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Short communication

Effects of anandamide and arachidonic acid on specific binding of (+)-PN200-110, diltiazem and (−)-desmethoxyverapamil to L-type Ca^{2+} channel

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Abstract

We examined the effects of anandamide (*N*-arachidonoylethanolamine) on the binding of three types of Ca^{2+} channel antagonists for L-type Ca^{2+} channel, i.e., 1,4-dihydropyridine, 1,5-benzothiazepine and phenylalkylamine, to rabbit skeletal muscle membranes. Anandamide inhibited the binding of all three ligands. Arachidonic acid, a putative metabolite or a precursor of anandamide, inhibited 1,4-dihydropyridine binding, whereas it augmented both 1,5-benzothiazepine and phenylalkylamine binding. The involvement of prostaglandins synthesized from arachidonic acid was considered to be minor. These findings indicate that both anandamide and arachidonic acid interact not only with 1,4-dihydropyridine but also with 1,5-benzothiazepine and phenylalkylamine binding sites as a common feature of unsaturated lipids.

Keywords: Anandamide; Ca^{2+} channel; Arachidonic acid; [^{3}H](+)-PN200-110; [^{3}H]Diltiazem; [^{3}H](−)-Desmethoxyverapamil

1. Introduction

Anandamide (*N*-arachidonoylethanolamine) was isolated and identified as an endogenous ligand for cannabinoid receptor (Devane et al., 1992). This compound has been shown to affect the function of N-type calcium channel via cannabinoid receptor (Mackie et al., 1993).

In the course of the search for endogenous modulators of voltage-dependent L-type Ca^{2+} channel, Johnson et al. (1993) identified their putative channel modulator as anandamide. They reported that anandamide displaced 1,4-dihydropyridine binding to rat cortex membranes with IC_{50} value of about 15 μM and slope factor of about 2. However, there have been no reports concerning the interactions between anandamide and other sites, i.e., 1,5-benzothiazepine and phenylalkylamine binding sites. It should be of interest to examine if anandamide affects binding sites other than those for 1,4-dihydropyridine, since it is well known that allosteric interactions exist among these three binding sites (Glossman et al., 1985).

2. Materials and methods

2.1. Animals and chemicals

Male New Zealand white rabbits (1.2–1.3 kg) were obtained from Nihon Ikagaku Co. [^{3}H](+)-PN200-110 (87 Ci/mmol) and [^{3}H](−)-desmethoxyverapamil (80–85 Ci/mmol) were purchased from Amersham, and [^{3}H]diltiazem (85–85.5 Ci/mmol) was from New England Nuclear. Anandamide was purchased from Research Biochemicals International. Diltiazem was a gift from Tanabe Seiyaku Co. Verapamil was from Nacalai Tesque. Arachidonic acid, indomethacin, and nicardipine were from Sigma. All other materials were of reagent grade quality and obtained from standard sources.

As anandamide and arachidonic acid were poorly soluble in water, it was added to assay tubes as ethanol solutions such that the final concentration of ethanol became 0.7% v/v. It has been established in our labo-

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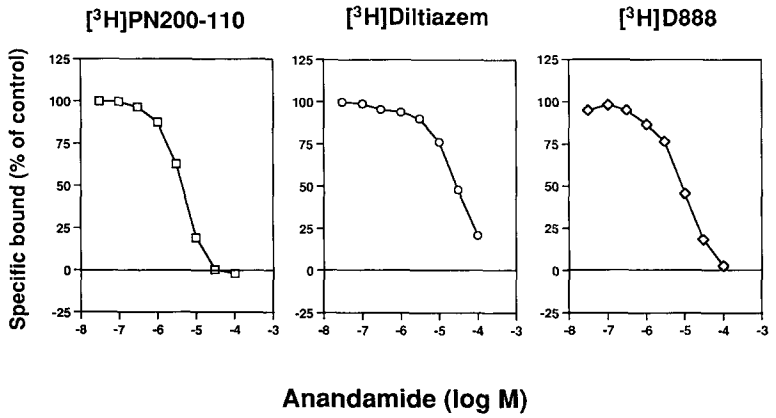

Fig. 1. Displacement curves of [^{3}H]PN200-110, [^{3}H]diltiazem and [^{3}H]D888 binding to rabbit skeletal muscle membranes by anandamide. Data are expressed as the mean ± S.E. of 4 experiments. Error bars are not visible since they are smaller than the symbols.

ratory that such concentration of ethanol does not affect binding of Ca^{2+} channel antagonists to L-type channels.

2.2. Binding assays

Membranes were isolated from rabbit skeletal muscle and binding assay was performed according to Glossman et al. (1985), Naito et al. (1989) and Ikeda et al. (1991) using [^{3}H](+)-PN200-110 ([^{3}H]PN200-110), [^{3}H]diltiazem or [^{3}H](−)-desmethoxyverapamil ([^{3}H]D888), as a ligand. For [^{3}H]PN200-110 binding, 0.5 ml of 50 mM Tris-HCl (pH = 7.4) containing 10 μg of membrane proteins and 1 nM of radioligand were incubated for 30 min at 37°C. For [^{3}H]diltiazem binding, 0.3 ml of 50 mM Tris-HCl (pH = 7.4) containing 60 μg of membrane protein and 30 nM of radioligand were incubated for 60 min at 37°C. For [^{3}H]D888 binding, 0.5 ml of 50 mM Tris-HCl (pH = 7.4) containing 30 μg of membrane protein and 3 nM of radioligand were incubated for 30 min at 37°C. Non-specific binding was determined in the presence of 1 μM of nicardipine, 100 μM of diltiazem, and 30 μM of (±)-verapamil, respectively, for each ligand. At the end of the incubation, samples were diluted to 2.5 ml with ice-cold washing buffer (50 mM Tris-HCl, pH = 7.4) and rapidly filtered through Whatman GF/C filters, which had been pretreated with 0.5% polyethyleneimine for 2 h or more, with the aid of a Brandel Cell Harvester. The filters were then washed 3 times with 2.5 ml of the ice-cold washing buffer, and the radioactivity on the filters was counted. For [^{3}H]diltiazem binding, both 0.5% polyethyleneimine and washing buffer contained 20 μM diltiazem according to Balwierczak et al. (1986). With the ligand concentrations used here, it has been reproducible in our labora-

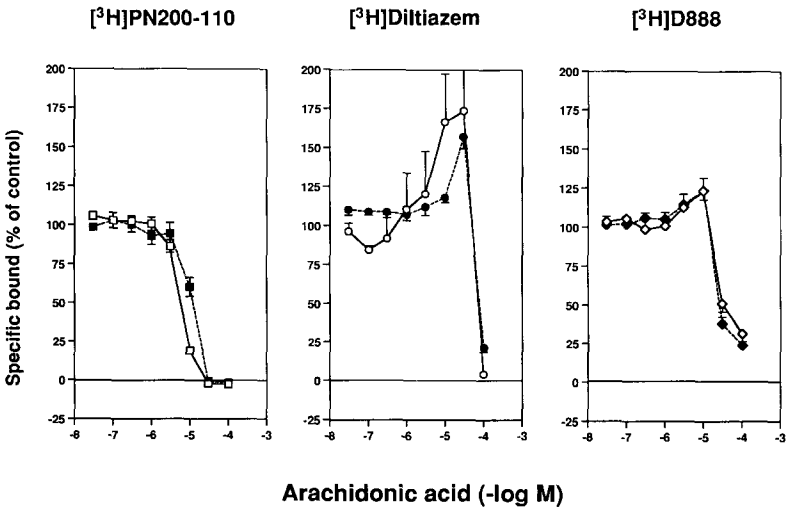

Fig. 2. Displacement curves of [^{3}H]PN200-110, [^{3}H]diltiazem and [^{3}H]D888 binding to rabbit skeletal muscle membranes by arachidonic acid in the absence (open symbols) or presence (filled symbols) of 3 μM of indomethacin. Data are expressed as the mean ± S.E. of 4 experiments.

tory with this preparation that the specific binding of [^{3}H]PN200-110, [^{3}H]diltiazem and [^{3}H]D888 is competitively inhibited by the corresponding chemical class ligands, and that the interactions between the three typical sites are consistent with the allosteric model of Ca^{2+} channel antagonist binding sites.

Binding assays were performed in duplicate manner and the results were expressed as means ± S.E. of 4 separate experiments. The slope factors, the IC_{50} or the EC_{50} values were calculated by computer fitting (GraphPad InPlot).

3. Results

Anandamide displaced the specific binding of [^{3}H]PN200-110, [^{3}H]diltiazem or [^{3}H]D888, to rabbit skeletal muscle membranes in a dose-dependent manner, as shown in Fig. 1. The specific binding of [^{3}H]PN200-110 was completely inhibited by 30 μM of anandamide with IC_{50} value of 4.04 ± 0.25 μM and slope factor of 1.62 ± 0.07. On the other hand, binding of [^{3}H]diltiazem was not completely inhibited by anandamide. More than 20% of specific binding remained even in the presence of 0.1 mM anandamide. Higher concentration could not be applied because of the limitation of solubility. IC_{50} of anandamide for [^{3}H]diltiazem binding was 28.8 ± 0.6 μM and slope factor was 1.01 ± 0.03. Binding of [^{3}H]D888 was inhibited almost completely (97%) by 0.1 mM anandamide. IC_{50} and slope factor were 8.15 ± 0.28 μM and 1.08 ± 0.05, respectively ($n = 4$).

As shown in Fig. 2, arachidonic acid showed a dose-dependent displacement of [^{3}H]PN200-110 binding as well. A complete inhibition of binding was attained at 30 μM of arachidonic acid with IC_{50} and slope factor of 5.63 ± 0.25 μM and 2.63 ± 0.11, respectively. In order to examine a possible involvement of endogenous prostaglandin production, we included 3 μM of indomethacin in the binding medium. Some changes in the effects of arachidonic acid were observed, namely, the IC_{50} was increased to 9.82 ± 0.18 μM, and the displacement curve became steeper. The slope factor in the presence of indomethacin was calculated to be 7.2 ± 2.1, but this value should be incorrect, since the displacement curves were too steep in some cases to obtain reasonable curve fittings.

In contrast to [^{3}H]PN200-110, the specific binding of both [^{3}H]diltiazem and [^{3}H]D888 was dose-dependently augmented by arachidonic acid up to 30 ([^{3}H]-diltiazem) or 10 ([^{3}H]D888) μM, and the maximum increments at these points were both statistically significant ($P < 0.05$, one sample t-test), while at the concentrations exceeding these points, these effects turned to inhibition (Fig. 2). The effect on [^{3}H]diltiazem was more prominent, but associated with larger deviations than the effect observed on [^{3}H]D888. The EC_{50} value and the maximal increment for the stimulatory phase, and the maximal inhibition (at 0.1 mM), for [^{3}H]diltiazem were 4.4 ± 1.3 μM, 174%, and 95.5%, respectively. In the case of [^{3}H]D888, the maximal increment was 128% with EC_{50} value of 2.8 ± 0.4 μM, and the maximal inhibition was 68.5% at 0.1 mM. As shown by filled symbols in Fig. 2, indomethacin at 3 μM did not affect the binding of [^{3}H]D888 but tended to attenuate both stimulatory and inhibitory phase of [^{3}H]diltiazem binding. In the presence of indomethacin, the EC_{50} value and the maximal increment for the stimulatory phase, and the maximal inhibition (at 0.1 mM), for [^{3}H]D888 were 2.6 ± 0.4 μM, 124%, and 76.1%, respectively, whereas those for [^{3}H]diltiazem were 13.4 ± 1.9 μM, 157%, and 78.3%, respectively.

4. Discussion

In the present study, it was revealed that anandamide inhibited not only 1,4-dihydropyridine binding but also both 1,5-benzothiazepine and phenylalkylamine binding to L-type Ca^{2+} channel of skeletal muscle. The effective concentration range, about 10 μM, was similar to that obtained by Johnson et al. (1993) for 1,4-dihydropyridine site. As allosteric interactions exist among the three binding sites on L-type Ca^{2+} channel for Ca^{2+} channel antagonists (Glossman et al., 1985), it is possible that anandamide acts on one of these sites allosterically affecting other sites. Alternatively, anandamide could affect the L-type Ca^{2+} channel via a site which interacts with phospholipids resulting in allosteric effects on the binding sites for Ca^{2+} channel antagonists. In fact, it has been reported that the binding of Ca^{2+} channel antagonists to the reconstituted L-type channel was affected by the phospholipids used (Glossman and Ferry, 1983). The latter possibility is further supported by the present experiments using arachidonic acid.

We used arachidonic acid in order to elucidate whether the effects of anandamide on the binding of Ca^{2+} channel antagonists to L-type channel were specific or not. Arachidonic acid showed almost the same effect as that of anandamide on 1,4-dihydropyridine binding, while it augmented the binding of both 1,5-benzothiazepine and phenylalkylamine. It has been reported that a positive allosteric interaction is observed between ligands for 1,4-dihydropyridine and 1,5-benzothiazepine (Glossman et al., 1985). However, arachidonic acid is the first compound which augments bindings of two types of ligands.

From the experiments using indomethacin, the involvement of prostaglandins synthesized from arachidonic acid during the binding assay seemed to be minor, if any. However, more work is necessary to

elucidate the possible involvement of lipoxygenase products.

This is also the first report of the effects of arachidonic acid on the binding of Ca^{2+} channel antagonists to L-type Ca^{2+} channel. Huang et al. (1992) reported the effects of several fatty acids on the function of L-type Ca^{2+} channel using isolated cardiac cells. They showed that long chain saturated and unsaturated fatty acids including arachidonic acid (3 to 30 μM) increased the voltage-dependent Ca^{2+} current possibly by acting on the channel directly. On the other hand, Pepe et al. (1994) reported that docosahexaenoic acid, but not arachidonic acid, abolished the effects of 1,4-dihydropyridine on the Ca^{2+} channel function in rat cardiac cells.

As to the biosynthesis of anandamide, there have been two hypotheses, i.e., it is produced by the condensation between arachidonic acid and ethanolamine (Devane and Axelrod, 1994; Kruszka and Gross, 1994), and it is produced by the degradation of a precursor, arachidonylphosphatidylethanolamine (Di Marzo et al., 1994). From the present experiments showing the effects of arachidonic acid on 1,5-benzothiazepine or phenylalkylamine binding, the production of anandamide from arachidonic acid, or its reversal, might modify the possible effect of the fatty acids on L-type channel. In the present study, we exclusively performed binding experiments. In future work, it is clearly necessary to elucidate the physiological role of these compounds.

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