Identification and Quantification of Neuroactive *N*-Acylethanolamines in Cottonseed Processing Fractions

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ABSTRACT: *N*-Acylethanolamines (NAE) include lipid species that have been identified as potent endocannabinoid mediators in mammalian physiology. Here we have isolated, identified, and quantified the NAE naturally present in cottonseed refining fractions. Among the fractions examined, NAE were most prevalent in finished meal, totaling approximately 8.5 μg/g fresh weight. These NAE were identified by GC–MS as ethanolamides of myristic (14:0), palmitic (16:0), stearic (18:0), oleic (18:1), and linoleic (18:2) acids, with NAE18:2 and NAE16:0 being the most abundant. NAE were not detected in refined, bleached, and deodorized oil, although they were present in less-purified oil fractions. At micromolar concentrations, both NAE18:2 and NAE16:0 exhibited neuromodulatory activities in murine neuronal networks cultured and evaluated on microelectrode arrays. It follows that oilseed processing fractions may represent a novel, natural source of bioactive NAE.

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KEY WORDS: *N*-Acylethanolamines, cottonseed products, endocannabinoids, lipid mediators, microelectrode arrays, neurotransmission.

Studies of the effects of ischemic injury on membrane lipids of mammalian tissues revealed that relatively large amounts of an unusual lipid fraction accumulated dramatically in infarcted areas (1). This fraction was identified as a mixture of *N*-acylphosphatidylethanolamines (NAPE) and *N*-acylethanolamines (NAE). These lipids are now identified as natural constituents of vertebrates, invertebrates, certain microorganisms, and higher plants (1,2). Accumulation of NAPE and NAE was initially attributed to physiological conditions leading to tissue degeneration and phospholipid degradation, and hence these lipids were presumed to play a role in membrane protection and to promote cellular survival (1,2).

In recent years, NAE were shown to bind and activate cannabinoid receptors CB1 and CB2, and these lipids are considered components of the endocannabinoid signaling system that mediates an array of physiological processes in animals (3,4). In addition, NAE appear to influence the activity of vanilloid receptors, protein kinases, ion channels, and nitric oxide synthase (3,4). Thus, the tight regulation of endogenous NAE levels in animals is important to the maintenance of normal physiological functions. Indeed, genetic studies to alter either the levels or the perception of NAE in transgenic animals emphasized the pleiotrophic effects of these lipid mediators (5,6). Perhaps the most extensively studied physiological role for the endocannabinoid signaling pathway to date is the regulation of neurotransmission by NAE, in which release of anandamide (NAE20:4) from postsynaptic neurons modulates presynaptic neurotransmitter release (7).

Our previous work with NAPE and NAE metabolism in plants indicated that desiccated seeds were enriched in NAE (e.g., total content of about 1.6 μg/g fresh weight in cottonseed; 8). These NAE were metabolized rapidly during seed imbibition and germination by two competing pathways, one involving a 13-lipoxygenase for the formation of NAE-derived oxylipins, and one involving an amidohydrolase for the hydrolysis of NAE (9), implying a physiological function for NAE in regulating seed germination. The occurrence of NAE in various types of desiccated oilseeds (including corn, soy, peanut, and cottonseed; 8) and reports by others of NAE presence in chocolate (10,11), prompted us to ask whether oilseed refining fractions contain measurable quantities of these bioactive lipids.

Here, we identify and quantify the NAE types in various samples typical of the industrial processing of raw cottonseed. Our results indicate that "finished" cottonseed meal was highest in total NAE content (about 8.5 μg/g fresh weight). NAE18:2 and NAE16:0 were most abundant in all fractions, and were assayed for neuromodulatory activity in spinal cord neuronal networks cultured on microelectrode arrays. With approximately three million metric tons (3×10^9 kg) of cottonseed crushed in the United States annually (12,13), cottonseed (and perhaps other oilseed) processing fractions may provide a plentiful, natural source for the recovery of *N*-acylethanolamines.

MATERIALS AND METHODS

NAE isolation and analyses. Cottonseed processing fractions were provided through the coordination of David Kinard (National Cottonseed Products Association) from a refinery in west Texas. Efforts were made to collect samples and seed from the same processing stream. Total lipids were extracted from all samples (except oil fractions), filtered, and subjected to normal-phase HPLC as described previously (8). Oil samples were dissolved in chloroform (1:1 vol/vol), filtered, and subjected to HPLC. This dilution was accounted for in final

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values reported for NAE. HPLC fractions were evaporated to dryness under a stream of N_2 gas, and the lipid residue was subjected to derivatization in bis(trimethylsilyl) trifluoroacetamide (BSTFA) at 50°C (30 min). Decachlorobiphenyl (DCB) was added as an internal standard to derivatized samples. NAE17:0, not present in cottonseeds, was added during lipid extraction to monitor recovery of NAE. Trimethylsilyl derivatives were analyzed by GC–MS, and NAE were identified by chromatographic retention time and EI mass spectra. NAE in samples were quantified according to standard curves developed for each NAE type (1 to 100 ng) using the mass ratio of diagnostic ions of the respective analytes to those of DCB. Synthetic NAE for GC–MS standards were synthesized from ethanolamine and the corresponding acylchloride according to the method of Hillard *et al.* (14) and were generally greater than 95% pure.

Spinal cord cultures on microelectrode arrays (MEA). The techniques used to fabricate and prepare MEA have been previously described (15–17). Briefly, MEA consisted of an array of 64 substrate-integrated electrodes covering a central 0.8 mm^2 recording area. Spinal cord tissue harvested from embryonic day 14 HSD:IRC mice was minced, triturated, mixed with Dulbecco's modified Eagle's medium (DMEM) containing 5% horse serum and 5% FBS (18), and seeded onto the recording area. The tissue was incubated at 37°C in a 10% CO_2 atmosphere and fed three times weekly with half-medium exchanges using DMEM containing 5% horse serum until ready for use. Spinal cord cultures form confluent glial and neuronal monolayer carpets by day 4, make functional synaptic connections, and develop spontaneous electrical activity by day 7. Cultures were generally used for experiments between 3 wk and 3 mon after seeding and remained viable and pharmacologically responsive for more than 6 mon (19).

Extracellular recording procedures and data analysis. MEA were removed from the incubator and transferred to a constant-temperature-bath stainless steel recording chamber (19,20) on a microscope stage at 37°C. pH 7.4 was maintained with a continuous stream of filtered, humidified, 10% CO_2. Extracellular signals of action potentials (spikes) were recorded with a two-stage amplifier system (Plexon Inc., Dallas, TX), consisting of 64 channels, with a total system gain of approximately 12,000 and digitized in real time. The effects of NAE18:2 and NAE16:0 were interpreted in terms of two network activity variables, spike and burst rate, and were analyzed using commercially available software (Neuroexplorer, Littleton, MA). Single-unit spike and burst activity per minute (60 s bin) was averaged across the entire network and plotted to show temporal changes.

NAE administration. Stock solutions of synthetic 18:2 and 16:0 ethanolamines were kept in DMSO at 10 mM concentration, protected from light, and under dry nitrogen at 4°C. To facilitate NAE solubility in culture medium, aliquots of stock NAE (never exceeding 0.7% of total bath) were dissolved in 0.5 mL of culture medium previously removed from the experimental neuronal network. The medium was then sonicated for 5 min, heated to 37°C, and returned to the network to give the appropriate final NAE concentration. All pipet tips and glass vials used were siliconized to prevent adhesion of NAE during applications.

RESULTS AND DISCUSSION

NAE in cottonseed processing samples were identified by retention time in GC (Table 1) and by their mass spectra (e.g., Fig. 1; NAE16:0), and were quantified by characteristic fragment ions (Table 1) according to standard curves developed for each NAE species. Methods adapted previously (8) for the isolation of NAE from crude plant lipid extracts by normal-phase HPLC effectively enriched all NAE types irrespective of abundance, chain length, and unsaturation level (Fig. 2). Even in complex mixtures such as those derived from "finished" cottonseed meal, NAE were quantitatively eluted between 11 and 15 min under these HPLC conditions.

We identified and quantified NAE in desiccated seed of various plants, including a variety of oilseeds (8), but somewhat surprisingly, we found no detectable levels of NAE in commercially refined vegetable oils from a variety of sources (data not shown). Cottonseed oil is solvent-extracted from cooked expander pellets into hexane and refined by removing polar lipids and other impurities through several steps including bleaching and steam distillation (13). The hexane-insoluble meal is dried and, in the case of cottonseed, is used as a feed for ruminant livestock. The normal cottonseed processing practice is to return the soapstock (removed from the oil) back to meal as an added nutritional supplement since is contains FA and other constituents (22). Lipid-soluble vitamins (e.g., vitamin E) are recovered from distillate fractions (13). A detailed examination of selected fractions from a typical cottonseed processing facility indicated that NAE were present in samples of whole cottonseed, kernels, flakes, and expander pellets but were present at highest levels in the "finished meal" (Fig. 3). Considerable amounts of NAE were quantified in crude oil and alkali-refined oil samples, but the final steam-distillation step appeared to remove all NAE to below detectable levels from the fully refined oil (Fig. 3). Although it is difficult to fully account for NAE in the industrially processed fractions due to continual removal of impurities during the refining process, it is likely that the higher than

TABLE 1
Summary of GC Retention Times and Fragmentation Ions Used for Identification/Quantification of Different NAE Types in Seed Lipid Fractions[a]

NAE type	Retention time/TMS derivative (min)	Fragmentation ions/TMS derivative (*m/z*)
NAE12:0	19.4	300, 272, 256, 225
NAE14:0	22.4	328, 300, 284, 253
NAE16:0	25.2	356, 328, 312, 281
NAE17:0[a]	26.7	370, 342, 326, 295
NAE18:2	27.9	395, 381, 380
NAE18:1	28.0	397, 382, 354, 307
NAE18:0	28.4	384, 340, 309, 256

[a]NAE, *N*-acylethanolamine; TMS, trimethylsilyl.

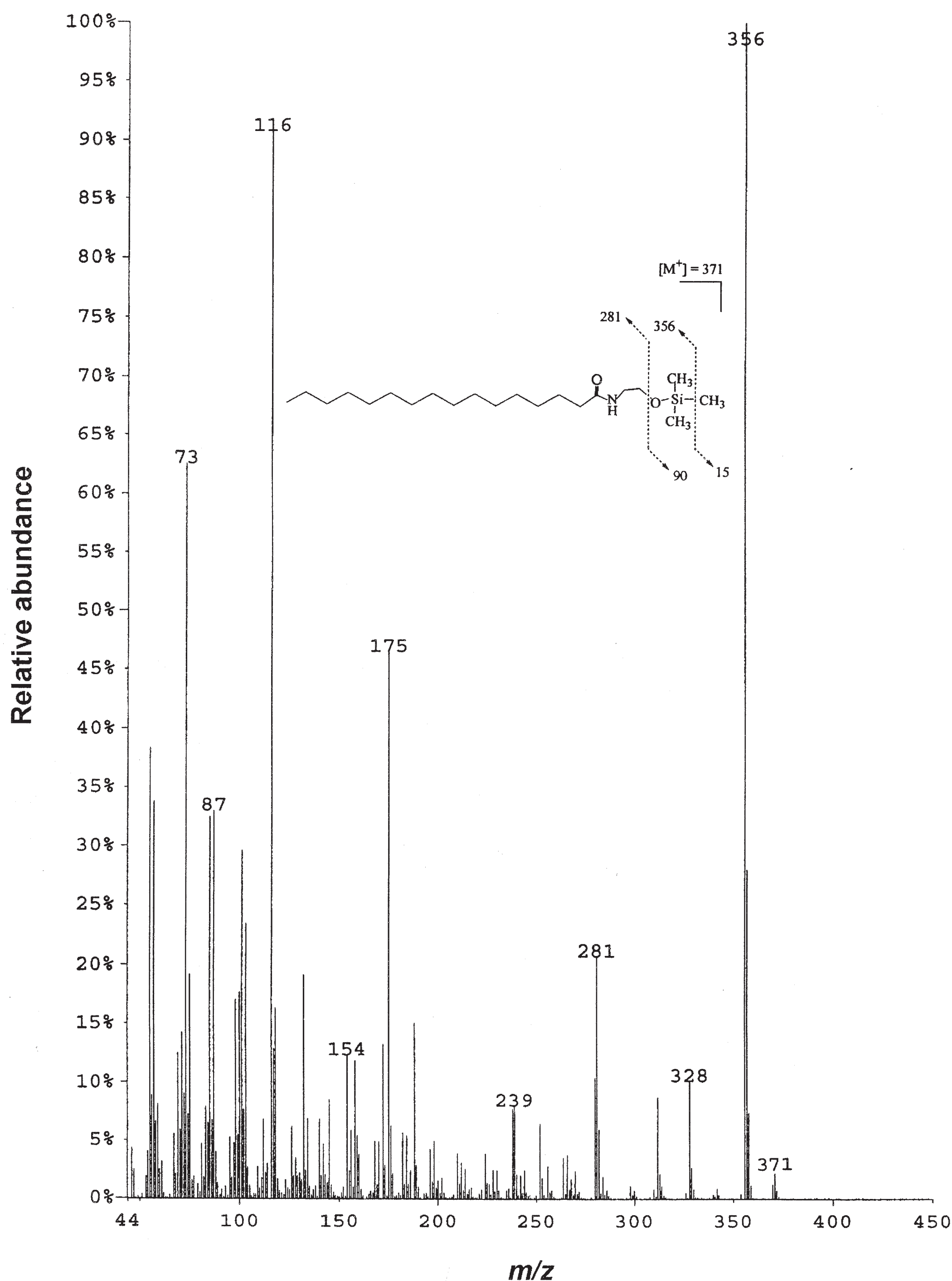

FIG. 1. EI mass spectrum of the trimethylsilyl (TMS) ether derivative of *N*-palmitoylethanolamine. The molecular ion M^+ is at *m/z* 371, and several fragment ions used for quantification are indicated on the structural diagram.

expected NAE levels in finished meal were a result of NAE being added back from soapstock, as is normal practice with mills that refine cottonseed oil (Kinard, D., National Cottonseed Processing Association, personal communication).

Others have reported NAE in plant-derived foods such as chocolate, soybeans, hazelnuts, and oatmeal (10,11). In these cases, although there was some debate as to the occurrence of NAE20:4, both NAE18:2 and NAE18:1 were identified in amounts similar to what we found for cottonseed fractions. In addition, we consistently found high levels of NAE16:0 in all cottonseed fractions (Fig. 3) and in all desiccated seed samples examined (8). Many believe that NAE16:0, NAE18:1, and NAE18:2 do not act directly in animal tissues through binding to cannabinoid receptors (3). Rather, these NAE act as inhibitors of endogenous anandamide (NAE20:4) degradation (4) and thereby exhibit cannabimimetic properties when

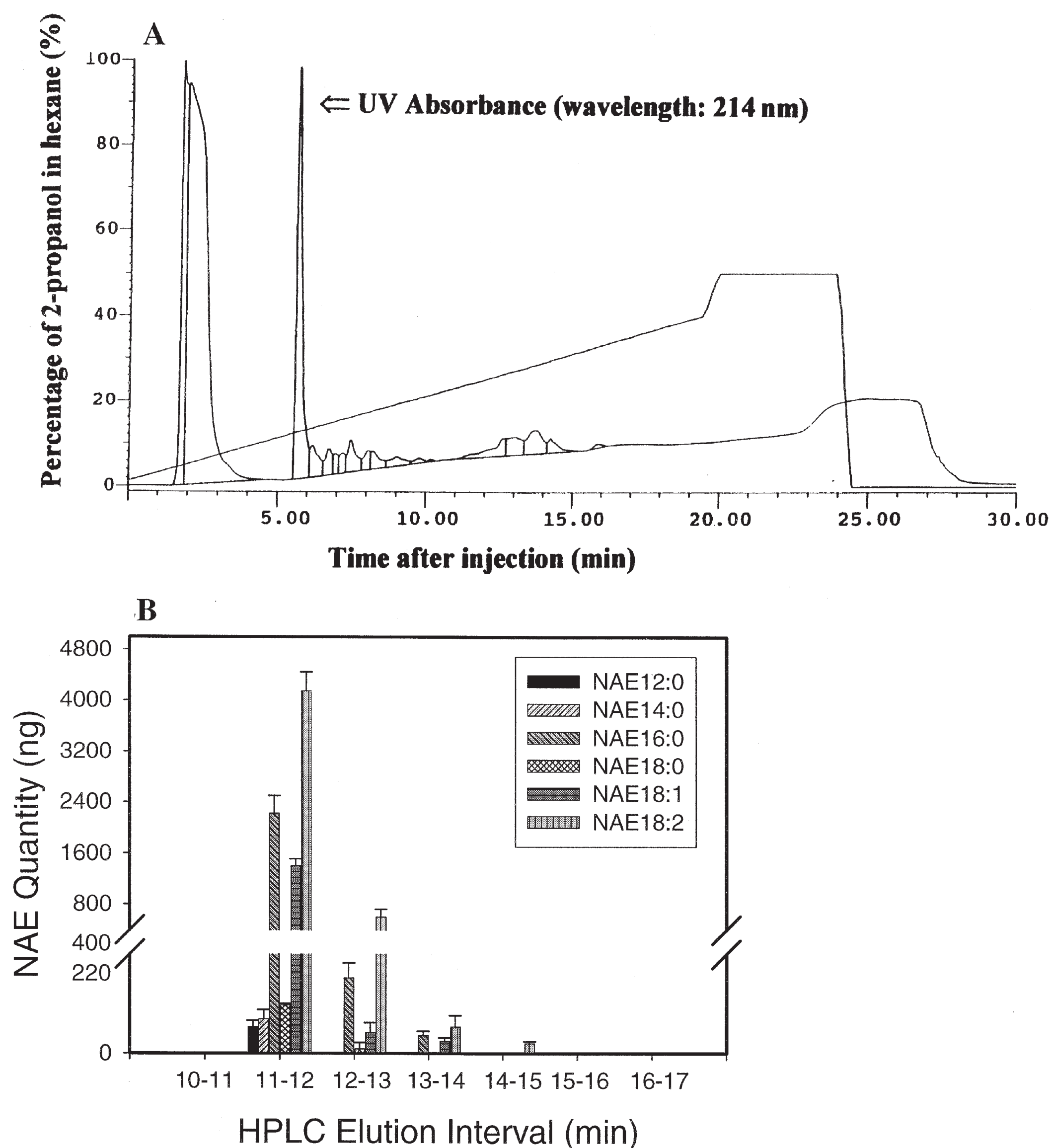

FIG. 2. Representative normal-phase HPLC fractionation of lipids extracted from cottonseed meal. The HPLC fractionation scheme was based on that developed originally by Piomelli and co-workers for *N*-acylethanolamine (NAE) analyses in animal tissues (21), and modified somewhat for the fractionation of plant lipids by Chapman *et al.* (8). Lipids dissolved in chloroform were subjected to normal-phase HPLC (4.6 × 250 mm Partisil 5 column, Whatman; model 712 HPLC system, Gilson) and eluted with a linear gradient of 2-propanol (0 to 40% over 20 min) in hexane (A). Eluting material was monitored by UV absorbance at 214 nm, and NAE types quantitatively eluted between 11 and 15 min (B). NAE types were identified and quantified by GC–MS as described in the Materials and Methods section. Error bars represent SD (n = 3).

administered to animals through their diet (11) or by injection (23).

The abundant NAE (NAE16:0 and NAE18:2) identified in cottonseed fractions inhibited spontaneous electrical activity in cultured primary neuronal networks in a dose-dependent manner (Figs. 4, 5). Previous research in our laboratory established that primary CNS (central nervous system) cultures retain functional CB1 receptors and respond histiotypically to CB1 receptor agonists and antagonists (24). Here, we tested the effects of NAE16:0 and NAE18:2 on extracellularly recorded action potentials (spikes) in spontaneously active spinal cord cultures. In Figure 4, two activity variables, spike and burst rate, are represented as 1-min averages from 64 discriminated nerve cells recorded from a single network. The minute means were graphed over time to show the temporal evolution of activity. Burst rate represents the grouping of high-frequency spikes and generally follows spike rates. The effective concentration range of NAE16:0 in spinal cord cultures was 5 to 60 µM, with an EC_{50} of approximately 33 µM. NAE18:2 was similarly potent, with an EC_{50} (effective con-

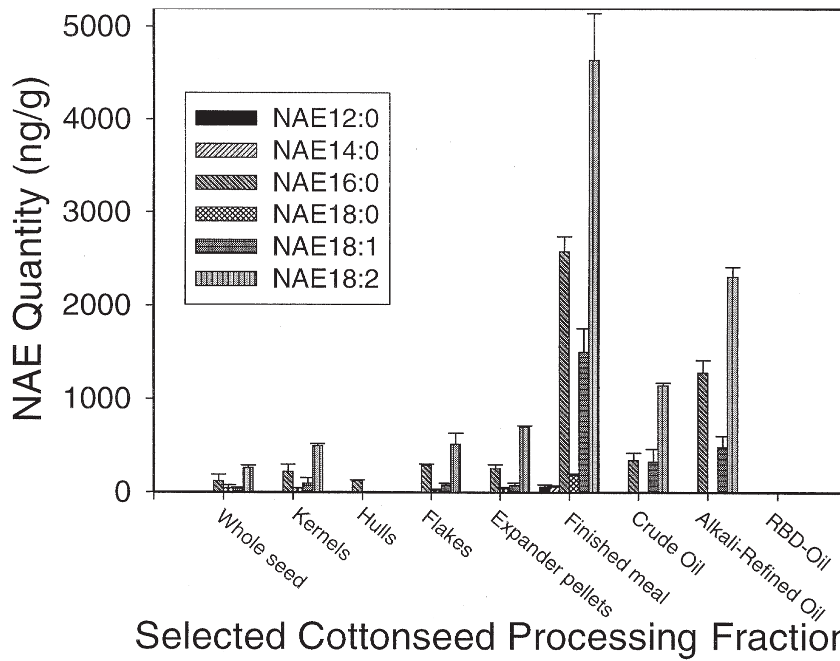

FIG. 3. NAE types identified and quantified in selected samples from a cottonseed processing stream. RBD, refined, bleached, deodorized; for other abbreviation and error bars see Figure 2.

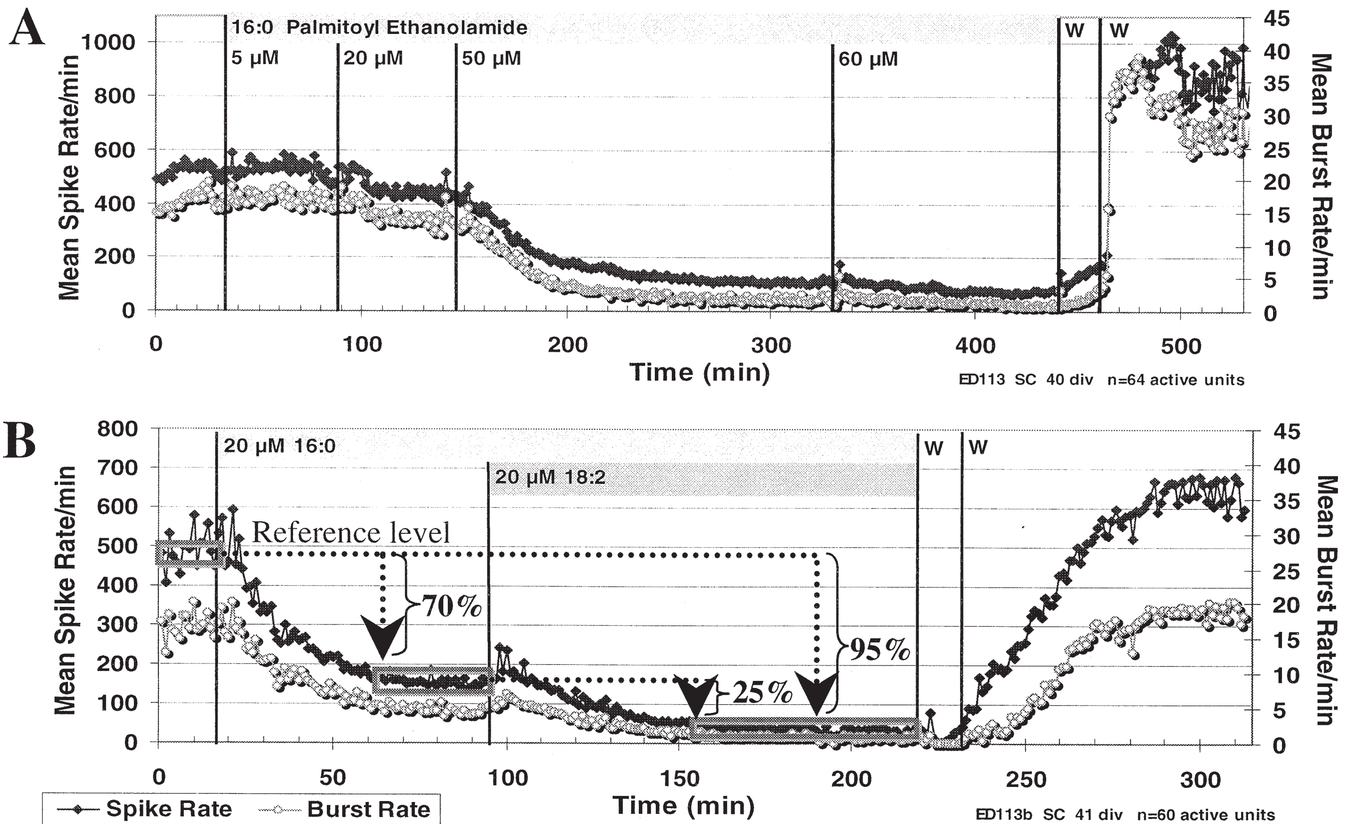

FIG. 4. Effect of NAE16:0 (palmitoylethanolamide) on spontaneous activity of a spinal cord network *in vitro*. (A) NAE16:0 decreased spike and burst rates in a dose-dependent manner, with an EC_{50} of approximately 33 µM. The effects were reversible with two full medium changes (W). Spike and burst rates increased about twofold following the medium changes, revealing possible lasting network sensitization effects of NAE16:0. Twelve hours after medium changes, both spike and burst rates returned to reference levels (continuation of experiment 12 h later in B). (B) NAE18:2 and NAE16:0 exhibited additive effects on network activity (same network as in A). NAE16:0 (20 µM) decreased spike and burst rates by 70% (compared with ~20% in A), reaching maximal effect 40 min after application, and attained a stable activity plateau. At 97 min NAE18:2 was applied to the network, resulting in a combined reduction of 95% from reference activity, 60 min after application. Two medium changes (W) returned the burst rate to reference levels and increased spike rates 23% over reference. For abbreviation see Figure 2.

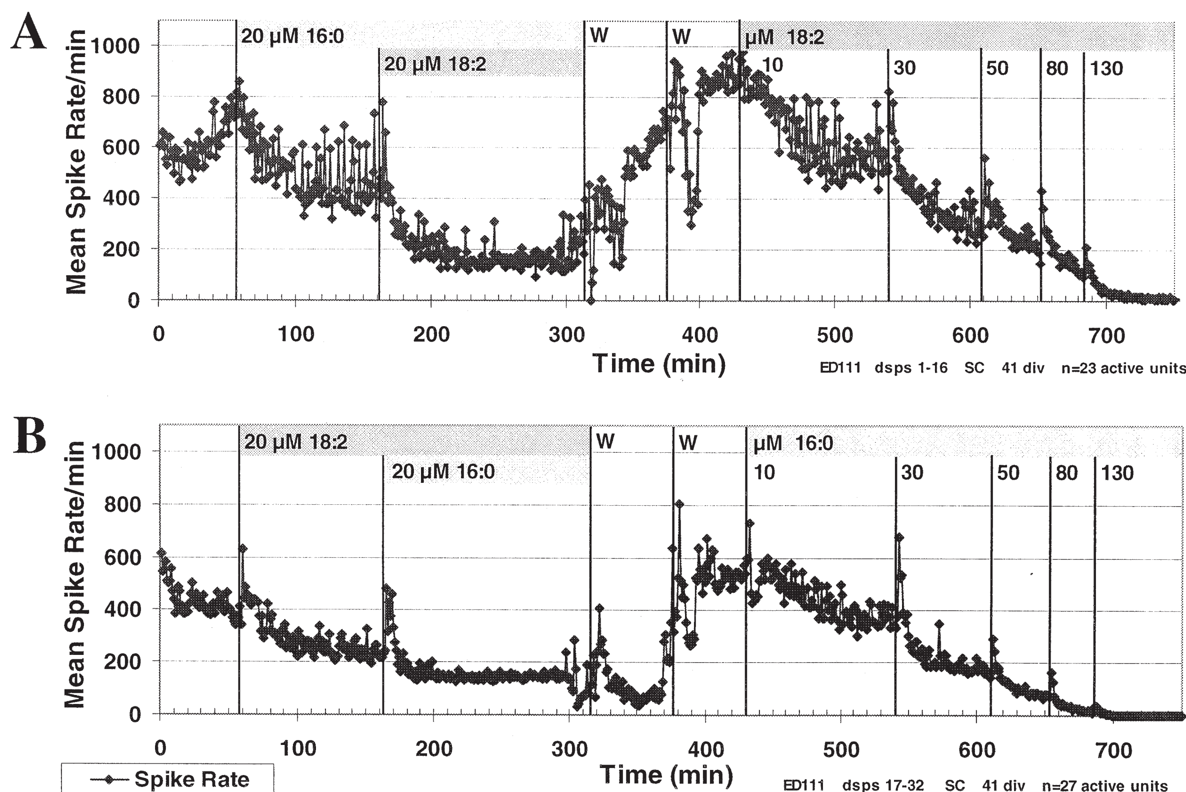

FIG. 5. Combination effects and dose responses of NAE16:0 and NAE18:2 on network spontaneous activity in spinal cord cultures. Burst activity generally tracked spike rates, so only spike data are presented here. (A) NAE16:0 (20 μM) was applied at 57 min, resulting in a spike rate decrease of 25%. The spike rate was allowed to reach a stable plateau (100 to 160 min), after which NAE18:2 (20 μM) was applied to the same network resulting in a further 50% spike rate decrease (a total of 75% from reference activity). (B) The reverse order of NAE applications was performed in another spinal cord culture, with a total spike rate decrease of 66% from reference. The inhibitory effect of NAE was removed by two medium changes (W) in both A and B, and resulted in a 20–25% spike rate increase over reference activity. After reaching a stable activity plateau, 10–130 μM NAE18:2 (A) and NAE16:0 (B) were applied to evaluate continued responsiveness of cultures to NAE treatment.

centration at which a 50% response is observed) of approximately 21 μM (Fig. 5A). Spike inhibition from NAE16:0 and NAE18:2 was global, affecting over 90% of the units recorded in culture (individual unit data not shown). Both NAE16:0 and NAE18:2 exerted their maximal effects 20 to 50 min after application, and all effects were reversible with two exchanges of the medium, indicating that inhibition was not due to nonspecific toxicity.

A combination of NAE16:0 and NAE18:2 was evaluated for possible synergistic/additive interactions (Figs. 4, 5). Figure 5A represents data collected from a one-step application of 20 μM NAE16:0 followed by 20 μM NAE18:2 100 min later. In a subsequent trial, NAE were added in the reverse order (Fig. 5B). The order of application did not significantly influence the potency of either NAE (also compare to Fig. 4B), and the combined effects were found to be additive. Interestingly, following prolonged exogenous NAE treatment, two full medium exchanges without NAE resulted in higher spike and burst rates when compared to reference activity (Figs. 4, 5), which we generally do not observe in these spinal cord cultures under non-NAE treated conditions (data not shown). This "disinhibition" phenomenon may represent an acclimation of the cultures to the higher levels of exogenously applied NAE such that when these inhibitory NAE are removed, the spontaneous network activity is higher than was established prior to NAE treatment. Although the precise mechanism of NAE16:0 and NAE18:2 action on endocannabinoid signaling within these neuronal networks remains to be clearly defined, our results indicate that NAE identified in cottonseeds have potent neurological activity in spinal cord microelectrode arrays. Consequently, oilseed processing fractions, such as cottonseed meal, may represent a plentiful, natural source for the recovery of neuroactive NAE.

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