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European Journal of Pharmacology 374 (1999) 157–160

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Short communication

Thromboxane A_2-induced phosphatidylcholine hydrolysis in porcine vascular smooth muscle cells

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Received 23 March 1999; received in revised form 28 April 1999; accepted 30 April 1999

Abstract

The effect of 9,11-epithio-11,12-methanothromboxane A_2 (STA_2), a thromboxane A_2 receptor agonist, on phosphatidylcholine hydrolysis was examined in porcine vascular smooth muscle cells. Although STA_2 stimulated diacylglycerol production in a concentration-dependent manner, it only caused a slight accumulation of [^{3}H]phosphatidylethanol in the presence of 0.5% ethanol, reflecting its weak stimulation of phosphatidylcholine-specific phospholipase D. STA_2-induced diacylglycerol production was potently and concentration dependently inhibited by potassium tricyclo-[5.2.1.0^{2,6}]-decyl-(9[8])-xanthogenate (D609), an inhibitor of phosphatidylcholine-specific phospholipase C. These results suggest that the thromboxane A_2 receptor in vascular smooth muscles is functionally coupled to phosphatidylcholine-specific phospholipase C to yield diacylglycerol. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Phosphatidylcholine-specific phospholipase C; D609; Thromboxane A_2 receptor; Phosphatidylcholine-specific phospholipase D

1. Introduction

Thromboxane A_2 causes platelet aggregation and vascular smooth muscle contraction, resulting in thrombus formation in blood vessels. It has been shown that different thromboxane A_2 receptor subtypes are expressed in vascular smooth muscles and platelets (Mais et al., 1985; Masuda et al., 1991; Krauss et al., 1996). Borg et al. (1994) reported that an antibody against a peptide having the amino acid sequence of a part of the thromboxane A_2 receptor recognized two thromboxane A_2 receptors in vascular smooth muscles. Although there are two isoforms (endothelial and placental) of the thromboxane A_2 receptor, each thromboxane A_2 receptor isoform shows the same binding affinity for [^{3}H]5Z-7-(3-endo-phenylsulfonylamino-(2,2,1)-bicyclohept-2exo-yl)heptenoic acid (S-145) (Hirata et al., 1996) and mobilizes intracellular Ca^{2+} in a similar way through phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis (Habib et al., 1997).

It is generally accepted that stimulation of the thromboxane A_2 receptor results in activation of the Gq family G protein followed by activation of PIP_2-specific phospholipase C-β (Nakahata et al., 1989; Baldassare et al., 1993; Ohkubo et al., 1996). Inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol produced by cleavage of PIP_2 by phospholipase C (Berridge, 1984) possibly contribute to changes in cell function in response to thromboxane A_2. However, our previous results demonstrated that PIP_2 hydrolysis was insufficient to explain the tonic phase of the contractile response to thromboxane A_2 in vascular smooth muscles (Yamamoto et al., 1995). The thromboxane A_2 receptor-mediated long-lasting contraction was inhibited by inhibitors of protein kinase C, suggesting that there is another pathway of diacylglycerol production besides PIP_2-specific phospholipase C.

Recently, phosphatidylcholine-specific phospholipase D, which causes the hydrolysis of phosphatidylcholine to yield phosphatidic acid, was extensively investigated (Exton, 1997). In spite of rapid advances in research into phosphatidylcholine-specific phospholipase D, phosphatidylcholine-specific phospholipase C is still under investigation and its physiological significance remains to be solved.

The present study was undertaken to investigate another signaling pathway besides PIP_2-specific phospholipase C that is activated by stimulation of thromboxane A_2 receptor in vascular smooth muscles.

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0014-2999/99/$ - see front matter
PII: S0014-2999(99)00331-3

2. Methods

2.1. Materials

Eagle's minimum essential medium (EMEM) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Nissui Pharmaceuticals (Tokyo, Japan). 9,11-Epithio-11,12-methanothromboxane A_2 (STA_2) was provided by Ono Pharmaceutical (Osaka, Japan). Phorbol 12, 13-dibutyrate (PDB), angiotensin II and phosphatidic acid were purchased from Sigma (St. Louis, MO. USA). Potassium tricyclo-[5.2.1.$0^{2.6}$]-decyl-(9[8])-xanthogenate (D609) was obtained from Kamiya Biomedical (Thousand Oaks, CA, USA). [^{3}H]Palmitic acid was from NEN/DuPont (Boston, MA, USA). [γ-^{32}P]ATP was from Amersham International (Buckinghamshire, England). Other chemicals and drugs were of reagent grade or the highest quality available.

2.2. Cell culture

Primary cultured cells of porcine aortic smooth muscle were obtained by the explant method and were grown on 150-mm culture dishes in DMEM containing 10% fetal calf serum, penicillin (50 U/ml) and streptomycin (50 μg/ml). The cultured cells were maintained in a 37°C humidified incubator in an atmosphere of 5% CO_2 in air. After 2–3 weeks, smooth muscle cells were separated from the remaining tissues by treatment with trypsin. Cells of two or three passages were used for analysis after 3 days of culture in DMEM containing 0.5% fetal calf serum.

2.3. Analysis of phosphatidylcholine-specific phospholipase D activity

Phosphatidylcholine-specific phospholipase D activity was determined by measuring [^{3}H]phosphatidylethanol (Tsukii et al., 1996). In brief, 48 h after differentiation by culture in DMEM containing 0.5% fetal calf serum, the cells were further cultivated in DMEM containing 0.5% fetal calf serum and 5 μCi/ml of [^{3}H]palmitic acid for an additional 24 h. After being washed 3 times with EMEM–20 mM HEPES, pH 7.35 (EMEM–HEPES), the cells were preincubated in the EMEM–HEPES containing 0.5% ethanol for 10 min and the drug was added to the medium. The reaction was carried out for 15 min and was terminated by addition of 1.5 ml of chloroform/methanol (1:2). The lower chloroform phase was dried and spotted on LK5D silica gel plates (Whatman). The samples were developed by using the upper phase of a solvent system consisting of ethyl acetate/isooctane/acetic acid/water (110:50:20:100, by volume). Authentic phosphatidylethanol was used as a standard and visualized with phosphomolybdate. Spots corresponding to phosphatidylethanol were scraped off and counted by liquid scintillation counting.

2.4. Assay of diacylglycerol

The cells were cultivated in a 12-well plate at the density of 10^5/ml in a well for 72 h. After the cells were washed three times with the EMEM–HEPES, the reaction was initiated by addition of drugs and terminated by addition of ice-cold methanol (0.5 ml) after aspiration of the medium. The cells were scraped off and rinsed with 0.5 ml of methanol. The sample was mixed with 1 ml of chloroform and 1 ml of 1 M NaCl, and centrifuged at $1500 \times g$ for 5 min. The lower chloroform phase was transferred to a tube and dried under a stream of N_2 gas. Diacylglycerol was determined as [^{32}P]phosphatidic acid after conversion by diacylglycerol kinase and [γ-^{32}P]ATP, using a diacylglycerol assay reagent system (Amersham International, UK). In brief, the sample or standard (31.25–1000 pmol) was incubated with diacylglycerol kinase and 500 μM [γ-^{32}P]ATP (1 μCi/tube) at 25°C for 30 min in the buffer. The buffer contained 2 mM DTT, 1.5% *n*-octyl-β-glucopyranoside, 1 mM cardiolipin, 0.25 mM diethylenetriamine-pentaacetic acid, 1% glycerol, 0.1 mM β-mercaptoethanol, 1 mM EGTA, 12.5 mM $MgCl_2$, 50 mM NaCl and 50 mM imidazole/HCl (pH 6.6) in a final volume of 100 μl. The reaction was terminated by addition of 1 ml of chloroform/methanol/1 N HCl (1:2:0.1, by volume), and 0.3 ml chloroform and 0.2 ml of 0.2 M KCl and 5 mM EDTA in 0.1 N HCl were further added. The lower chloroform phase was dried and spotted on LK5D slica gel plates (Whatman). The samples were developed by using the upper phase of a solvent system consisting of ethyl acetate/isooctane/acetic acid/water (110:50:20:100, by volume). Spots corresponding to phosphatidic acid were scraped off and counted by liquid scintillation counting.

2.5. Data analysis and protein determination

The statistical difference of the values was determined by unpaired Student's *t*-test. The protein concentration was measured by a dye-binding method, using bovine serum albumin as a standard.

3. Results

3.1. Effect of STA_2 on production of diacylglycerol and phosphatidylethanol

To examine STA_2-induced phospholipid breakdown, the production of diacylglycerol, a direct metabolite of PIP_2-specific phospholipase C and phosphatidylcholine-specific phospholipase C, was determined. Incubation of the cells with STA_2 for 15 min resulted in diacylglycerol production in a concentration-dependent manner (Fig. 1A). In the presence of 0.5% ethanol, STA_2 caused a slight but significant accumulation of [^{3}H]phosphatidylethanol in the

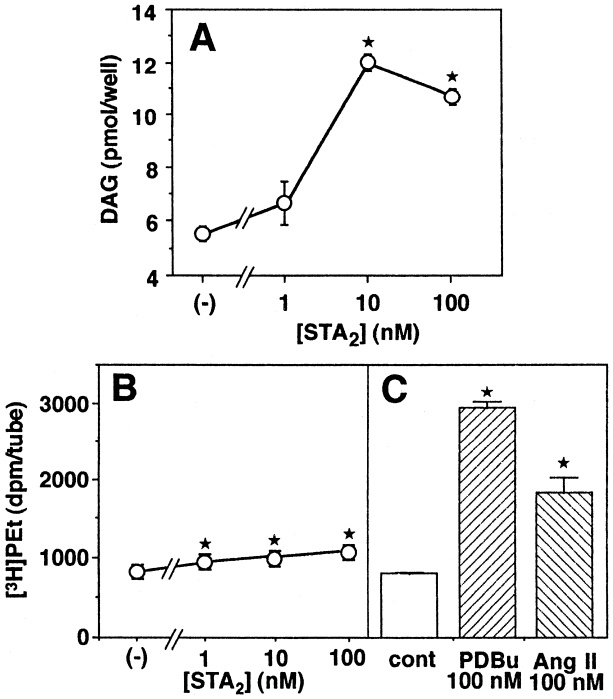

Fig. 1. Effect of STA_2 on the production of diacylglycerol and phosphatidylethanol. (A) The cells were incubated with 1–100 nM STA_2 for 15 min. Diacylglycerol was determined by conversion to phosphatidic acid as described in detail in Section 2. (B,C) Effects of STA_2, PDB and angiotensin II on phosphatidylethanol production. The [^3H]palmitic acid-labeled cells were incubated with 1–100 nM STA_2 (B), 0.1 μM PDB or 0.1 μM angiotensin II (Ang II) (C) for 15 min in the presence of 0.5% ethanol. [^3H]Phosphatidylethanol (PEt) was determined as described in Section 2. Each point or column represents the mean with S.E.M of triplicate determinations. The data are representative of two separate experiments. * Significant difference from control ($P < 0.05$).

vascular smooth muscle cells labeled with [^{3}H]palmitic acid (Fig. 1B), reflecting activation of phosphatidylcholine-specific phospholipase D. In contrast, PDB and angiotensin II resulted in a greater accumulation of [^{3}H]phosphatidylethanol (Fig. 1C). STA_2 was much less effective in causing the production of [^{3}H]phosphatidylethanol than PDB and angiotensin II.

3.2. Effects of D609 on STA_2-induced diacylglycerol production

D609, an inhibitor of phosphatidylcholine-specific phospholipase C (Schutze et al., 1992), was used for examining the involvement of phosphatidylcholine-specific phospholipase C in STA_2-induced diacylglycerol production. D609 inhibited STA_2-induced diacylglycerol production in a concentration-dependent manner with an IC_{50} value of approximately 11 μM (Fig. 2A). In contrast, D609 partly inhibited PDB-induced diacylglycerol production, and it had no effect on angiotensin II-induced production (Fig. 2B).

4. Discussion

Since the tonic contractile response to thromboxane A_2 is inhibited by protein kinase C inhibitors (Yamamoto et al., 1995), it was assumed that diacylglycerol might be produced in response to thromboxane A_2 receptor stimulation in vascular smooth muscles. Thus, we examined phosphatidylcholine hydrolysis in cultured aortic smooth muscle cells. Thromboxane A_2 receptor stimulation caused the production of [^{3}H]phosphatidylethanol in the presence of 0.5% ethanol, via phosphatidylcholine-specific phospholipase D. However, the potency of STA_2 in causing the production of [^{3}H]phosphatidylethanol was much lower than that of PDB and angiotensin II. Thus, it is assumed

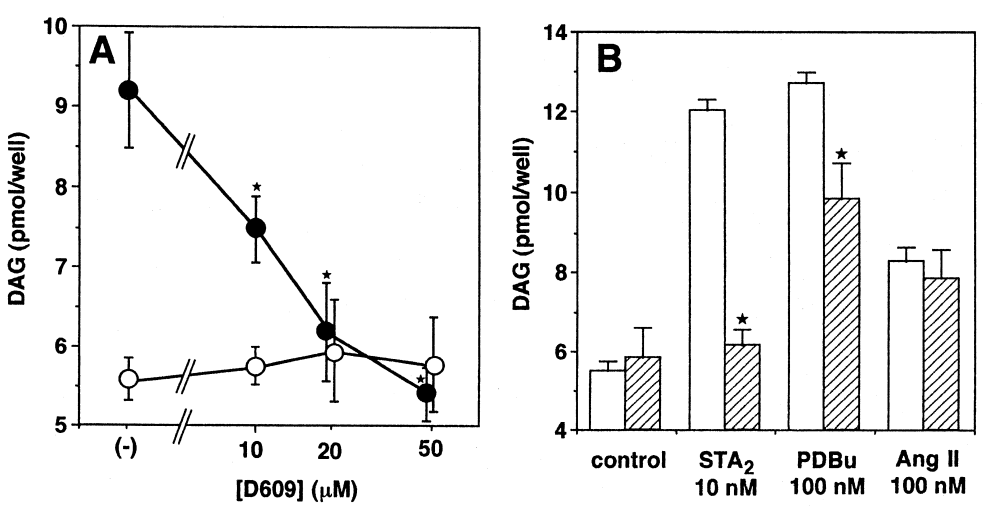

Fig. 2. Effect of D609 on diacylglycerol production. (A) Effect of D609 on STA_2-induced diacylglycerol production. The cells were preincubated with 10–50 μM D609 for 15 min, and incubated with 10 nM STA_2 (●) or vehicle (○) for 15 min. (B) Comparison of D609 effects on STA_2-, PDB- and angiotensin II-induced diacylglycerol production. The cells were preincubated with 20 μM D609 (hatched column) or vehicle (open column) for 15 min, and incubated with 10 nM STA_2, 0.1 μM PDB or 0.1 μM angiotensin II (Ang II) for 15 min. Each point or column represents the mean with S.E.M of three determinations. Data are representative in two separate experiments. * Significant difference from each stimulant alone ($P < 0.05$).

that the thromboxane A_2 receptor is not mainly coupled to phosphatidylcholine-specific phospholipase D. In spite of the weak coupling to phosphatidylcholine-specific phospholipase D, STA_2-induced diacylglycerol production was clearly and potently inhibited by D609, an inhibitor of phosphatidylcholine-specific phospholipase C. It has been shown that D609 inhibits not only phosphatidylcholine-specific phospholipase C but also phosphatidylcholine-specific phospholipase D (Kiss and Tomono, 1995). Since D609 potentially inhibited diacylglycerol production induced by STA_2, which slightly activated phosphatidylcholine-specific phospholipase D, D609 is assumed to act as a specific inhibitor of phosphatidylcholine-specific phospholipase C. In fact, D609 has a different inhibitory effect on diacylglycerol production induced by STA_2, PDB and angiotensin II. Our finding of the functional coupling of the thromboxane A_2 receptor with phosphatidylcholine-specific phospholipase C is important for the characterization of receptor-mediated activation of phosphatidylcholine-specific phospholipase C, because there are few reports concerning the receptor-mediated stimulation of phosphatidylcholine-specific phospholipase C.

In conclusion, we show for the first time that the stimulation of thromboxane A_2 receptors causes phosphatidylcholine breakdown to yield diacylglycerol through phosphatidylcholine-specific phospholipase C in vascular smooth muscle cells. This may underlie the tonic contraction mediated by protein kinase C.

Acknowledgements

Authors are grateful to Ono Pharmaceutical for providing STA_2. This work was supported in part by Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Culture of Japan.

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