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Authors
Beltramo, Massimiliano
di Tomaso, Emmanuelle
Piomelli, Daniele

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Inhibition of anandamide hydrolysis in rat brain tissue by (E)-6-(bromomethylene) tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one

Massimiliano Beltramo[1], Emmanuelle di Tomaso[1], Daniele Piomelli*

The Neurosciences Institute, 10640 J.J. Hopkins Drive, San Diego, CA 92121, USA

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Abstract Anandamide, an endogenous cannabinoid substance, is hydrolyzed by an amidohydrolase activity present in rat brain and liver. We report that the bromoenol lactone, (E)-6-(bromomethylene) tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (BTNP), is a potent inhibitor of this enzyme activity. BTNP prevented anandamide hydrolysis in rat brain microsomes with an IC_{50} of 0.8 ± 0.3 μM. Kinetic and dialysis experiments indicated that this effect was non-competitive and irreversible. After chromatographic fractionation of the enzyme activity, BTNP was still effective, suggesting that it interacts directly with the enzyme. Anandamide hydrolysis was 12-fold greater in rat cortical neurons (1.94 ± 0.1 pmol/min/mg protein) than in cortical astrocytes (0.16 ± 0.01 pmol/min/mg protein) and, in either cell type, it was inhibited by BTNP ($IC_{50} = 0.1$ μM in neurons). These results suggest that BTNP may provide a useful lead for the development of novel inhibitors of anandamide hydrolysis.

Key words: Bromoenol lactone; Phospholipase A_2; Amidohydrolase; Anandamide; Cannabinoid

1. Introduction

To ensure that chemical transmission in the nervous system occurs with high spatial and temporal resolution, neurotransmitters released into the synaptic cleft are rapidly disposed of by a variety of mechanisms of inactivation. These fall into two main categories: reuptake into cells mediated by transporter proteins terminates the synaptic effects of biogenic amines, glutamate and γ-aminobutyrate [1,2], while breakdown by extracellular hydrolyzing enzymes terminates the effects of acetylcholine [3], and participates in the inactivation of many neuropeptides [3–5]. Because of these essential regulatory functions, neurotransmitter-inactivating processes have provided targets for drugs of both experimental and clinical significance, ranging from amine reuptake blockers to acetylcholinesterase inhibitors.

The abundant expression of cannabinoid receptors in brain and the behavioral consequences of their stimulation by drugs have led to suggest that an endogenous cannabinoid system exists in brain, which may participate in modulating such diverse brain functions as cognition, emotion and movement [6,7]. Anandamide (*N*-arachidonoylethanolamine), a cannabinoid receptor agonist isolated from brain tissue, is thought to be a central component of this modulatory system [8]. Anandamide mimics many of the pharmacological effects of cannabinoid drugs both in vitro and in vivo [8,9], and is produced in and released from neurons in an activity-dependent manner [10]. Two potential mechanisms of anandamide inactivation have been described. An enzyme activity, abundant in brain and liver, catalyses the hydrolysis of anandamide to arachidonic acid and ethanolamine [11–13]. This amidohydrolase activity is enriched in areas of the rat central nervous system that contain high densities of cannabinoid receptors, suggesting that it may contribute to anandamide degradation at its sites of action [12]. In addition to enzymatic hydrolysis, evidence for the existence of an anandamide transport system has been provided in primary cultures of rat brain neurons [10]. Do these mechanisms participate in the physiological inactivation of anandamide and, if so, what are their relative roles? These questions have been difficult to address, partly because selective pharmacological blockers of anandamide inactivation are not yet available.

In the present study we show that the bromoenol lactone, (E)-6-(bromomethylene) tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (BTNP, Fig. 1), a mechanism-based inhibitor of Ca^{2+}-independent phospholipase A_2 [14], is also potent in preventing anandamide hydrolysis both in vitro and in situ. Its distinctive chemical structure and pharmacological properties may make it an interesting lead for the development of more potent and selective inhibitors of anandamide hydrolysis.

2. Experimental procedures

2.1. Materials

Radiolabeled anandamide (221 Ci/mmol, 8177 GBq/mmol) was purchased from New England Nuclear (Wilmington, DE), and BTNP from Biomol Inc. (Plymouth Meeting, PA). Arachidonoyltrifluoromethylketone (AATFMK) was synthesized as previously described [15]. Briefly, arachidonoylchloride (Nu-Chek Prep, Elysian, MN) was allowed to react with trifluoroacetic anhydryde in dichloromethane containing pyridine for 1 h at room temperature. After extraction with chloroform, identity and purity of the reaction product were confirmed by gas-chromatography/mass spectrometry.

2.2. Anandamide amidohydrolase assays

Microsome fractions were prepared from brain or liver tissues of Wistar rats, as previously described [12]. Standard anandamide amidohydrolase assays were carried out for 10 min at 37°C in 0.5 ml of Tris-HCl buffer (50 mM, pH 8) containing microsomes (0.2 mg of protein) and [^{3}H]anandamide (1 nM [^{3}H]anandamide brought to 100 nM with unlabeled anandamide). The reactions were stopped by adding 1 ml of cold methanol, followed by extraction with 2 ml of chloroform. The chloroform phases were dried under a stream of nitrogen and fractionated by silica gel G thin-layer chromatography (TLC) (Analtech, Newark, DE), using a solvent system of chloroform/methanol/ammonium hydroxide (90:10:1, v/v/v). The lipids were visualized by spraying the TLC plates with a solution of phosphomolybdic acid in ethanol (10%, w/v). The bands comigrating with authentic free arachidonic acid (AA) were scraped off the plates, and radioactivity measured by liquid scintillation counting. BTNP and AATFMK were

*Corresponding author. Fax: (1) (619) 626-2199.
E-mail: piomelli@nsi.edu

[1] *Contributed equally to this work.*

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diluted, from a stock solution of 100 mM in DMSO, with Tris-HCl buffer (50 mM) to a working solution (430 μM), which was used for subsequent serial dilutions. Samples were incubated with the drugs for 10 min at 37°C prior to the enzyme assay.

2.3. Amidohydrolase fractionation by Fast Protein Liquid Chromatography (FPLC)

Samples of brain or liver microsome fractions (1 mg of protein) were applied, at a flow rate of 2 ml/min, to a MonoQ HR 10/10 column (Pharmacia, Piscataway, NJ), which had been equilibrated with Tris-HCl (20 mM, pH 8) containing NaCl (50 mM). Anandamide amidohydrolase activity was eluted from the column with a gradient of NaCl in Tris-HCl (20 mM), as illustrated in Fig. 3. The column effluent was collected in 2-ml fractions, 0.5 ml of which were used for the enzyme assays.

2.4. Preparation of neuron and astrocyte cultures

Cortical neurons devoid of glial cells were prepared in a serum-free culture medium, essentially as previously described [16]. Briefly, cerebral cortices of 18-day-old Wistar rat embryos were dissected, cells were dissociated mechanically and seeded at a density of 10^6 cells/ml in culture dishes which had been previously coated with polyornithine (10 μg/ml) and polylysine (100 μg/ml). The culture medium was composed of Dulbecco's modified Eagle's medium (DMEM) supplemented with streptomycin (100 μg/ml), penicillin (60 μg/ml), insulin (25 μg/ml), transferrin (100 μg/ml), putrescine (60 μM), progesterone (20 nM), sodium selenite (30 nM), $NaHCO_3$ (10 mM) and HEPES (10 mM, pH 7.3).

Cortical astrocytes were prepared from 18-day-old Wistar rat embryos. Cerebral cortices were dissected and cells were mechanically dissociated in an Earle's balanced salt solution containing bovine serum albumin (BSA, 0.2%), trypsin inhibitor (0.2%), and DNAse (50 Kunitz units/ml). Cells were plated at a concentration of 25×10^6 cells per dish (90 mm diameter) using a culture medium composed of a Minimum Essential Medium (44%), F-12 nutrient mixture (44%), glucose (0.6%), fetal bovine serum (5%), fetal horse serum (5%), glutamine (1 mM) and phenol red (0.0015%). Cells were replated twice, after 1 and 2 weeks in culture, and grown in a medium in which fetal horse serum had been replaced by fetal bovine serum.

2.5. Anandamide amidohydrolase activity in neurons and astrocytes in culture

Enzyme assays were carried out with neurons after 5 to 6 days in culture, and with astrocytes after 3 to 4 weeks in culture. Cells were rinsed twice with Krebs-Tris buffer (NaCl 136 mM, KCl 5 mM, $MgCl_2$ 1.2 mM, $CaCl_2$ 2.5 mM, glucose 10 mM, Trizma base 20 mM; pH 7.4). [^{3}H]Anandamide (0.45 nM) was dissolved in Krebs-Tris buffer and mixed with unlabeled anandamide to reach a final concentration of 100 nM. When testing the effects of BTNP, cells were preincubated for 10 min with the drug (0.018–10 μM). The same concentration of BTNP was also added during the incubation period. After 20 min of incubation at 37°C, the medium was removed and the cells were rinsed with Krebs-Tris buffer containing fatty acid-free BSA (0.1%). The cells were collected in an ice-cold mixture of Krebs-Tris buffer/methanol (5:2; v/v), and lipids were extracted by adding chloroform to reach a final ratio of buffer/methanol/chloroform of 5:2:3 (v/v/v). The chloroform phases were split into two equal samples. One was used for phospholipid analysis, which was carried out on plastic-backed TLC plates (Alltech, Deerfield, IL) by using a solvent system of chloroform/methanol/ammonia/water (65:25:2.7:0.7; v/v/v). The second was used for anandamide analysis, which was carried out by open-bed column chromatography followed by TLC using a solvent system of chloroform/methanol/ammonia (80:20:1; v/v/v). In either case, bands from the plastic-backed TLC plates were cut at 1-cm intervals, and radioactivity measured by liquid scintillation counting.

2.6. Anandamide amidohydrolase activity in neuron and astrocyte homogenates

Neurons and astrocytes were grown in culture as described above. The cells were incubated twice at 37°C for 10 min with HEPES buffer (pH 7.4), lysed by adding ice-cold Tris-HCl (50 mM, pH 8), and homogenized. Anandamide amidohydrolase activity was measured under standard conditions.

3. Results and discussion

We incubated rat brain microsomes with exogenous [^{3}H]anandamide (100 nM) for 10 min, purified by TLC the free [^{3}H]arachidonic acid produced during the incubations, and quantified it by liquid scintillation counting. At this substrate concentration, chosen to approximate physiological conditions and well below the enzyme K_m [12], anandamide amidohydrolase activity was on average 2.85 ± 0.13 pmol/min/mg of protein (mean ± SEM, $n = 34$). When we included BTNP (20 μM) in the incubation buffer, the radioactivity associated with arachidonic acid was reduced to background levels, indicating a complete inhibition of the enzyme activity (data not shown). Fig. 2A demonstrates that this inhibitory effect was concentration-dependent: in three experiments the median inhibitory concentration (IC_{50}) of BTNP was 0.8 ± 0.3 μM.

Such potent inhibition of anandamide amidohydrolase activity was reminiscent of that produced by BTNP on cytosolic Ca^{2+}-independent phospholipase A_2 activity, which was inactivated with an IC_{50} of approximately 0.1 μM [14]. Inhibition of this phospholipase by BTNP was thought to result from the covalent binding of the drug at or near the enzyme's active site [14]. To examine the mechanism of anandamide amidohydrolase inhibition by BTNP, we incubated rat brain microsomes with a maximally effective concentration of the drug (25 μM) in the presence of varying concentrations of [^{3}H]anandamide (from 12.5 to 200 μM). Lineweaver-Burk analysis of the results, illustrated in Fig. 2B, showed that the inhibitory effect of BTNP was non-competitive, as expected of an irreversible enzyme inhibitor. In the absence of BTNP, we obtained a K_m for anandamide of 33 ± 5.6 μM and a V_{max} of 2038 ± 302 pmol/min/mg of protein ($n = 3$). In the presence of BTNP (25 μM), we obtained a K_m of 39 ± 11 μM and a V_{max} of 149 ± 49 pmol/min/mg of protein ($n = 3$). In further support of an irreversible mechanism, we noted that extensive dialysis of the microsomal incubates (1 l/ml of sample for 4 h) did not reverse the BTNP inhibition (Table 1).

There is both experimental and therapeutic interest in developing potent and selective inhibitors of anandamide hydrolysis. This has recently led to identify a group of polyunsaturated fatty acyl trifluoromethylketones which may act as

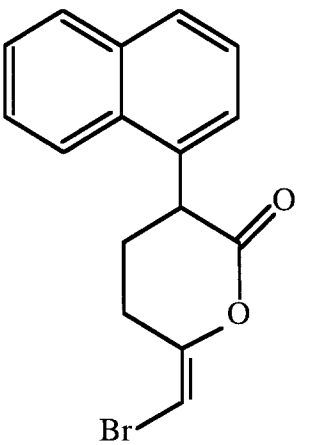

Fig. 1. Chemical structure of (E)-6-(bromomethylene) tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (BTNP).

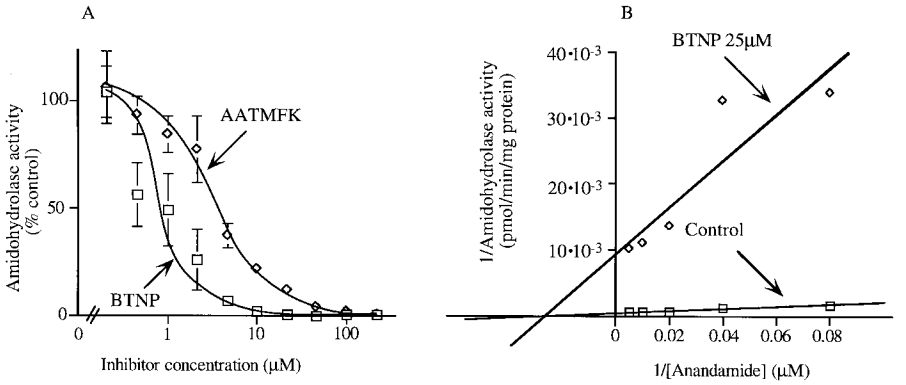

Fig. 2. Inhibition of [^{3}H]anandamide hydrolysis in rat brain microsomes. A: Concentration-dependent inhibition with BTNP and arachidonoyltrifluoromethylketone (AATFMK). Results are from three experiments. B: Lineweaver-Burk analysis of BTNP inhibition. Results are from one experiment representative of three.

transition-state inhibitors of this enzyme activity [17]. One such compound, AATFMK, prevents anandamide hydrolysis in subcellular fractions of neuroblastoma N18TG2 cells, where it displays an IC_{50} of 0.7–3 μM [18]. Under our experimental conditions, AATFMK effectively inhibited anandamide amidohydrolase activity, although 5-fold less potently than BTNP ($IC_{50} = 4 \pm 1$ μM, $n = 3$) (Fig. 2A).

To obtain evidence that BTNP acts through a direct interaction with the enzyme responsible for anandamide hydrolysis, we examined the inhibitory effects of this drug after fractionation of the enzyme activity by FPLC. In three experiments, we applied samples of non-solubilized rat brain microsomes to a MonoQ column, eluted the proteins with a NaCl gradient, and measured anandamide amidohydrolase activity in the column eluate. As shown by the representative experiment depicted in Fig. 3, four peaks of enzyme activity were resolved by MonoQ chromatography (lower tracing, control), which were all effectively inhibited by BTNP (10 μM) (lower tracing, BTNP). Multiple peaks of anandamide amidohydrolase activity have been already observed after partial purification from pig brain microsomes, but the significance of these putative isoforms is still unknown [19]. The recent cloning of an hydrolase involved in the degradation of long-chain fatty acid amides, including anandamide, should help shed light on this question [20].

In the rat, anandamide amidohydrolase activity is mainly localized in liver and brain [12]. When tested on crude microsomes prepared from rat liver tissue, BTNP inhibited anandamide hydrolysis with an IC_{50} that was about 100-fold greater than that measured in brain microsomes (79.5 ± 12.9 μM, $n = 3$). However, this marked difference likely resulted from BTNP degradation by liver enzymes, rather than from the existence of tissue-specific amidohydrolase isoenzymes. Two findings support such conclusion. First, after FPLC fractionation, anandamide amidohydrolase activity from liver was inhibited by BTNP as effectively as the activity from brain (data not shown). Second, incubation of BTNP with a mixture of brain and liver microsomes for 10 min at 37°C prevented the inhibition of anandamide hydrolysis (data not shown).

The regional distribution of anandamide hydrolysis in the rat central nervous system, paralleling that of CB_1 cannabinoid receptors, suggests that this enzymatic reaction may be at least in part responsible for the biological disposition of anandamide [12]. Yet, it is still unclear whether anandamide amidohydrolase activity is localized in neurons (which are thought to express the majority of brain CB_1 receptors) [21,22], in glial cells or in both. We prepared cell-type specific cultures of neurons or astrocytes from the cortex of embryonic rats, and measured anandamide amidohydrolase activity in homogenates of these cultures. Supporting a preferential neuronal localization, we found that the enzyme activity was 12-fold greater in neurons (1.94 ± 0.1 pmol/min/mg protein, $n = 7$) than in astrocytes (0.16 ± 0.01 pmol/min/mg protein, $n = 6$). BTNP was equally potent in inhibiting anandamide amidohydrolase activity, producing in either cell type an inhibition of approximately 80% at 0.5 μM (neurons: control 9472 ± 466 dpm/min/mg of protein; BTNP treated 1807 ± 78 dpm/min/mg of protein, $n = 4$; astrocytes: control 919 ± 17 dpm/min/mg of protein; BTNP treated 197 ± 21 dpm/min/mg of protein, $n = 3$. Data are from one experiment representative of three). Noteworthy, Shivachar and coworkers have previously reported that cultures of rat cortical astrocytes contain negligible levels of anandamide amidohydrolase activity [23]. This discrepancy may be due to different culture conditions and/or assay sensitivity.

To determine whether BTNP inhibits anandamide hydrolysis in intact cells, we tested its effects on primary cultures of rat cortical neurons, incubated for 4 min in a medium containing [^{3}H]anandamide. As previously noted, [^{3}H]anandamide was readily hydrolyzed by the neurons, and virtually all of the [^{3}H]arachidonate produced in this reaction was found esterified into cellular phospholipids, most prominently into phosphatidylcholine (PC) and phosphatidylethanolamine (PE) [10]. BTNP prevented [^{3}H]anandamide hydrolysis by the

Table 1

	Microsomes		Microsomes+10 μM BNTP		% Residual activity
	(dpm/min/mg protein ± SEM) ($n = 3$)		(dpm/min/mg protein ± SEM) ($n = 3$)		
Before dialysis	16797	(±681)	201.6	(±36.72)	0.01
After dialysis	13071.5	(±863.5)	69.88	(± 4.5)	0.005

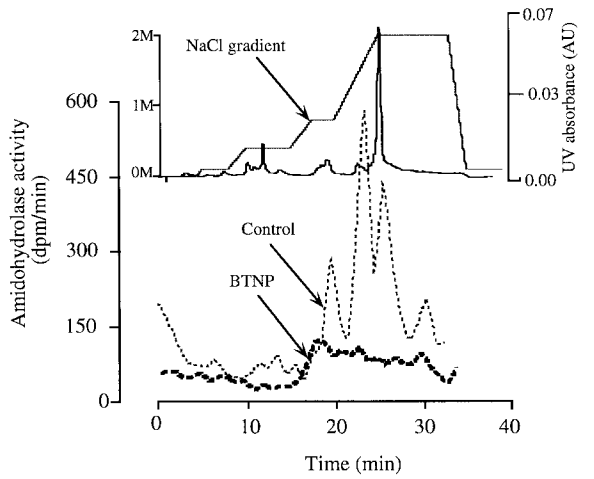

Fig. 3. Fractionation of anandamide amidohydrolase activity by MonoQ column chromatography. Proteins were eluted with a NaCl gradient, as illustrated in the upper tracing, and elution was monitored by UV detection (A_{280}, middle tracing). Fractions of the column eluate were collected and assayed for enzyme activity under standard conditions with or without BTNP (10 μM) (lower tracings). Results are from one experiment, representative of three.

neurons in a concentration-dependent manner, as indicated by a reduced incorporation of [^{3}H]arachidonate into PC and PE, as well as by an increased intracellular accumulation of unmetabolized [^{3}H]anandamide. Both effects occurred with an IC_{50} close to 0.1 μM (Fig. 4).

We have also investigated the effects of BTNP on the enzymes involved in anandamide biosynthesis. In mixed cortical cultures, formation of anandamide and other *N*-acylethanolamines is thought to be mediated by a D-type phospholipase activity, and is stimulated by the Ca^{2+} ionophore ionomycin [10,24,25]. To determine whether BTNP inhibits *N*-acylethanolamine formation we labeled cortical cultures overnight with [^{3}H]ethanolamine, and stimulated them with ionomycin (1 μM) either in the presence or in the absence of BTNP (25 μM). Radioactivity in the *N*-acylethanolamine fractions, determined after TLC fractionation, was: control 83 ± 41 dpm, ionomycin 123 ± 9 dpm, ionomycin plus BTNP 248 ± 35 dpm ($n = 3$). These results suggest that BTNP inhibits anandamide degradation without affecting the formation of anandamide and other *N*-acylethanolamines [10]. Next, we measured the effects of BTNP on the biosynthesis of *N*-arachidoyl PE, a putative anadamide precursor. Particulate fraction of the rat brain tissue were incubated at 37°C for 60 min in the presence of di[^{14}C]arachidonoyl phosphatidylethanolamine and the *N*-arachidonoyl PE produced was fractionated by TLC. Under these conditions, BTNP inhibited *N*-arachidonoyl PE with an IC_{50} of approximately 2 μM [24].

4. Conclusion

Our results indicate that BTNP is a potent and irreversible inhibitor of anandamide hydrolysis. BTNP was previously shown to inhibit chymotrypsin-like proteases and, very potently, cytosolic Ca^{2+}-independent phospholipase A_2. Despite this relative lack of selectivity, the potency of BTNP in inhibiting anandamide hydrolysis, its efficacy both in vitro and in situ, and its distinctive chemical structure — different from previously described amidohydrolase inhibitors — suggest

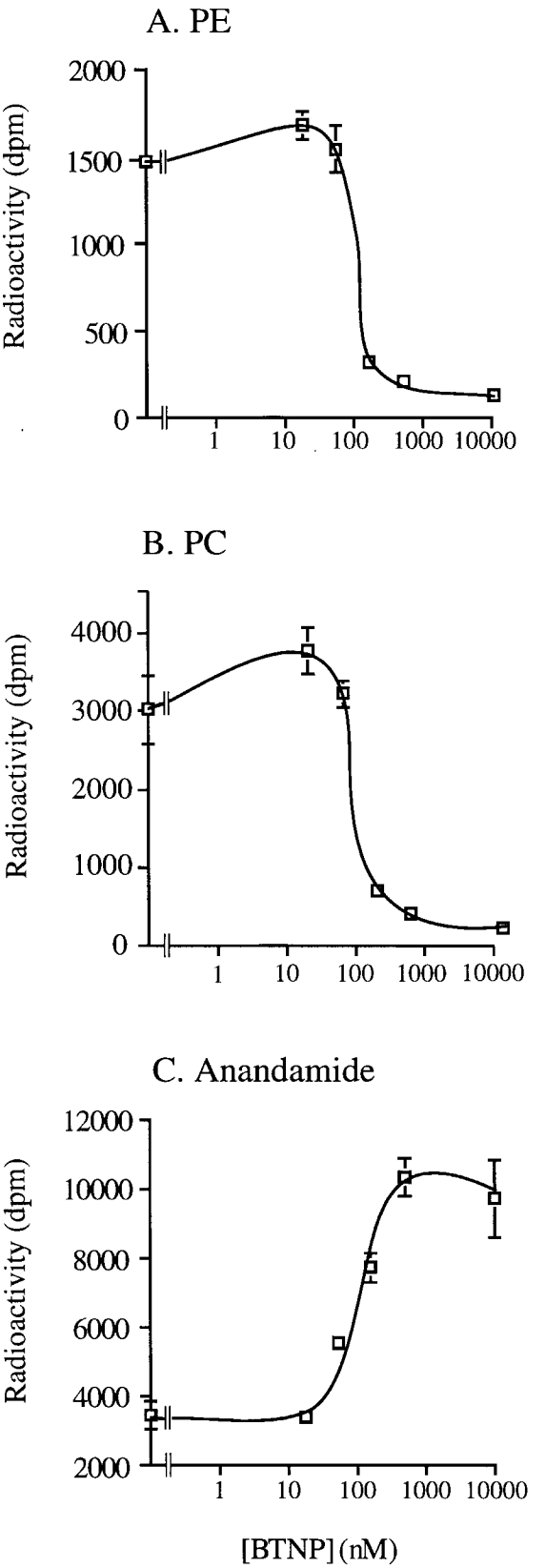

Fig. 4. Inhibition of [^{3}H]anandamide hydrolysis by BTNP in neurons. Cultures of rat cortical neurons were incubated in a medium containing [^{3}H]anandamide with or without BTNP. [^{3}H]anandamide, [^{3}H]phosphatidylethanolamine (PE) and [^{3}H]phosphatidylcholine (PC) were purified and quantified by liquid scintillation counting. Points represent the mean ± SEM of 3–6 independent determinations.

that this drug may provide a useful lead for the development of novel pharmacological agents that interfere with anandamide inactivation. Such drugs may help elucidate the roles of the endogenous cannabinoid system, and may find applications in pathological conditions where an indirect activation of this system is expected to cause a desirable pharmacological effect.

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