A neuronal membrane protein, neuropathy target esterase (NTE), reacts with those organophosphates that initiate a syndrome of axonal degeneration. NTE has homologues in Drosophila and yeast and is detected in vitro by assays with non-physiological ester substrate, phenyl valerate. We report that NEST, the recombinant esterase domain of NTE (residues 727–1216) purified from bacterial lysates, can catalyze hydrolysis of several naturally occurring membrane-associated lipids. The active site regions of NEST and calcium-independent phospholipase A2 (iPLA2) share sequence similarity, and the phenyl valerate hydrolysis activity of NEST is inhibited by low concentrations of iPLA2 inhibitors. However, on incubation with NEST, fatty acid was liberated only extremely slowly from the sn-2 position of phospholipids (V_{max} = 0.01 \mu mol/min/mg and K_{m} = 0.4 \text{ mM} for 1-palmitoyl, 2-oleoylphosphatidylcholine). Comparison of the NEST-mediated generation of $^{14}$C-labeled products from two differentially labeled $^{14}$C-phospholipid substrates suggested that a rate-limiting sn-2 cleavage was followed very rapidly by hydrolysis of the resulting lyso-phospholipid. Among the various naturally occurring lipids tested with NEST, lysophospholipids were by far the most avidly hydrolyzed substrates ($V_{max} = 20 \mu mol/min/mg$ and $K_{m} = 0.05 \text{ mM}$ for 1-palmitoyl-lysophosphatidylcholine). NEST also catalyzed the hydrolysis of monoacylglycerols, preferring the 1-acyl to the 2-acyl isomer ($V_{max} = 1 \mu mol/min/mg$ and $K_{m} = 0.4 \text{ mM}$ for 1-palmitoyl- glycerol). NEST did not catalyze hydrolysis of di- or triacylglycerols or fatty acid amides. This demonstration that membrane lipids are its putative cellular substrates raises the possibility that NTE and its homologues may be involved in intracellular membrane trafficking.

**Neuropathy target esterase (NTE)** is an integral membrane protein in neurons and some non-neural cell types; it was originally identified as the primary site of action for those organophosphates which, in humans and other vertebrates, cause a syndrome characterized by axonal degeneration (1). The NTE homologue in Drosophila, the swiss cheese protein, is essential for fly brain development (2). In the mouse, NTE is present in neurons from their earliest appearance in the nervous system and so is well placed to play a similar role in mammalian neural development (3). NTE also has a homologue in yeast (4), suggesting that it has functions beyond the nervous system and mediates a biochemical reaction highly conserved through evolution.

In keeping with its reactivity with organophosphates, NTE belongs to the serine hydrolase class of enzymes. Since its discovery more than 30 years ago (5), NTE has been detected in vitro by assays with non-physiological ester substrates, most commonly with phenyl valerate (6). Clues to the cellular functions of NTE might be provided by identifying its natural substrate. Data on this point are sparse, but using artificial substrates, NTE activity in brain homogenates has been shown to catalyze hydrolysis of ester rather than peptide or amide bonds (7, 8). In addition, inhibition of the phenyl valerate hydrolysis activity of NTE by a homologous series of alkyl saligenin cyclic phosphonates (9) and alkyl-thiatrifluoromethyl ketones (10) suggests that the active site of the enzyme has a preference for esters of carboxylic acids with an alkyl chain length of at least 9–10 carbon atoms (1). This hints that the natural substrate of NTE may be an ester of a fatty acid, such as a phospholipid or an acylglycerol.

Human NTE is a polypeptide of 1327 residues and has two major domains (4) as follows: a C-terminal catalytic domain containing the active site serine residue (Ser-966) that reacts with organophosphates and phenyl valerate; and an N-terminal putative regulatory domain that contains sequences similar to cyclic AMP-binding proteins. We have expressed in Escherichia coli a recombinant protein (corresponding to NTE residues 727–1216) called NEST which comprises the esterase domain of NTE (11). NEST is very hydrophobic, and the purified protein must be incorporated into phospholipid liposomes to acquire a conformation with full phenyl valerate hydrolytic activity (12). In this respect, NEST appears similar to enzymes such as cytoplasmic phospholipase A$_2$ (13) and triacylglycerol lipase (14) which show the phenomenon of interfacial activation, i.e. they exhibit full catalytic activity only when at a lipid-water interface. Here, we use NEST to investigate the possibility that NTE has lipase- or phospholipase-A-like activity with membrane-associated lipids as its potential substrates.

## EXPERIMENTAL PROCEDURES

**Materials**—1-Palmitoyl, 2-oleoyl-sn-glycero-3-phosphatidylcholine (52 mCi/mmol) and dioleoyl-sn-glycero-3-phosphocholine (104 mCi/mmol) were from PerkinElmer Life Sciences. Methyl arachidonoyl fluorophosphonate (MAFP), 2-arachidonoylgllycerol, anandamide, and N-arachidonoyl ethanolamine were from CN Biosciences; bromoelaidoyl lactone and arachidonoyl trifluoromethyl ketone (–TFMK) were from Affiniti.
All other lipid substrates, putative lipase inhibitors, and bee venom secretory phospholipase A₂ were purchased from Sigma. Lipid substrates were prepared by evaporating chloroform solutions under a stream of nitrogen gas and then resuspending the residue in PEN buffer (50 mM sodium phosphate, 0.5 mM EDTA, 300 mM NaCl, pH 7.8) by sonication (MSE Sanyo Soniprep 150 probe sonicator) at maximum power for 10 min to give stock suspensions at a concentration of 5 mM. Free fatty acid assay kit (half-micro test) was from Roche Diagnostics. The sources of all other materials have been described previously (11).

Expression and Isolation of NEST—NEST (human NTE residues 727–1216 with N-terminal T7 and C-terminal His₆ tags) was expressed in E. coli BL21 pLysS, extracted, and isolated by nickel chelate and gel filtration chromatography in solutions containing CHAPS as described previously (11). NEST, with its catalytic serine residue mutated to alanine (11), was expressed and isolated in identical fashion. Before assaying its phenyl valerate hydrolase activity, purified NEST was diluted with 0.3% CHAPS to a protein concentration of 0.1 mg/ml, mixed with dioleoylphosphatidylcholine (DOPC; 10 mg/ml in 10% (w/v) CHAPS) in a ratio of 1:4 (w/w), and then dialyzed overnight at room temperature against PEN buffer.

Enzyme Assays—Phenyl valerate hydrolase activity was determined by the method of Johnson (6) as described previously (11). Purified NEST-DOPC complexes were diluted in 50 mM Tris-HCl, 1 mM EDTA to a protein concentration of 20–80 ng/ml and then incubated for 20 min at 37 °C with inhibitors before addition of phenyl valerate in Triton X-100 (final concentrations of 1.4 and 0.24 mM, respectively) and incubation for a further 20 min.

NEST lipase activity was assayed by determining the liberation of free fatty acid from various lipid substrates. For lysophospholipids and monoacylglycerols, incubations contained 2.5 mM substrate, NEST (0.1–3.0 µg of protein), and 2.4 mM CHAPS in a final volume of 0.05 or 0.1 ml of PEN buffer. Incubations were at 37 °C and were terminated at the times indicated in the figures (including zero time) by addition of MAFP to a final concentration of 20 µM. Free fatty acid was determined using a kit from Roche Diagnostics based on the coupled enzyme assay of Shimizu et al. (15). Zero time values (i.e. MAFP added before NEST) were subtracted from values determined at all subsequent time points. Data shown are the means of at least two separate experiments.

**Fig. 1.** Sequence similarity in the active site regions of iPLA₂ and NTE. a, iPLA₂, NTE, and NEST (the recombinant esterase domain of NTE) are represented as horizontal bars with the region of homology shaded. The location of the putative N-terminal transmembrane helix of the NTE is indicated by a vertical bar. b, the region of homology is shown with identical residues indicated white-on-black, similar residues in bold, and residues essential for the catalytic activity of NEST marked with an arrowhead.
lase activity of NEST-DOPC preparations. Bromoenol lactone inhibits iPLA2 with an IC50 value (after a 5-min preincubation) of 60 nM (18). Under standard conditions for assay of NEST-DOPC phenyl valerate hydrolase activity (see “Experimental Procedures”), we determined an IC50 value of 94 ± 20 nM (n = 5) for bromoenol lactone. MAFP inhibits iPLA2 with an IC50 in the submicromolar range (19) and NEST-DOPC phenyl valerate hydrolase activity with an IC50 of 2.0 ± 0.9 nM (n = 3). Finally, micromolar concentrations of two trifluoromethyl ketones (TFMK) inhibit iPLA2 activity, with palmitoyl-TFMK 4 times more potent than arachidonoyl-TFMK (18). These two compounds also inhibited NEST-DOPC phenyl valerate hydrolase activity but, in contrast to iPLA2, arachidonoyl-TFMK (IC50 = 2.5 nM; n = 2) was 10 times more potent than palmitoyl-TFMK (IC50 = 28 nM; n = 2).

Inhibition of the phenyl valerate hydrolase activity of NEST-DOPC preparations by fatty acid TFMKs led us to ask whether fatty acids themselves would be inhibitory. Preincubating NEST-DOPC with 50 μM oleic acid abolished phenyl valerate hydrolase activity, whereas palmitic and arachidonic acids at this concentration had marginal (25% inhibition) or no effect, respectively (Fig. 2a). Similarly, palmitate and arachidonate at concentrations of 30 μM did not inhibit iPLA2, although this study did not report the effect of oleic acid (18).

As free fatty acid and lysophospholipid are the products of PLA2 activity, we examined possible effects of lysophospholipid.
Neuropathy Target Esterase Has Lipase Activity

**FIG. 4.** NEST liberates free fatty acid from phospholipids. NEST was incubated with 3.2 mM CHAPS and 2 mM substrate (dioleoyl phospholipids (a) and phosphatidylcholines (b)), and at the indicated times reaction was stopped by addition of MAFP, and free fatty acid was quantified. (a) NEST was incubated with 3.2 mM CHAPS and 2 mM substrate (dioleoyl phospholipids, 1-palmitoyl, 2-oleoyl-[oleoyl-1-14C]PC; 1-O-H; 2-O-PC, 1-O-H; 2-A-PC, 1-O-hexadecyl, 2-arachidonoyl-PC).

**FIG. 5.** Release of [14C]oleic acid from sn-2-labeled phospholipid, time course and dependence on substrate concentration. NEST (9.3 μg) was incubated (37 °C) in 0.05 ml of PEN buffer with 3.6 mM CHAPS and 1-palmitoyl, 2-oleoyl-[oleoyl-1-14C]PC (POPC) at either 0.1 or 2.1 mM (a) or concentrations from 0.36 to 2.16 mM (b). Reactions were stopped after 5, 15, and 45 min (a) or 15 min (b), and [14C]oleic acid was quantified as described under "Experimental Procedures."
esterase activity to purified NEST requires its incorporation into phospholipid (DOPC) liposomes concomitant with removal of CHAPS by overnight dialysis at room temperature. Determination of free oleic acid concentration in the dialyzed samples yielded a value of 0.12 ± 0.02 mM (n = 7), compared with an original DOPC concentration of −0.5 mM. Free oleic acid was not detected in dialyzed mixtures of DOPC and detergent with S966A mutant NEST, indicating that generation of the fatty acid required catalytic activity.

We established conditions (2.5 mM phospholipid, 3.2 mM CHAPS) for assaying the lipase activity of NEST as described under “Experimental Procedures.” After 1 h at 37 °C, −0.8 μmol of oleic acid was liberated from DOPC per mg of NEST (Fig. 4a). This slow rate of hydrolysis was not substantially altered by several changes to the incubation conditions as follows: varying CHAPS concentration between 1.6 and 8 mM; by adding calcium or magnesium ions; or by adding ATP, which DOPC/dioleoylethanolamine/sphingomyelin/cholesterol); by incorporating DOPC into mixed lipid vesicles (various ratios of 2-arachidonoyl-PC and was 4–5 times faster than rates with DOPC, 1-palmitoyl, 2-oleoyl-PC, and dipalmitoyl-PC (Fig. 4b).

Whereas 1-O-hexadecanoyl, 2-arachidonoyl-PC is a substrate for iPLA₂ (20), it was not detectably hydrolyzed by NEST (Fig. 4b). This raised the possibility that NEST mediates selective cleavage of the sn-1 bond of phospholipids; to examine this issue we incubated NEST with differentially labeled [14C]phosphatidylycholines (Table I). Approximately twice as much [14C]-labeled oleic acid was liberated from substrate labeled in both the sn-1 and sn-2 positions (1.75 ± 0.05 nmol) as from substrate labeled exclusively at the sn-2 position (0.83 ± 0.01 nmol). Furthermore, no [14C]-labeled LPC was detected after reaction with the [(sn-1 + -2)-14C]PC substrate. By contrast, incubation of bee venom secretory PLA₂ with the sn-2-labeled substrate resulted in essentially quantitative conversion to [14C]oleic acid and, with the (sn-1 + -2)-labeled substrate, quantitative conversion to [14C]oleic acid and [14C]LPC (Table I). These results strongly suggest that NEST mediates a very slow cleavage of the sn-2 bond of phospholipid followed by a rapid hydrolysis of the resulting lysophospholipid product, possibly before this product is released from the enzyme. Cleavage of the sn-2 bond of 1-O-hexadecyl, 2-arachidonoyl-PC by NEST would yield a non-hydrolyzable lysophospholipid product; the latter may dissociate only very slowly from the enzyme and hence inhibit binding of further phospholipid substrate, giving rise to the result observed in Fig. 4b.

NEST liberated [14C]oleic acid from sn-2-labeled PC at rates that were approximately linear over a period of 15 min (Fig.
Fig. 8. NEST-catalyzed hydrolysis of monoacylglycerols. NEST was incubated with 2.4 mM CHAPS and 2.5 mM substrate (a, 1-, and 2-palmitoylglycerols (1-PG, 2-PG); b, 2-palmitoyl-, 1-oleoyl-, 2-oleoyl-, 2-arachidonoylglycerols (1-oleoylglycerol (1-OG), 2-oleoylglycerol (2-OG), 2-arachidonoylglycerol (2-AG))) and at the indicated times the reaction was stopped with MAEP and free fatty acid determined as under “Experimental Procedures.” Note different scales in a and b.

Fig. 9. NEST-catalyzed hydrolysis of 1-palmitoylglycerol: linear rate conditions (a) and dependence on substrate concentration (b). NEST (4 µg) was incubated (37°C) in 0.5 ml of PEN buffer with 2.4 mM CHAPS and 1-palmitoylglycerol at the concentrations (mM) indicated in the figure. At the times shown the reaction was stopped and free fatty acid determined as under “Experimental Procedures.” Portions of the time course (a) with linear reaction rates were used to derive data for the 1/v versus 1/v plot (b).

Other Naturally Occurring Lipid Substrates for NEST—NEST hydrolyzed monoacylglycerols, with a marked preference for the sn-1 isomer. After a 5-min incubation, 8–10 times more fatty acid was liberated from 1-palmitoylglycerol than from the 2-isomer (Fig. 8a). Although the first 2–5 min of NEST-catalyzed hydrolysis of 1- and 2-oleoylglycerol and 2-arachidonoylglycerol were at least as rapid as for 2-palmitoylglycerol, the rate diminished dramatically after this time (Fig. 8b). Thus, with certain members of each class of lipid substrate (phospholipids, lysophospholipids, and acylglycerols), NEST-catalyzed release of fatty acid shows an initial burst of variable duration and then slows markedly. A similar, poorly understood stalling in reaction rate has been reported for several phospholipases (21–23). Nevertheless, we were able to establish conditions under which approximately linear rates of hydrolysis of 1-palmitoylglycerol could be measured (Fig. 9a). Lineweaver-Burk transformations of these rates (Fig. 9b) allowed determination of values for $V_{\text{max}} = 1.37 \pm 0.47 \mu\text{mol/min/mg}$ and $K_m = 0.37 \pm 0.09$ (from three experiments).

In contrast to the relatively brisk hydrolysis of monoacylglycerols, NEST did not catalyze detectable fatty acid release from di- or triacylglycerols nor from a variety of acyl amides including sphingomyelin, anandamide, and N-oleylethanolamine (data not shown). Free fatty acid was slowly liberated when a suspension of cholesterol olate was incubated with NEST, at rates similar to those with DOPC (data not shown).

We established conditions under which linear rates of hydrolysis of 1-palmitoyl-LPC could be observed (Fig. 7a). Lineweaver-Burk transformation of data obtained under these conditions (Fig. 7b) allowed the determination of values for $V_{\text{max}} = 20.8 \pm 2.5 \mu\text{mol/min/mg}$ and $K_m = 0.054 \pm 0.006$ mM (from four experiments).

Lysophospholipids Are the Most Acidly Hydrolyzed Lipid Substrate for NEST—Fatty acid was generated rapidly when NEST was incubated with LPC. With 1-palmitoyl-LPC, initial rates of ~20 µmol/min/mg were about twice those observed with 1-oleoyl-LPC (Fig. 6a) and were ~200 times faster than those with 1-palmitoyl, 2-arachidonoyl-LPC (cf. Fig. 6b). As with the diacylphospholipid substrates, rates declined after 2–5 min, and this was not due to substrate depletion.

Among the lipid substrates tested with NEST, lysophospholipids gave by far the fastest initial rates. The affinity of the active site of NEST for lysophospholipids may underlie the potent inhibition by these compounds and their ether analogues of phenyl valerate hydrolase activity (cf. Figs. 2b and 3b). However, unlike phenyl valerate hydrolase activity, NEST lipase activity toward lysophospholipid (Fig. 6b) and other lipid substrates did not require incorporation into DOPC vesicles to achieve interfacial activation, suggesting that in these cases the substrate itself fulfills this requirement.

We established conditions under which linear rates of hydrolysis of 1-palmitoyl-LPC could be observed (Fig. 7a). Lineweaver-Burk transformation of data obtained under these conditions (Fig. 7b) allowed the determination of values for $V_{\text{max}} = 20.8 \pm 2.5 \mu\text{mol/min/mg}$ and $K_m = 0.054 \pm 0.006$ mM (from four experiments).
DISCUSSION

We have shown here that NEST, the recombinant esterase domain of NTE, liberates fatty acid from phospholipids, monoaoylglycerols, and lysophospholipids. Thus, more than 30 years after the discovery of NTE, we have identified naturally occurring, membrane-associated lipids as its potential endogenous substrates. This demonstration that NTE is a putative lipase provides new clues to its possible cellular functions.

Although the active site of NTE has primary sequence similarity to iPLA₂, it hydrolyzes diacylphospholipids much more slowly. Using sn-2-labeled \(^{[14]}\text{C}\)PC we found a \(V_{\text{max}} \sim 0.01\) \(\mu\text{mol/min/mg}\) NEST, whereas initial rates of 1–5 \(\mu\text{mol/min/mg}\) have been reported for iPLA₂ itself (20, 24). When assayed with relatively low detergent concentrations, both iPLA₂ and calcium-dependent cytosolic PLA₂ can display lysophospholipase activity of about the same magnitude (a few \(\mu\text{mol/min/mg}\)) as their maximal PLÀ₂ activity (21, 28, 29). NEST shows no significant sequence similarity to the cytosolic 25-kDa lysophospholipid-specific lysophospholipases (25, 26), and yet it catalyzes hydrolysis of 1-palmitoyl-LPC (\(V_{\text{max}} \sim 20\) \(\mu\text{mol/min/mg}\)) much faster than initial rates (1–2 \(\mu\text{mol/min/mg}\)) reported for recombinant lysophospholipase (27).

Under the experimental conditions in this report, lysophospholipids were by far the most avidly hydrolyzed substrate for NEST. It has been stressed repeatedly that the rates and selectivities of bond cleavage observed in lipase assays in vitro are profoundly affected by the physicochemical nature of the substrate (14, 16, 20, 23). Nevertheless, it seems reasonable to consider the possibility that lysophospholipids may be a physiological substrate for NTE.

The presence of a homologue of NTE in yeast (4) suggests that this enzyme mediates a cellular process conserved throughout much of eukaryotic evolution. NTE itself is firmly associated with intracellular membranes, and from a combination of esterase assays on brain subcellular fractions (30), immunohistochemistry on brain sections (31), and distribution of green fluorescent protein-tagged NTE in transfected COS-7 cells,² we have tentatively concluded that NTE may be localized predominantly in the endoplasmic reticulum and Golgi complex. What might be the biological significance of NTE-mediated lysophospholipase activity in the endoplasmic reticulum/Golgi of various cells from yeast to neurons?

There is a growing recognition that, along with other factors, the relative concentrations of diacyl- and lysophospholipids contribute to the degree of curvature of biological membranes and that this is an important determinant in the process of membrane fission, tubulation, and fusion (32–34). Addition to cultured mammalian cells of various PLÀ₂ inhibitors, including bromoelactone and arachidonoyl-TFMK, causes reversible fragmentation of the Golgi complex (35, 36). It has been suggested that a PLÀ₂ activity may be required for maintenance of Golgi complex architecture (35); this would result in continuous production of lysophospholipid within the Golgi. Indeed, in Golgi membranes (of rat kidney and liver), lysophospholipids compose 3–4% of the total phospholipid (37). It has been dem-

² Y. Li and P. Glynn, unpublished data.