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Membrane localization of *N*-acylphosphatidylethanolamine in central neurons: studies with exogenous phospholipases

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Abstract

We studied the localization of *N*-acyl phosphatidylethanolamine (NAPE), a putative cannabinoid precursor, in primary cultures of striatal and cortical neurons from the rat brain. We probed intact neurons with various exogenous phospholipases, including *S. chromofuscus* phospholipase D (PLD). *S. chromofuscus* PLD does not penetrate into neurons (as demonstrated by a lack of internalization of ^{125}I-labeled PLD), and does not cause gross damage to the neuronal membrane (as demonstrated by a lack of effect of PLD on [^{3}H]γ-aminobutyric acid release). When neurons, labeled to isotopic equilibrium with [^{3}H]ethanolamine, were incubated for 10 min with *S. chromofuscus* PLD, approximately 50% of neuronal NAPE was hydrolysed. This hydrolysis was accompanied by the release of a family of *N*-acyl ethanolamines (NAE) (assessed by high performance liquid chromatography), which included the cannabinoid receptor agonist, anandamide. Exogenous phospholipase A_2 (PLA_2) (*Apis mellifera*) and PLC (*B. cereus*) mobilized [^{3}H]arachidonate and [^{3}H]diacylglycerol, respectively, but had no effect on NAE formation under these conditions. These experiments indicate that $\approx 50\%$ of neuronal NAPE is localized in a compartment that is easily accessible to extracellular PLD, possibly the plasmalemma, where it would also be easily hydrolyzed upon stimulation to produce NAE.

Keywords: Cannabinoid; *N*-acylethanolamine; Anandamide; Phospholipase D; Neuromodulation

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1. Introduction

Since the pioneering studies of Schmid and coworkers, it has been recognized that mammalian tissues have the ability to produce small quantities of saturated and unsaturated fatty acid ethanolamides (*N*-acylethanolamines, NAEs) (reviewed in Schmid et al., 1990). The biological roles of these lipids have remained elusive, however, until the isolation of *N*-arachidonoylethanolamine (anandamide), and the discovery that this naturally-occurring NAE binds to and activates CB1-type cannabinoid receptors in the brain (Devane et al., 1993; reviewed in Mechoulam et al., 1994).

That anandamide may be the long-sought endogenous cannabinoid substance is indicated not only by its remarkable pharmacological properties, largely overlapping with those of plant-derived or synthetic cannabinoid receptor agonists, but also by a growing body of biochemical evidence. Anandamide is produced in and released from central neurons, but not from astrocytes, in a calcium ion-dependent manner (Di Marzo et al., 1994). Also, anandamide is rapidly and selectively taken up into neurons and astrocytes (Di Marzo et al., 1994), and hydrolyzed to ethanolamine and arachidonate by a selective anandamide amidohydrolase activity (Desarnaud et al., 1995). These properties (neuronal synthesis, calcium-dependent release, local inactivation) are characteristic of brain signaling molecules, supporting the hypothesis that anandamide may serve such a function in the mammalian central nervous system. In an important sequel to these findings, it has now been suggested that the saturated congener of anandamide, *N*-palmitoylethanolamine, may exert a parallel action on non-neuronal cells, by binding selectively to CB2-type cannabinoid receptors, which have been identified thus far in mastocytes and other immune cells (Facci et al., 1995; Munro et al., 1993).

While the case for a signaling role of anandamide and of related NAEs is gaining support, the molecular mechanisms underlying the generation of these lipids in tissues remain the object of debate. Two mechanisms have been proposed: the energy-independent condensation of ethanolamine and arachidonate, catalysed by anandamide synthetase activity (Deutsch and Chin, 1993; Devane and Axelrod, 1994; Kruszka and Gross, 1994); and the phospholipase D (PLD)-mediated cleavage of a membrane precursor phospholipid, *N*-acyl phosphatidylethanolamine (NAPE) (Di Marzo et al., 1994; Cadas et al., 1996). Although these two mechanisms need not be mutually exclusive (multiple paths of biosynthesis exist for many biologically active molecules, including several lipids) it would certainly be desirable to define their respective physiological roles in the formation of cannabimimetic NAEs.

In primary cultures of rat brain neurons, NAPE may serve as the precursor for anandamide, *N*-palmitoylethanolamine and other NAEs. Evidence supporting this possibility includes the existence of an enzymatic activity that catalyses the formation of NAEs from exogenous NAPE, as well as the occurrence of endogenous NAPE in unstimulated neurons (Di Marzo et al., 1994). To serve as a precursor for extracellular messengers such as anandamide and *N*-palmitoylethanolamine, NAPE is expected to be located in a membrane compartment from which newly-formed NAEs can readily access the extracellular space. In the present study, we have investigated the cellular localization of NAPE in intact neurons in culture by probing their plasma membrane composition with exogenous phospholipases.

2. Experimental procedures

Primary cultures of rat brain striatal neurons were prepared, kept in culture and labeled with [^{3}H]ethanolamine or [^{3}H]arachidonic acid as previously described (Di Marzo et al., 1994). The neurons (plated at a density of 2.5×10^7 cells/dish in 90 mm dishes) were used after 10 days in vitro. The labeled neurons were washed with Dulbecco Modified Eagle's Medium (DMEM) and incubated for 10 min in DMEM containing one of the following phospholipases: phospholipase D (PLD), from *Streptomyces chromofuscus* (12.5 U/ml, Boehringer Mannheim or Sigma), phospholipase A_2 (PLA_2), from *Apis mellifera* (6.8 U/ml, Sigma) and phospholipase C (PLC), from *Bacillus cereus* (40 U/ml, Boehringer Mannheim). Incubations were stopped by adding methanol, immediately followed by chloroform extraction. Analyses of the [^{3}H]NAEs present in the lipid extracts were carried out as described (Fontana et al., 1995) [^{3}H]NAPE was isolated by thin-layer chromatography (TLC), using plastic-backed silica gel G plates eluted with a solvent system of chloroform/methanol/ammonia (80/20/1, v/v/v). Lipids were visualized with phosphomolybdic acid, and radioactivity measured by liquid scintillation counting after cutting the plates. [^{3}H]Ethanolamine was determined in the aqueous phase of neuronal extracts by fractionation on polydivinylbenzene minicolumns, as described (Desarnaud et al., 1995). In some experiments, we measured [^{3}H]arachidonic acid and [^{3}H]diacylglycerol in the extracts by using standard TLC methods (Hamilton and Hamilton, 1992). To measure [^{3}H]γ-aminobutyric acid (GABA) release, the neurons were incubated with [^{3}H]GABA (Amersham) for 30 min at 37°C, washed 5 times with DMEM, and then incubated in DMEM containing PLD (10 U/ml) for 10 min at 37°C.

3. Results and discussion

Our main objective in carrying out the present experiments was to determine whether the plasma membrane of brain neurons contains NAPE. Interest in this question lies in the potential role of NAPE as precursor for anandamide and *N*-palmitoylethanolamine, two NAEs that are thought to be released from neurons (Di Marzo et al., 1994; Cadas et al., 1996) and to act extracellularly by binding to cannabinoid receptors on neighbouring neuronal or non-neuronal cells (Mechoulam et al., 1994; Facci et al., 1995).

Because cellular fractionation methods require substantial quantities of biological material and are therefore poorly applicable to neurons in primary culture, we took an alternative course. By applying an approach extensively used with circulating blood cells (reviewed in Waite, 1987), we incubated prelabeled rat brain striatal neurons in culture with purified PLD (from *S. chromofuscus*) and determined the effect of this treatment on the levels of [^{3}H]NAPE and on the formation of [^{3}H]NAEs.

We found that incubation with PLD resulted in the formation of large amounts of [^{3}H]NAEs, which we identified by a combination of TLC and normal-phase HPLC (Fig. 1a and Fig. 1d). Analysis of these [^{3}H]NAEs by two sequential reversed-phase HPLC steps revealed a family of saturated and unsaturated congeners (Fig. 1d and

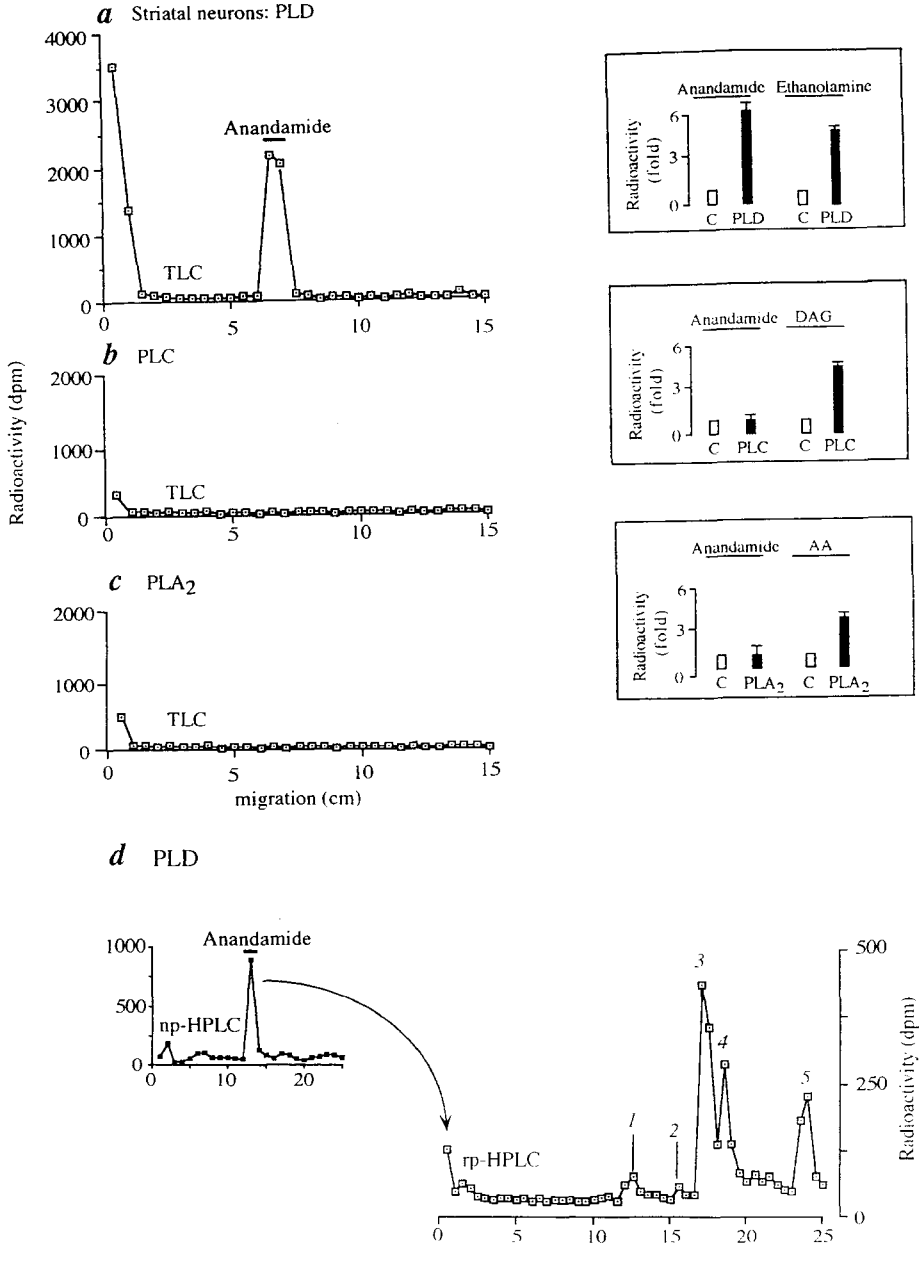
a Striatal neurons: PLD
Anandamide
TLC
b PLC
TLC
c PLA2
TLC
Radioactivity (dpm)
migration (cm)
Anandamide Ethanolamine
Radioactivity (fold)
C PLD C PLD
Anandamide DAG
C PLC C PLC
Anandamide AA
C PLA2 C PLA2
d PLD
Anandamide
np-HPLC
rp-HPLC
Radioactivity (dpm)

Fig. 1.

Table 1
Compositions of the [^{3}H]NAEs produced by striatal neurons in primary culture, incubated in the presence of exogenous PLD. Analyses were carried out by two sequential reversed-phase HPLC steps, as described (Fontana et al., 1995). Data are expressed as percent of total [^{3}H]NAEs recovered

	(% of total)
N-arachidonoylethanolamine	0.2
N-γ-linolenoylethanolamine	5.0
N-linoleoylethanolamine	0.2
N-oleoylethanolamine	27
N-stearoylethanolamine	20
N-palmitoylethanolamine	52

Table 1). These were similar in qualitative composition to those produced by stimulation of striatal neurons with membrane-depolarizing agents or by PLD treatment of purified NAPE, but showed a greater percentage of saturated species (Di Marzo et al., 1994). This discrepancy may be explained by the fact that, unlike saturated NAEs, anandamide and other polyunsaturated NAEs are good substrates for anandamide amidohydrolase activity, and are subject to rapid hydrolytic degradation (Desarnaud et al., 1995). As expected, [^{3}H]NAE formation was accompanied by release of [^{3}H]ethanolamine, a product of PLD hydrolysis, from the neurons. By contrast, formation of [^{3}H]arachidonate or [^{3}H]diacylglycerol was not observed (Fig. 1a).

The effect of PLD on [^{3}H]NAE formation did not result from membrane damage. In support of this conclusion, we found that treating the neurons with PLD had no effect on either exclusion of the vital dye, Trypan blue (data not shown), or release of the striatal neurotransmitter, [^{3}H]GABA. Extracellularly released [^{3}H]GABA was $0.283 \pm 0.03\%$ of total [^{3}H]GABA in control neurons, and $0.276 \pm 0.09\%$ in neurons with PLD (mean $\pm$ SEM, $n = 5$). Also, incubation with PLD was not accompanied by internalization of this phospholipase. When the neurons were incubated for 10

Fig. 1. Effect of exogenous phospholipases on [^{3}H]NAE formation in rat brain striatal neurons. PLD stimulates the formation of NAEs. Representative TLC fractionation of samples from the 10-min incubation of neurons (one dish): a, with PLD (*S. chromofuscus*, 12.5 U/ml); b, with PLC (*B. Cereus*, 40 U/ml); c, with PLA_2 (*A. mellifera*, 6.8 U/ml). Bars indicate the mobility of synthetic anandamide. Radioactivity associated with the TLC origin is composed of [^{3}H]ethanolamine-labeled phospholipids, which are expected to be different after treatment with PLD, PLC or PLA_2. Bar graphs on the right show the mean $\pm$ SEM of five experiments in which we measured the radioactive material in NAE TLC fractions. The graph also shows the effects of PLD, PLC and PLA_2 on the formation of products characteristic of their respective enzymatic activities, using neurons labeled with either [^{3}H]ethanolamine or [^{3}H]arachidonic acid: a, free [^{3}H]ethanolamine and related water-soluble products (for PLD); b, [^{3}H]diacylglycerol (DAG, for PLC); c, [^{3}H]arachidonic acid (AA, for PLA_2); d, identification of the [^{3}H]NAEs produced by PLD treatment. Representative normal-phase HPLC (np-HPLC) and reversed-phase HPLC (rp-HPLC) fractionations of samples from the incubation of striatal neurons with PLD. Numbers indicate the retention times of the following synthetic NAEs: 1, *N*-γ-linolenoylethanolamine; 2, *N*-linoleoylethanolamine plus *N*-arachidonoylethanolamine (anandamide); 3, *N*-palmitoylethanolamine; 4, *N*-oleoylethanolamine; 5, *N*-stearoylethanolamine. Further purification of component 2 was obtained by an additional rp-HPLC step (Fontana et al., 1995).

min with [^{125}I]PLD (3.6×10^6 dpm/dish), little radioactivity above background was recovered in association with the cells (2000 ± 560 dpm/dish, or 0.06% of total, $n = 5$). Finally, the effect of PLD on [^{3}H]NAE formation was specific. Exogenous PLA_2 (from *A. mellifera*) and PLC (from *B. Cereus*) were potent in producing [^{3}H]arachidonate and [^{3}H]diacylglycerol, respectively, but had no effect on the formation of [^{3}H]NAEs (Fig. 1b and Fig. 1c).

To determine whether NAPE served as substrate for exogenous PLD under our experimental conditions, we measured the levels of [^{3}H]NAPE by TLC. We found that incubating the neurons with PLD for 10 min resulted in a marked decrease in the quantity of [^{3}H]NAPE recovered (Fig. 2). When the neurons were disrupted and

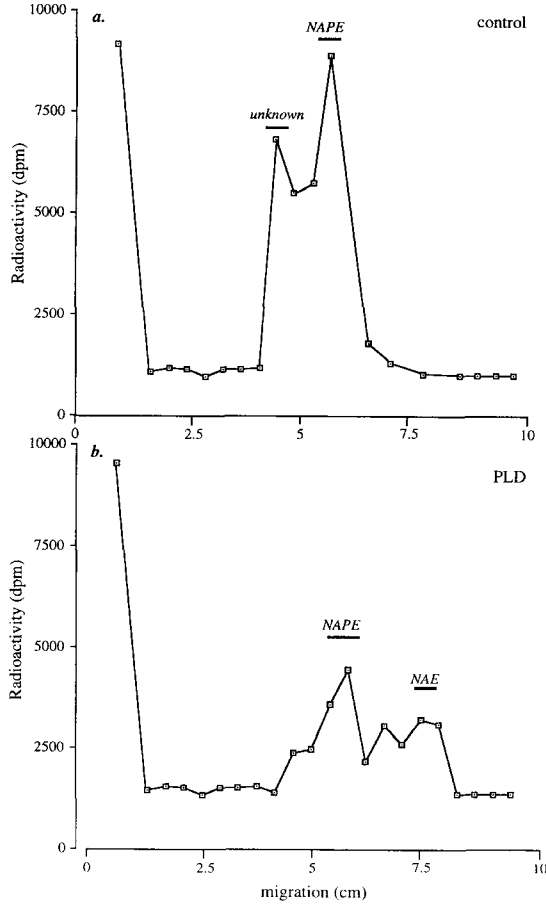

Fig. 2. Effect of exogenous PLD on [^{3}H]NAPE levels in striatal neurons: PLD catalyses the hydrolysis of endogenous [^{3}H]NAPE. Representative TLC fractionation of samples from the 10-min incubation of neurons (one dish): a, without PLD; b, with PLD. Bars indicate the mobilities of synthetic NAPE and NAEs, as well as that of an unknown [^{3}H]ethanolamine-containing lipid component.

endogenous [^{3}H]NAPE was purified and subjected to digestion with PLD, [^{3}H]NAPE was hydrolysed quantitatively (data not shown).

The finding that *S. chromofuscus* PLD does not penetrate neurons and yet is able to catalyse the hydrolysis of endogenous [^{3}H]NAPE, indicates that [^{3}H]NAPE is present in the plasma membrane of striatal neurons in culture. We estimate from our results that up to $\approx 50\%$ of neuronal [^{3}H]NAPE is sensitive to PLD treatment, and may be localized therefore in this membrane compartment. However, because the extent to which *S. chromofuscus* PLD penetrates the lipid bilayer in these neurons is unknown, we cannot conclude that [^{3}H]NAPE is restricted to the outer leaflet of this membrane. While further experiments will be necessary to address this issue (linked to that of the subcellular localization of NAPE-metabolizing enzymes), the presence of NAPE in neuronal plasma membranes lends support to the proposed role of this phospholipid as a cellular precursor for anandamide, *N*-palmitoylethanolamine and other NAEs that may act as intercellular messenger molecules. Treatment with PLD revealed also that release of [^{3}H]NAEs can take place independently of release of [^{3}H]GABA, an inhibitory neurotransmitter released by the majority of striatal neurons both in vivo and in vitro. Our results thus underscore the distinction between cannabimimetic signaling via cleavage of precursor NAPE and vesicular release of classical neurotransmitters.

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