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Activation of *N*-acylethanolamine-releasing phospholipase D by polyamines

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Abstract

N-acylethanolamines including anandamide (an endogenous ligand of cannabinoid receptors) are biosynthesized from *N*-acyl-phosphatidylethanolamine (PE) by a phosphodiesterase of the phospholipase D type. The enzyme partially purified from the particulate fraction of rat heart hydrolyzed *N*-palmitoyl-PE to *N*-palmitoylethanolamine with a specific activity of 50 nmol/min per mg protein at 37 °C in the presence of 10 mM $CaCl_2$. We found that the enzyme was highly activated in dose-dependent manner by polyamines like spermine, spermidine, and putrescine. Spermine was the most potent with an EC_{50} value around 0.1 mM, and increased the specific enzyme activity 27 fold up to 53 nmol/min per mg protein. However, a synergistic effect of spermine and the known activator (Ca^{2+} or Triton X-100) was not observed. The spermine-stimulated enzyme was also active with *N*-arachidonoyl-PE (a precursor of anandamide). Thus, polyamines may function as endogenous activators to control the biosynthesis of anandamide and other *N*-acylethanolamines.

Keywords: Phospholipase D; *N*-acylphosphatidylethanolamine; *N*-acylethanolamine; Anandamide; Polyamine; Spermine

1. Introduction

Ethanolamides of long-chain fatty acids, collectively referred to as *N*-acylethanolamines, are present in various mammalian tissues (Bachur et al., 1965; Schmid et al., 1990). *N*-acylethanolamines markedly increase in degenerating tissues or cells such as infarcted myocardium (Epps et al., 1979), glutamate-treated neocortical neurons (Hansen et al., 1995), cadmium chloride-administered rat testis (Kondo et al., 1998), and mouse epidermal cells exposed to stress (UVB irradiation and serum deprivation) (Berdyshev et al., 2000). These compounds were suggested to serve a cytoprotective role in injured cells because of their anti-inflammatory effect, membrane-stabilizing effect and other biological activities (Schmid et al., 1990; Lambert and Di Marzo, 1999). Moreover, *N*-arachidonoylethanol-

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amine (anandamide) was found to be an endogenous ligand of the cannabinoid receptors (Devane et al., 1992), and exhibit a variety of cannabimimetic activities (Di Marzo, 1998). Very recently, it was reported that saturated and monounsaturated *N*-acylethanolamines as well as anandamide stimulated phosphorylation of extracellular-signal-regulated protein kinase (ERK) independent of cannabinoid receptor and enhanced activator protein-1 (AP-1)-dependent transcriptional activity (Berdyshev et al., 2001).

N-acylethanolamines are biosynthesized from phosphatidylethanolamine (PE) via *N*-acyl-PE (Schmid et al., 1990; Hansen et al., 2000; Schmid, 2000). A phosphodiesterase of the phospholipase D type catalyzes the hydrolysis of *N*-acyl-PE to *N*-acylethanolamine and phosphatidic acid (Schmid et al., 1990; Hansen et al., 2000; Schmid, 2000). The enzyme is widely distributed in mammalian tissues, and has been characterized with crude enzyme preparations such as microsomes (Schmid et al., 1983; Natarajan et al., 1984; Sugiura et al., 1996; Petersen and Hansen, 1999; Petersen et al., 2000). Recently, we partially purified the phosphodiesterase from the particulate fraction of rat heart to a specific activity of 17 nmol/min per mg protein at 37 °C for *N*-palmitoyl-PE as the substrate. With the partially purified enzyme we found a potent activation of the enzyme by Ca^{2+} and several other divalent cations in addition to Triton X-100 (a non-ionic detergent) previously reported as an activator (Ueda et al., 2001). However, since millimolar concentrations of Ca^{2+} were required for the significant activation of the enzyme, the physiological importance of Ca^{2+} as an activator is not clear.

Polyamines like spermine, spermidine and putrescine are organic polycations which are present in all living organisms (Bachrach, 1973; Morgan, 1999). These compounds interact with nucleic acids and other negatively charged biomolecules, and are involved in a variety of cellular functions such as growth and differentiation. Furthermore, polyamines stabilize membrane, modulate activity of many enzymes, and regulate intracellular free calcium (Schuber, 1989; Morgan, 1999). Since several phospholipases were reported to be stimulated by polyamines (Eichberg et al., 1981; Sagawa et al., 1983; Haber et al., 1991; Cordella-Miele et al., 1993; Madesh and Balasubramanian, 1997), we were interested in the effect of polyamines on the *N*-acyl-PE-hydrolyzing phosphodiesterase.

In the present studies, we improved the purification method of the phosphodiesterase from rat heart, and investigated the stimulatory effect of polyamines on the partially purified enzyme.

2. Experimental procedures

2.1. Materials

[1-^{14}C]Palmitic acid was purchased from Du Pont NEN (Boston, MA, USA), [1-^{14}C]arachidonic acid, HiTrap SP HP and HiTrap Q from Amersham Pharmacia Biotech (Amersham, UK), arachidonic acid from Nu-Chek-Prep (Elysian, MN, USA), palmitic acid and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine from Sigma (St. Louis, MO, USA), spermine, spermidine, putrescine and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) from Wako Pure Chemical Company (Osaka, Japan), Triton X-100 from Nacalai Tesque (Kyoto, Japan), *n*-octyl β-D-glucoside and ethanolamine hydrochloride from Katayama Chemical (Osaka, Japan), methyl arachidonyl fluorophosphonate (MAFP) from Cayman Chemical (Ann Arbor, MI, USA), protein assay dye reagent concentrate from Bio-Rad (Hercules, CA, USA), and precoated silica gel 60 F_{254} aluminum sheets for thin-layer chromatography (20×20 cm, 0.2 mm thickness) from Merck (Darmstadt, Germany). *N*-[^{14}C]Palmitoyl-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine and *N*-[^{14}C]arachidonoyl-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine were prepared from 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine and [1-^{14}C]palmitic acid or [1-^{14}C]arachidonic acid according to the method of Schmid et al. (Schmid et al., 1983). The products were purified by thin-layer chromatography with a mixture of chloroform/methanol/28% ammonium hydroxide (80:20:2, v/v).

2.2. Enzyme preparation

Wistar rats (250–500 g weight, Charles River, Japan) were anesthetized with diethyl ether, and sacrificed by cervical dislocation. Hearts were removed, cut into small pieces, and then homogenized in five times the volume (v/w) of ice-cold 20 mM Tris–HCl (pH 7.4) containing 0.32 M sucrose by a Polytron homogenizer. The homogenate was centrifuged at $800 \times g$ for 15 min. The supernatant was further centrifuged at $105\,000 \times g$ for 50 min, and the resultant pellet (referred to as particulate fraction) was suspended in phosphate-buffered saline (PBS; pH 7.4). After one cycle of freezing and thawing, the sample was centrifuged at $105\,000 \times g$ for 50 min. The pellet was suspended in 20 mM Tris–HCl (pH 7.4) containing 1% (w/v) octyl glucoside, and centrifuged again at $105\,000 \times g$ for 50 min. The resultant clear supernatant was frozen at −80 °C, and then thawed slowly at 4 °C. The developed precipitate was removed by centrifugation at $105\,000 \times g$ for 30 min. The supernatant (22 mg protein) was loaded on a HiTrap SP HP cation-exchange column (bed volume, 5 ml) preequilibrated with 20 mM Tris–HCl (pH 7.4) containing 1% (w/v) CHAPS (buffer A). After washing the gel with 20 ml of buffer A, the enzyme was eluted with 20 ml of buffer A containing 150 mM NaCl. The fractions with a high specific enzyme activity (2.3 mg protein) were combined, and diluted 3-fold (v/v) with buffer A. This diluted sample was loaded on a HiTrap Q anion-exchange column (bed volume, 1 ml) preequilibrated with buffer A containing 50 mM NaCl. After washing the column with 4 ml of buffer A containing 50 mM NaCl and 4 ml of buffer A containing 200 mM NaCl, the enzyme was eluted with 4 ml of buffer A containing 300 mM NaCl. Thus prepared partially purified enzyme was stored at −80 °C until use. Protein concentration was determined by the method of Bradford (Bradford, 1976). Bovine serum albumin was used as a standard, and the calibration curve was obtained in the presence of CHAPS.

2.3. Enzyme assay

The enzyme assay was performed as we described previously (Ueda et al., 2001). The enzyme was incubated with 100 μM *N*-[^{14}C]palmitoyl-PE or *N*-[^{14}C]arachidonoyl-PE (10 000 cpm in 5 μl of ethanol) in 100 μl of 50 mM Tris–HCl (pH 7.5) containing an activator at 37 °C for 30 min. A mixture of chloroform/methanol (2:1, v/v, 0.3 ml) was added to the reaction mixture to terminate the reaction. After centrifugation, 100 μl of the lower layer was spotted on a silica gel thin-layer plate (10 cm height). The sample was then developed in the organic phase of a mixture of isooctane/ethyl acetate/water/acetic acid (50:110:100:20, v/v) at room temperature for 20 min. Distribution of radioactivity on the plate was quantified by a BAS1500 bioimaging analyzer (Fujix, Tokyo, Japan).

3. Results

The phosphodiesterase hydrolyzing *N*-[^{14}C]palmitoyl-PE to *N*-[^{14}C]palmitoylethanolamine was solubilized from the particulate fraction of rat heart with the aid of 1% octyl glucoside. The solubilized enzyme was then partially purified by the combination of freezing and thawing, cation-exchange chromatography using a HiTrap SP HP column, and anion-exchange chromatography using a HiTrap Q column. As summarized in Table 1, when the enzyme assay was performed in the presence of 10 mM $CaCl_2$ and the product was analyzed by thin-layer chromatography, the specific enzyme activity increased about 260-fold up to 50 nmol/min per mg protein at 37 °C. As discussed later, spermine used as a representative polyamine was found to function as an activator, and the enzyme reaction in the presence of 1 mM spermine also gave specific activities similar to those with 10 mM $CaCl_2$ in each fraction. Interestingly, the activation by $CaCl_2$ or spermine was observed only in the enzyme preparations after solubilization.

With this partially purified enzyme we examined the effect of the polyamines, occurring ubiq-

Table 1
Purification of the *N*-palmitoyl-PE-hydrolyzing phosphodiesterase from the particulate fraction of rat heart

	Specific enzyme activity[a]		
	None	10 mM $CaCl_2$ (nmol/min per mg protein)	1 mM Spermine
Particulate fraction	0.22 ± 0.01	0.19 ± 0.01	0.16 ± 0.00
Protein solubilized with 1% octyl glucoside	0.07 ± 0.02	1.00 ± 0.03	0.79 ± 0.05
Soluble fraction after freezing and thawing	0.10 ± 0.02	3.25 ± 0.34	2.92 ± 0.43
Active fraction from a HiTrap SP HP column	1.23 ± 0.07	7.85 ± 0.43	5.70 ± 0.27
Active fraction from a HiTrap Q column	1.99 ± 0.06	50.18 ± 3.37	53.52 ± 0.19

[a] The enzyme was allowed to react with 100 μM *N*-[^{14}C]palmitoyl-PE in the presence of 10 mM $CaCl_2$ or 1 mM spermine or in their absence. Mean values ± S.D. are shown ($n = 3$).

uitously in animal tissues (spermine, spermidine and putrescine), on the *N*-palmitoyl-PE-hydrolyzing activity. As shown in Fig. 1, all the polyamines tested enhanced the enzyme activity dose-dependently. Spermine was the most potent with an EC_{50} value around 0.1 mM. Spermine at 10 mM activated the enzyme 27 fold. Spermidine was also potent although its EC_{50} value (about 1 mM) was higher than that of spermine. Putrescine was much less active, and the activity was increased only 6 fold at 10 mM. Contrary to polyamines, 0.1–10 mM ethanolamine used as a representative of monoamine hardly affected the activity.

In the presence of 1 mM spermine, the K_m value for *N*-palmitoyl-PE was as low as about 2 μM. The partially purified enzyme was also active with *N*-arachidonoyl-PE (a precursor of anandamide) in the presence of 1 mM spermine, and the hydrolysis rate with 100 μM *N*-arachidonoyl-PE was about 50% of that with 100 μM *N*-palmitoyl-PE (not shown).

The partially purified enzyme was also activated dose-dependently by Ca^{2+} (Fig. 2A) and Triton X-100 (Fig. 2B) in accordance with our previous results (Ueda et al., 2001). The EC_{50} of $CaCl_2$ was approximately 3 mM. Triton X-100 was maximally active at 0.05% (w/v) (equivalent to 0.8 mM), while higher concentrations of Triton X-100 reduced the activity. In order to examine synergistic effect of these substances and spermine, the enzyme was assayed in the presence of 1 mM spermine together with different concentrations of either $CaCl_2$ or Triton X-100 (Fig. 2A and B). However, increasing concentrations of $CaCl_2$ or Triton X-100 failed to further stimulate the spermine-activated enzyme, and more than 0.1% of Triton X-100 markedly inhibited the enzyme. We also tested the stimulatory effect of different concentrations of spermine in the presence of 10 mM $CaCl_2$ or 0.05% Triton X-100 (Fig. 2C). Again, synergistic activation of the enzyme by spermine and $CaCl_2$ or by spermine and Triton X-100 was not observed.

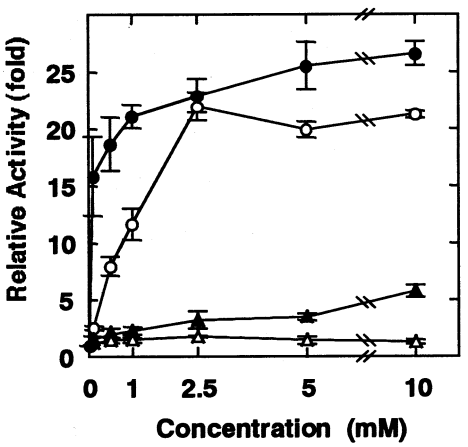

Fig. 1. Dose-dependent activation of the *N*-palmitoyl-PE-hydrolyzing phosphodiesterase by polyamines. The partially purified enzyme (1.6 μg protein) was allowed to react with 100 μM *N*-[^{14}C]palmitoyl-PE in the presence of the indicated concentrations of the following amines: spermine (closed circles), spermidine (open circles), putrescine (closed triangles) and ethanolamine (open triangles). The specific enzyme activity without amines (1.5 nmol/min per mg protein) was normalized to 1. Mean values ± S.D. are shown ($n = 3$).

As shown in Table 1, the specific enzyme activity increased 5 fold by the solubilization of the particulate fraction with 1% octyl glucoside and by the addition of 10 mM $CaCl_2$ to the reaction mixture. Therefore, these procedures seemed to be useful for precise determination of the enzyme activity in organs expressing a low level of the enzyme. We applied this assay method to investigate the distribution of the enzyme in rat organs. Fatty acid amide hydrolase (FAAH) to hydrolyze *N*-acylethanolamine to fatty acid and ethanolamine is widely distributed in rat tissues (Katayama et al., 1997). It is likely that *N*-palmitoylethanolamine produced by the phosphodiesterase is further degraded by FAAH in the same organ. MAFP inhibits FAAH potently (De Petrocellis et al., 1997; Deutsch et al., 1997), but shows much weaker inhibitory effect on the phosphodiesterase (Petersen and Hansen, 1999). Therefore, 1 μM MAFP was included in the reaction mixture. As presented in Fig. 3, the highest specific activity was observed in heart, followed by testis, cerebellum and cerebrum. Other organs tested also showed a low, but detectable activity.

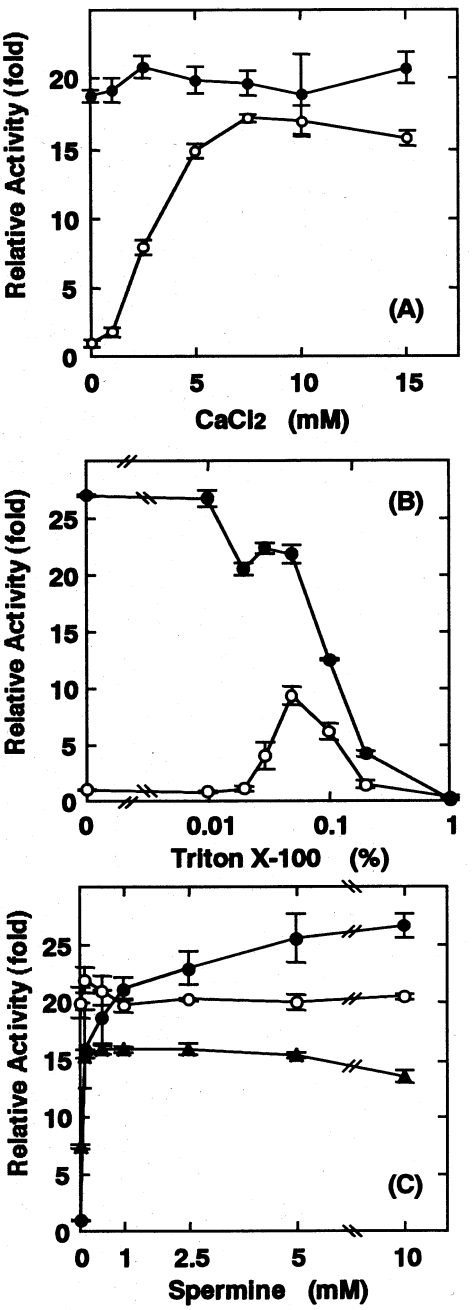

Fig. 2.

4. Discussion

N-Acyl-PE-hydrolyzing phospholipase D is involved in the biosynthesis of anandamide (an endogenous cannabinoid receptor ligand) and other bioactive *N*-acylethanolamines (Schmid et al., 1990; Hansen et al., 2000; Schmid, 2000). In spite of this physiological importance, attempts to purify the enzyme have not been reported until recently. We reported for the first time the partial purification of the enzyme from the particulate fraction of rat heart (Ueda et al., 2001). Furthermore, in the present study we refined and im-

Fig. 2. Activation of the phosphodiesterase by $CaCl_2$, Triton X-100, spermine, and their combinations. The partially purified enzyme (0.9–1.6 μg protein) was allowed to react with 100 μM *N*-[^{14}C]palmitoyl-PE in the presence of the indicated concentrations of $CaCl_2$ (A) or Triton X-100 (B). Spermine at 1 mM was also included (closed circles) or was absent (open circles) (A and B). (C) The enzyme activity was assayed in the reaction in the presence of the indicated concentrations of spermine. $CaCl_2$ at 10 mM (open circles), 0.05% Triton X-100 (closed triangles) or none (closed circles) were also included. The specific enzyme activity without any activators (1.5–2.0 nmol/min per mg protein) was normalized to 1. Mean values ± S.D. are shown ($n = 3$).

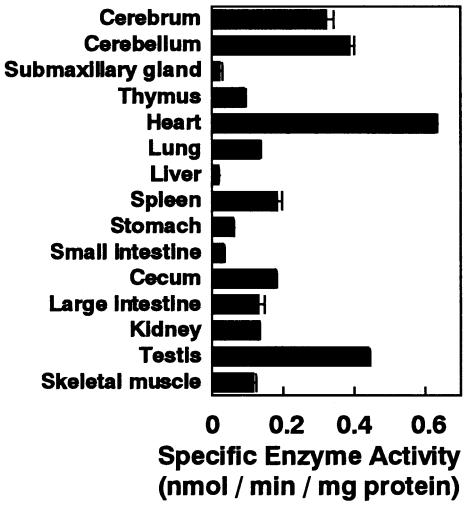

Fig. 3. Distribution of the phosphodiesterase in rat organs. The particulate fraction from various organs of male rats was solubilized with 1% octyl glucoside, and the solubilized protein (22–111 μg protein) was allowed to react with 100 μM *N*-[^{14}C]palmitoyl-PE in the presence of 10 mM $CaCl_2$ and 1 μM MAFP. Mean values ± S.D. are shown ($n = 3$).

proved the purification procedures. As a result, the specific activity of the partially purified enzyme increased three fold (about 50 nmol/min per mg protein) compared with the value which we reported earlier (Ueda et al., 2001). When this partially purified enzyme was subjected to SDS-polyacrylamide gel electrophoresis, followed by staining with silver nitrate, several major protein bands were detected. We are currently trying to further purify the enzyme for its molecular characterization.

In the previous work we found that Ca^{2+} and several other inorganic divalent cations potently activated the enzyme. However, a millimolar level, to be much higher than physiological intracellular concentration, of Ca^{2+} was required to cause significant activation of the enzyme (Ueda et al., 2001). Several phospholipases such as phosphoinositide-specific phospholipase Cδ and intestinal mitochondrial phospholipase D were reported to be stimulated by polyamines (Eichberg et al., 1981; Sagawa et al., 1983; Haber et al., 1991; Cordella-Miele et al., 1993; Madesh and Balasubramanian, 1997). Therefore, we examined the stimulatory effect of polyamines on the phospholipase D in this study. Using the partially purified enzyme, spermine, spermidine and putrescine were shown to activate the enzyme markedly in dose-dependent manner. Spermine was the most potent with an EC_{50} value around 0.1 mM. Spermidine was also potent but with a higher EC_{50} value (approximately 1 mM). Putrescine was much less active. The stimulatory effect on phospholipase Cδ was also reported to be more potent in the order of spermine (half-maximum activation, 0.15 mM) > spermidine (0.9 mM) > putrescine (6 mM) (Haber et al., 1991). It should be noted that the stimulatory effects increased with increases in amino groups, namely, number of amino groups is 2, 3, and 4 in putrescine, spermidine, and spermine, respectively.

Intracellular concentration of spermine was reported to be 635 μM in resting fibroblasts and 694 μM in SV-3T3 cells (Morgan, 1990). Therefore, physiological levels of spermine may contribute to the intracellular regulation of the enzyme. The partially purified enzyme was also active with *N*-arachidonoyl-PE (a precursor of anandamide) in the presence of 1 mM spermine. Polyamine levels may thus affect the generation of anandamide.

A synergistic activation by spermine and Ca^{2+} was not observed. This result suggests that these cations activate the enzyme by the same mechanism although their EC_{50} values are considerably different (0.1 mM versus 3 mM). It should also be noted that the K_m value for *N*-palmitoyl-PE in the presence of 1 mM spermine was as low as 2 μM. This value was similar to that in the presence of 10 mM $CaCl_2$ (about 8 μM), but was much lower than that in the presence of 0.04% Triton X-100 (about 45 μM) (Ueda et al., 2001). Polyamines as polycations are well known to interact with negatively charged biomolecules such as nucleic acids and acid phospholipids (Schuber, 1989). Thus, polyamines may form complexes with an *N*-acyl-PE substrate, resulting in the enhancement of affinity of the substrate to the enzyme. Alternatively, interaction of polyamines with negatively charged residues of the enzyme protein may lead to the enzyme activation.

Interestingly, we found that spermine as well as Ca^{2+} could stimulate the enzyme only after its

solubilization. The membrane-bound enzyme was insensitive to these activators. This finding may explain why in the earlier studies the membrane-bound enzyme in microsomes was activated slightly, if any, by Ca^{2+} (Schmid et al., 1983; Natarajan et al., 1984; Sugiura et al., 1996). Certain endogenous substances in crude preparations like microsomes may prevent exogenously added Ca^{2+} or spermine from activating the enzyme, or endogenous Ca^{2+}- or spermine-like activators may be present in the preparations. We also can not rule out a possibility that the solubilized enzyme is activated by direct interaction of Ca^{2+} or spermine with the enzyme protein while the membrane-bound native enzyme can not bind to the cations. Further studies will be required to clarify whether or not the membrane-bound enzyme is physiologically activated by polyamines and other cations in the cell.

We also found that heart exhibited the highest phosphodiesterase activity among various rat organs when assayed with octyl glucoside-solubilized proteins in the presence of 10 mM $CaCl_2$. Testis, cerebrum and cerebellum also showed a high specific enzyme activity. This distribution is in considerable agreement with the distribution examined earlier with homogenates from rat organs in the presence or absence of Triton X-100 (Schmid et al., 1983). Based on these results obtained under the different assay conditions, the same enzyme protein seems to be expressed in various organs.

In summary, we found a potent activation of the *N*-acyl-PE-hydrolyzing phospholipase D by polyamines. In particular, spermine caused significant activation within the physiological range of concentration. Polyamines may function as one of endogenous activators for the biosynthetic pathway of anandamide and other *N*-acylethanolamines.

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