in the K_D of [⁴⁰Ca]PN200-110 binding indicates an increase in the affinity of dihydropyridines to its specific Ca²⁺ channels, presumably the L-type Ca²⁺ channels, thereby suggesting that dihydropyridines may affect the Ca²⁺ channels effectively and therefore inhibit contractions mediated by Ca²⁺ influx (Table 1). The activation of PKC by phorbol esters may activate L-type Ca²⁺ channels, resulting in an increase of [⁴⁰Ca]PN200-110 binding affinity. These processes may be enhanced in the aorta isolated from Mg-deficient rats. The possibility that dietary Mg deficiency
may affect the distribution of PKC or the phosphorylation of the L-type Ca\(^{2+}\) channels via activation of PKC must be examined.

It has been shown that PKC has at least ten different isoforms, some of which like cPKC (a, b1, b11 and g) are Ca\(^{2+}\)-sensitive, whereas others (nPKC and aPKC) are Ca\(^{2+}\)-independent (28, 29). One can speculate that in rat aorta, activation of cPKC may be involved in the contraction induced by a low concentration of PDBu. Further biochemical studies are required to clarify which PKC isoforms contribute to the activation of L-type Ca\(^{2+}\) channels.

In conclusion, the present study suggests that activation of PKC by PDBu may participate in the opening of L-type Ca\(^{2+}\) channels, increasing both the affinity of \[^{3}\text{H}\]\-PN200-110 binding and magnitude of the external Ca\(^{2+}\)-dependent contraction. Dietary Mg deficiency may enhance these processes.

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