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Improved method for the routine analysis of acetylcholine release *in vivo*: quantitation in the presence and absence of esterase inhibitor

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ABSTRACT

An improved high-performance liquid chromatographic (HPLC) method using electrochemical detection (ED) is described capable of routinely measuring the low levels of acetylcholine (ACh) typically found in rat brain microdialysis samples. Microdialysis was performed in the striatum of the urethane anesthetized rat using a 4-mm membrane length, high recovery (40% at 1.0 μl/min; ambient conditions), loop-design probe perfused with an artificial cerebrospinal fluid (aCSF) solution containing physiologically normal calcium levels (1.2 m*M*). The HPLC method utilizes a polymeric stationary phase to resolve choline (Ch) from ACh. These analytes are then converted to hydrogen peroxide (H_2O_2) by a solid-phase reactor (containing immobilized choline oxidase and acetylcholinesterase enzymes). The H_2O_2 is detected amperometrically and quantitated on a platinum (Pt) working electrode (+300 mV; with a unique analytical cell featuring a solid-state palladium reference electrode). Two designs of the Pt working electrode were examined, differing only in the support material used (Kel-F or PEEK). The Kel-F/Pt electrode had a limit of detection (LOD) for both analytes of <30 fmol per 10 μl with a signal-to-noise ratio of 3:1. Striatal microdialysis perfusates were monitored for ACh and Ch over a 0–1000 n*M* range of neostigmine (NEO) in the CSF perfusion medium. Using the 4-mm probe, basal ACh and Ch levels were detected with a NEO level as low as 10 n*M* and were found to be 37 ± 3 fmol and 22 ± 1 pmol per 10 μl (mean ± S.E.M., $n = 6$ replicates) respectively. In similar experiments using 3-mm concentric probes comparable (lower) levels of ACh were found with the 50 and 1000 n*M* NEO doses ($n = 4$–21 animals). ACh could not be reliably quantitated when animals were perfused with the 10 n*M* dose of NEO ($n = 4$). The PEEK/Pt electrode had an improved LOD of < 20 fmol per 10 μl due to a two- to three-fold decrease in the background noise component. Basal striatal levels of ACh in the

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absence of NEO approached the LOD and were found to be 15 ± 2 fmol per 10 μl; Ch was 5 ± 1 pmol per 10 μl ($n = 2$, mean of five basal samples). The analytical system requires very little maintenance; a simple electrochemical electrode cleaning step eliminates the need for routine polishing of the Pt electrode and the mobile phase is stable for up to one week. Both ACh and Ch are resolved in under 7 min making this method highly suitable for analysis of microdialysis samples.

INTRODUCTION

Cholinergic neurons are located throughout the entire central nervous system [1,2] and are involved in such diverse behaviors as sleep, locomotion and aggression as well as learning and memory [3]. Continued interest in the cholinergic system stems from the need to better understand the cholinergic neuron's role in affective disorders [4] and in many debilitating neurodegenerative diseases including Alzheimer's disease, Huntington's disease and Parkinson's disease with prominent dementia [3,5–7]. Furthermore, many warfare agents (nerve gases) as well as commonly used insecticides specifically target and disrupt the cholinergic system often with dire consequences [8–10].

Historically, as with other neurotransmitters, many sampling procedures have been used to examine the levels of ACh and Ch including tissue homogenates, synaptosomes and brain slices in an attempt to better quantify ACh release [11–14]. However, interpretation of such data may be complicated due to sample preparation artifacts [12], loss of neurotransmitter compartmentalization, the necessary use of high levels of ACh esterase inhibitors (AChEI) [14–16] and the fact that all these procedures occur *ex vivo*. Recently, brain microdialysis has become the preferred sampling technique for ACh and Ch [17,18] overcoming many of the complications previously mentioned. With this method it is possible to dialyze discrete brain regions in the living organism repetitively and with minimal disruption of vital functions. Microdialysis coupled with HPLC/ED is now the most promising analytical method for the study of ACh neurotransmission *in vivo* [19].

Neuronal extracellular fluid (ECF) levels of ACh are typically in the fmol range (as a result of its highly efficient catabolism within the extracellular space) and, as a result, the lack of sensitive analytical equipment has made ACh measurement difficult. Researchers have attempted to "artificially" elevate ACh levels in the perfusate in order to overcome analytical system sensitivity constraints. Three basic approaches have typically been used: perfusion with aCSF containing high levels of AChEI (typically 100 n*M*–100 μ*M* [15,16,20–27]), perfusion with aCSF containing elevated Ca^{2+} levels (typically > 2.4 m*M* [20,24,28,29]) and finally the use of microdialysis probes with an enlarged membrane surface area (trans-striatal probe design [20,28]). Unfortunately, the elevation in ACh levels due to the use of inhibitor and/or elevated Ca^{2+} may interfere with normal physiological release and response to pharmacological agents [20,28,30]. Ideally, the analytical technique would have sufficient sensitivity allowing for the measurement of basal ECF ACh levels when using an aCSF which lacks inhibitors yet contains physiologically relevant Ca^{2+} levels. The microdialysis probe must also be capable of being easily placed into a discrete brain region.

Our method, based on that of Potter *et al.* [11], involves the separation of Ch and ACh on a normal bore polymeric reversed-phase analytical column, conversion of these analytes to electroactive H_2O_2 with a solid-phase reactor (SPR) and detection of H_2O_2 with a Pt-target working electrode. Two different Pt-target working electrode designs (Kel-F/Pt and PEEK/Pt) were examined and both enabled low fmol limits of detection (LODs). We demonstrate the utility of our method by examining basal release of ACh from the striatum of the anesthetized rat. Comparative data, obtained using 4-mm loop-type and 3-mm concentric-design microdialysis probes [both perfused with physiological Ca^{2+} levels (1.2 m*M*) and containing as little as 0–50 n*M* NEO], are presented and discussed. Both Ch and ACh are well resolved within 7 min, offering good temporal resolution for microdialysis experiments.

EXPERIMENTAL

Chemicals

All chemicals purchased were of the purest grade available. Tetramethylammonium chloride (TMACl) and octanesulfonic acid, sodium salt (OSA) were purchased from Eastman Kodak (Rochester, NY, USA). Anhydrous di-sodium hydrogen phosphate was purchased from Fluka Biochemika (Ronkonkoma, NY, USA). The microbicide "Reagent MB" was from ESA (Bedford, MA, USA). Acetylcholine chloride, choline chloride and NEO were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Purified water was obtained by first passing deionized water through a Milli-Q reagent water system (Millipore, Bedford, MA, USA) and then through a C_{18} solid-phase extraction column (Sep-Pak, Millipore) to remove trace organics. The HPLC grade phosphoric acid (85%) used to adjust the pH of the mobile phase was purchased from Fisher Scientific (Fairlawn, NJ, USA).

Instrumentation

ACh and Ch levels were measured in 10-μl samples using an isocratic HPLC–ED system. All equipment (including the guard column, analytical column and SPR) was obtained from ESA and consisted of a Model 580 pump with two pulse dampeners, a Model 460 autosampler, a Model 480 column oven and a Coulochem II Model 5200A electrochemical detector equipped with a Model 5040 solid state analytical cell, containing either a Kel-F/Pt or a PEEK/Pt target working electrode; chromatograms were recorded and integrated on a Model 450 data station which also provided remote RS-232 control of the autosampler and detector. Fig. 1 presents a cross-section of the 5040 analytical cell and shows the geometry of the solid-state palladium (Pd) reference electrode, the stainless steel counter electrode and the 7.9 mm^2 circular Pt working electrode. A 0.125-mm thick teflon gasket was used resulting in an analytical cell volume of approximately 3.4 μl.

Chromatographic and detector conditions

ACh and Ch were separated at a flow-rate of 0.35 ml/min on a polymeric reversed-phase column (ACH-3, 5 μm, 15 cm × 3 mm I.D.) equipped with an ACH-3-G guard cartridge. The mobile phase consisted of 100 m*M* sodium phosphate, 0.5 m*M* TMACl, 0.005% (v/v) Reagent MB and 2.0 m*M* OSA at a final pH of 8.0 and was continuously sparged with a light stream of helium (ultra-pure grade). The mobile phase was changed on a weekly basis. ACh and Ch were enzymatically converted to H_2O_2 by a post-column SPR (containing immobilized acetylcholinesterase and choline oxidase) and the H_2O_2, in turn, was measured electrochemically on a Pt-target working electrode maintained at +300 mV (*vs.* Pd reference). Consistent enzymatic efficiency and minimum changes in retention time were achieved by maintaining the column and SPR at 35°C. A prefilter containing a 0.45-μm graphite frit was inserted between the pump and pulse dampeners to protect the analytical column from micro-particulates.

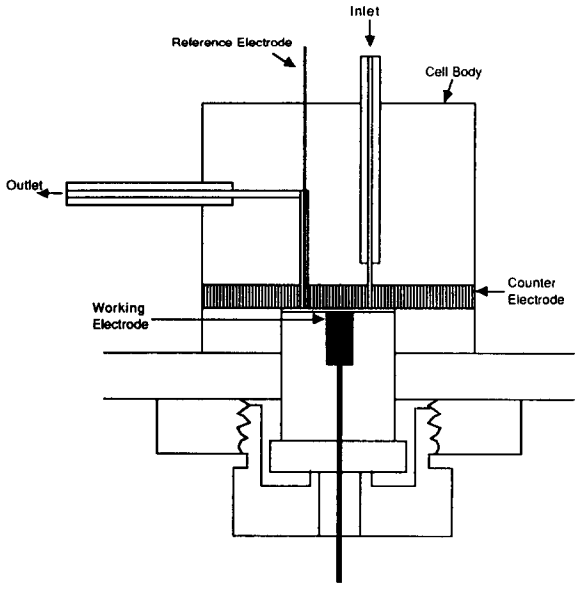

Fig. 1. Cross-section schematic of the analytical cell showing the solid-state Pd reference, stainless-steel counter and Pt working electrodes.

Microdialysis

Male Sprague–Dawley rats (250–350 g) were anesthetized with an 8-ml/kg i.p. dose of a 125-mg urethane/12.5-mg α-chloralose per ml solution; the depth of anesthesia was maintained by

additional doses when necessary. Animals were placed in a stereotaxic frame and a precalibrated (*in vitro*) 4-mm loop-type microdialysis probe (ESA, Inc.) was positioned within the right striatum through a hole drilled in the cranium (coordinates from Bregma: AP + 0.4 mm, L.R. 2.7 mm, DV-6.5 mm from dura). Probes were perfused at 1.0 μl/min with aCSF (145 m*M* NaCl, 2.7 m*M* KCl, 1.2 m*M* $CaCl_2$, 1.0 m*M* $MgCl_2$, 2.0 m*M* Na_2HPO_4 pH 7.4 [31]) containing 0 to 1000 n*M* NEO. Samples were collected every 15–20 min following a 150–180 min recovery period from "injury mediated" release. Samples were then collected for an additional two to three hours.

The loop-type probe consisted of an 8-mm length of regenerated cellulose dialysis membrane (225 μm O.D.) folded in half: consequently the probe's dimensions were 225 × 450 μm in cross-section with a membrane length of 4 mm. Typical *in vitro* recoveries of this probe were found to be approximately 40% for both ACh and Ch at a flow-rate of 1.0 μl/min under ambient conditions. The *in vitro* recoveries were only used to verify probe viability and probe-to-probe variability and were never used to calculate extracellular levels as this would grossly underestimate the actual ECF concentrations [32,33].

External standards

The initial standard solutions were prepared rapidly due to the hygroscopic nature of the ACh and Ch chlorides. When not in use, the powders were stored desiccated (over calcium chloride).

Initially, 10 m*M* standard solutions of ACh and Ch were prepared in purified water. These solutions were then individually diluted 1:10 (v/v) in water to provide 1 m*M* stock solutions which were aliquoted and stored at −20°C. These stock solutions were stable for at least 6 months at −20°C or one week at 4°C.

A 20-pmol per 10 μl working standard containing both ACh and Ch was prepared by diluting the stock solutions in either mobile phase or aCSF. This working standard was used for both *in vitro* probe recoveries and, with the appropriate dilutions, for external calibration. External standards (from 10 fmol to 20 pmol on column) were analyzed randomly throughout the assay period in order to monitor system efficiency.

RESULTS

Chromatography

The optimum electrochemical potential applied for the analysis of ACh and Ch was determined through the generation of hydrodynamic voltammograms (HDV) as presented in Fig. 2. The potential of +300 mV was selected as this offered the maximum signal response and minimum noise characteristics. On a daily basis, prior to analysis, an electrochemical cleaning of the working electrode was performed by applying a potential of −300 mV for 5 min with a subsequent re-equilibration time of 30 min at +300 mV. Such cleaning removes any film which may have been deposited on the electrode surface and overcomes the need for mechanical cleaning and polishing procedures.

A standard chromatogram showing resolution of 20 pmol/10 μl Ch and ACh standards in under seven minutes is presented in Fig. 3A. Fig. 3B illustrates the separation of 78 fmol/10 μl levels of both Ch and ACh standards approaching the LOD of the Kel-F/Pt-target working electrode. Analysis of aCSF showed no contaminant peaks capable of co-eluting with either analyte. Similarly, Fig. 3C shows quantitation of

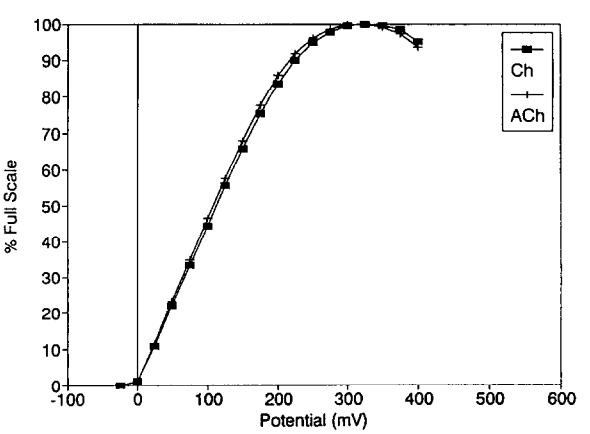

Fig. 2. Hydrodynamic voltammograms obtained from analysis of Ch and ACh standards at various applied potentials.

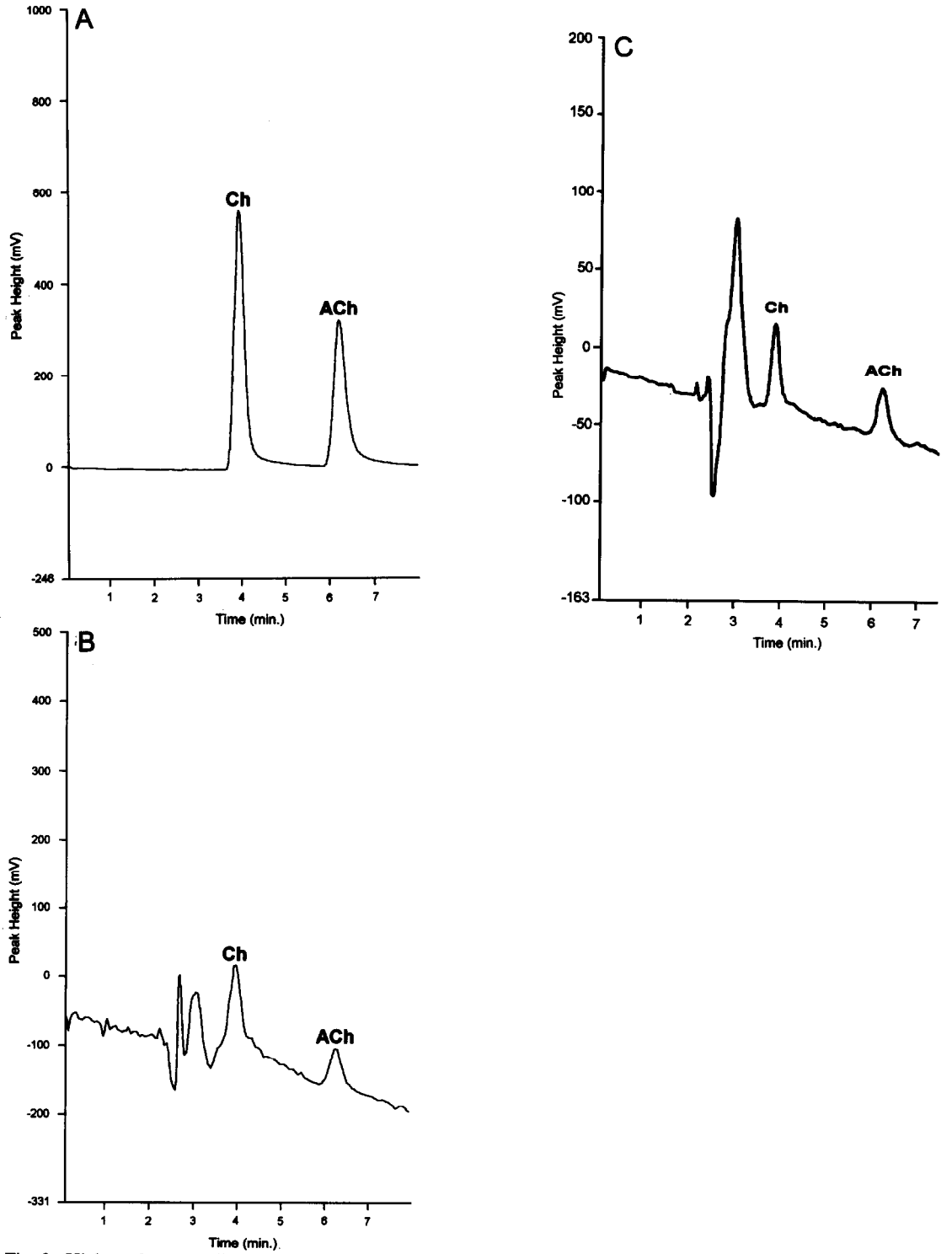

Fig. 3. High-performance liquid chromatograms of Ch and ACh standards. (A) Analysis of 20 pmol/10 μl standards (50 nA/1000 mV full scale) using the Kel-F/Pt working electrode. (B) Analysis of 78 fmol/10 μl standards (2 nA/1000 mV full scale) approaching the LOD of the analytical system when using the Kel-F/Pt working electrode. (C) Analysis of 39 fmol/10 μl standards (1 nA/1000 mV full scale) using the PEEK/Pt working electrode.

39 fmol/10 μl levels of analytes (1 nA/1000 mV) on the PEEK/Pt-target working electrode.

Standard linearity and limit of detection

Linear regression analysis of the peak height *versus* concentration demonstrated linearity for both ACh and Ch in the 0.0 to 20 pmol per 10 μl injection range. The r^2 values for ACh and Ch were 0.993 and 0.999 respectively. The average LOD for both compounds was determined to be <30 and <20 fmol (per 10 μl injection at a signal-to-noise (S/N) ratio of 3:1) for the Kel-F/Pt- and PEEK/Pt-target working electrodes respectively.

Retention time and peak-height precision

The within-day variation in retention time of the standards analyzed over a 12-h period was 3.77 ± 0.008, 0.2 and 6.49 ± 0.02, 0.3 min (mean ± S.D., R.S.D.; $n = 6$) for Ch and ACh respectively. The inter-day variation in retention time over a six day period was 3.77 ± 0.006, 0.2 and 6.48 ± 0.01, 0.2 min (mean ± S.D., R.S.D.) for Ch and ACh respectively. The inter-day peak-height precision for 20 pmol standards analyzed over six consecutive days, 30 min following electrochemical electrode cleaning, was 813 ± 63, 8 and 325 ± 38, 12 (mean ± S.D., R.S.D) for Ch and ACh respectively. External standards were analyzed before and during the experiment to monitor the electrode's response.

Analysis of striatal samples

Originally, when using the Kel-F/Pt working electrode, as little as 10 n*M* NEO could be used in the aCSF but the LOD of the system was approached (Fig. 4A). When perfused with 10 n*M* NEO, basal striatal levels of ACh and Ch were found to be 37 ± 3 fmol/10 μl and 22 ± 1 pmol/10 μl ($n = 6$ replicates) respectively. When perfused with 50 n*M* NEO, striatal ACh could be readily quantitated (Fig. 4B) and was found to be 313 ± 8 fmol/10 μl; Ch levels were 19 ± 2 pmol/10 μl ($n = 6$ replicates). When perfused with 1000 n*M* NEO, striatal levels of ACh and Ch were 2 ± 0.04 and 17 ± 1 pmol/10 μl ($n = 6$ replicates) respectively. In comparable experiments, 3-mm concentric probes (home-made) were used and striatal levels of ACh and Ch were determined when animals were perfused with aCSF containing 10, 50 or 1000 n*M* NEO (*in vitro* recovery was typically <20% [34]). When perfused with 50 or 1000 n*M* NEO basal levels of ACh were 100 ± 10 fmol/10 μl ($n = 21$ separate animals) and 780 ± 80 fmol/10 μl ($n = 4$ separate animals), respectively. With the 3-mm concentric probe ACh levels could not be reliably quantitated when animals were perfused with aCSF containing 10 n*M* NEO. Levels of Ch were 10 ± 1, 13 ± 1 and 12 ± 2 pmol/10 μl sample for the 10, 50 and 1000 n*M* NEO concentration. Analysis of striatal perfusates using both the 4-mm loop-type and 3-mm concentric design probes using aCSF lacking NEO failed to measure ACh, however no other endogenous compounds were found to co-elute with the ACh peak.

With the PEEK/Pt-target design, due to its better signal-to-noise characteristics, lower levels of NEO could be used. In the absence of NEO, ACh could still be quantitated but did approach the LOD of the system. ACh and Ch levels were 15 ± 2 fmol/10 μl sample and 5 ± 1 pmol/10 μl respectively ($n = 2$, mean of 5 basal samples). A chromatogram showing basal striatal ACh release in the absence of inhibitor is presented in Fig. 5.

DISCUSSION

Over the past several years, the study of central cholinergic transmission *in vivo* has been successfully accomplished using microdialysis techniques in conjunction with HPLC/ED [17–21,34–36]. However, due to lack of analytical system sensitivity, researchers have had to utilize several approaches to artificially elevate ACh release to measurable levels including the use of: AChEIs in the aCSF; elevated aCSF Ca^{2+} levels; a probe with a large membrane surface area.

The local perfusion of AChEI is often a prerequisite for routine ACh analysis. An 1000 fold concentration range of inhibitor has been reported in literature with some researchers using NEO concentrations up to 100 μ*M* [15,16,20–27]. Recently, several publications have addressed the validity of including AChEIs in the perfusion medium and have reported that

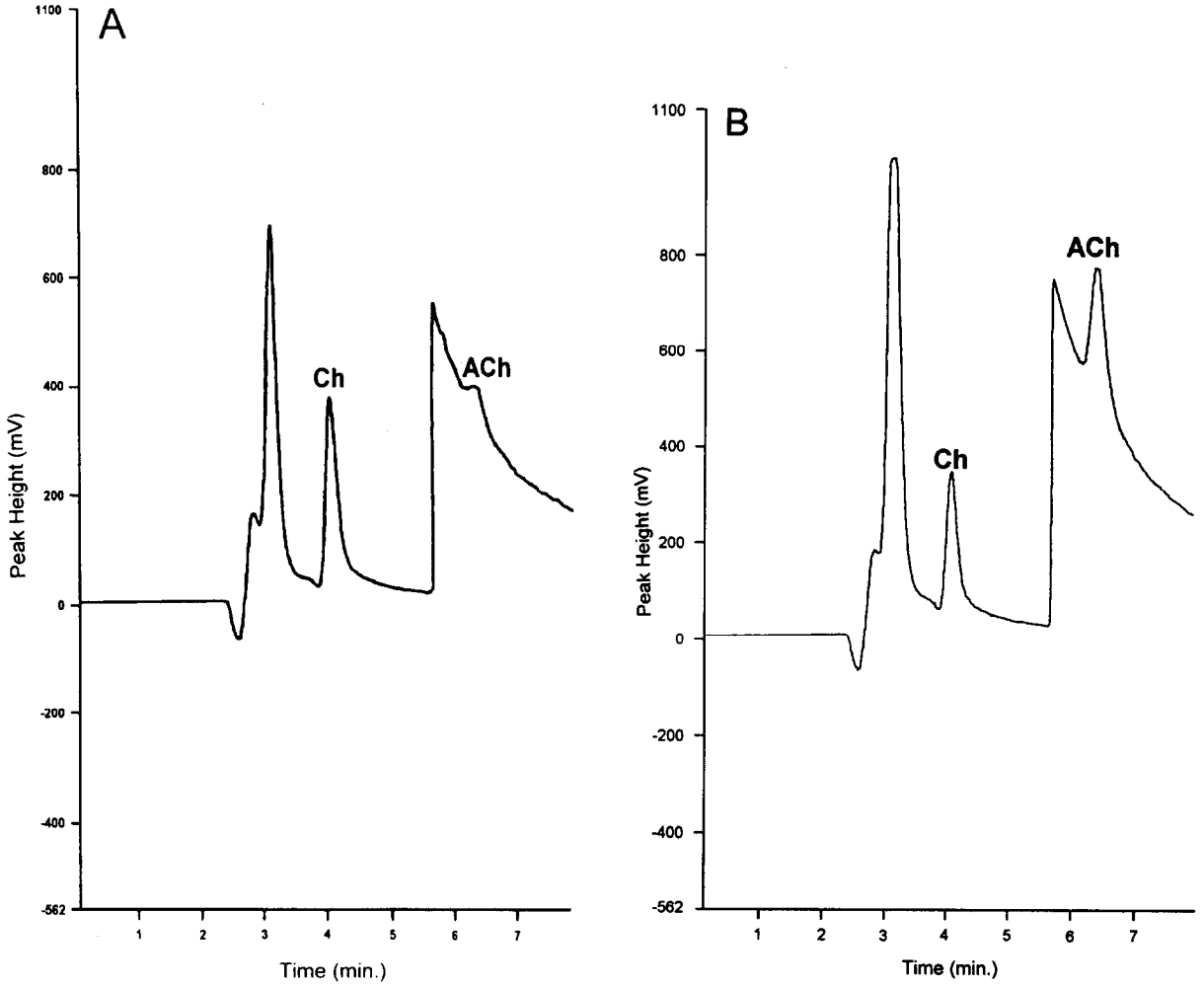

Fig. 4. High-performance liquid chromatograms of Ch and ACh in basal striatal ECF perfusates obtained from the urethane anesthetized rat. (A) Typical chromatogram when aCSF contained 10 n*M* NEO and analysis was performed using the Kel-F/Pt working electrode. Levels of Ch and ACh were 22 ± 1 pmol/10 μl and 37 ± 3 fmol/10 μl. The sensitivity was altered from 50 nA/1000 mV for Ch to 1 nA/1000 mV for ACh. (B) Typical chromatogram when aCSF contained 50 n*M* NEO and the analysis was performed using the Kel-F/Pt working electrode. Levels of Ch and ACh were 19 ± 2 pmol/10 μl and 313 ± 8 fmol/10 μl respectively. The change in sensitivity is as in (A) above.

artificially elevating ACh release can seriously interfere with the pharmacological responsiveness of the cholinergic neuronal system. For example, Damsma *et al.* [20] reported that perfusion with NEO (2 μM) prevented the expected decrease in ACh release when the striatum was perfused with aCSF lacking Ca^{2+} but that ACh output tended to zero in animals not receiving NEO. Likewise, although administration of a muscarinic antagonist, atropine, increased ECF levels of ACh while administration of a muscarinic agonist, oxotremorine, decreased ACh release, the magnitude and time course of the changes were dependent upon the level of inhibitor in the aCSF [20]. Similarly, de Boer *et al.* [28] and Kawashima *et al.* [30] suggested that the use of AChEIs to promote ACh release interferes with presynaptic ACh autoreceptors, integral to a physiological negative feed-back loop, designed to monitor and regulate synaptic ACh levels. More recently, perfusion with NEO has been reported to significantly affect ACh

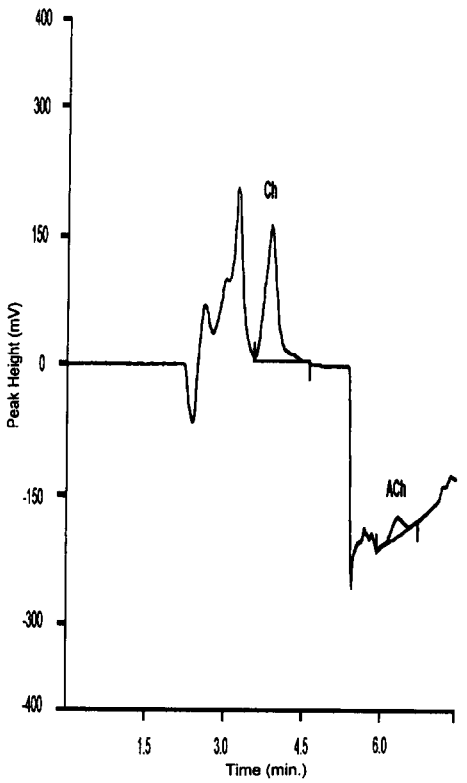

Fig. 5. High-performance liquid chromatogram of Ch and ACh in basal striatal ECF perfusates obtained from urethane anesthetized rats perfused with aCSF containing no NEO. Analysis was performed using the PEEK/Pt electrode. Levels of Ch and ACh were determined to be 5 ± 1 pmol/10 μl and 15 ± 2 fmol/10 μl respectively. The sensitivity was altered from 50 nA/1000 mV for Ch to 500 pA/1000 mV for ACh.

synthesis and the cholinergic neuron's responsiveness to its precursor Ch [34]. Finally, de Boer and Abercrombie have shown that the response of the cholinergic system to dopamine D_1 and D_2 agonists is dependent on the presence or absence of NEO in the perfusion medium [35]. Taken together, these reports strongly suggest that the perfusion level of AChEI should be minimized, or better still, be entirely absent if physiologically relevant data is to be obtained. For these reasons, we developed a sensitive assay capable of routinely measuring ACh release using 10 n*M* NEO or less.

The ionic composition of the aCSF used to perfuse the microdialysis probe is of critical importance [36]. The impulse driven release of the monoamine neurotransmitters is particularly affected by the level of Ca^{2+} within the perfusion medium. Perfusion of the striatum with aCSF lacking Ca^{2+} results in marked decreases in both dopamine [37–39] and ACh [22] release. Conversely, elevated Ca^{2+} levels within the perfusion medium can promote monoamine release [40]. For example, Moghaddam *et al.* reported that increasing Ca^{2+} from 1.2 to 3.4 m*M* not only increased striatal dopamine release by 70% over baseline but also altered the pharmacological responsiveness of the nigrostriatal dopamine system to synthesis inhibition [22]. Based on Moghaddam's paper, we used the physiologically relevant Ca^{2+} level of 1.2 m*M*.

To maximize *in vivo* ACh recovery, several authors have used permanently placed transversal microdialysis fibers with exposed membrane of at least 8 mm in length [20,28]. We have used a more practical high-recovery loop-design probe capable of being repetitively placed into more discrete brain regions. As our comparative data show (4-mm loop *vs.* 3-mm concentric probe designs), the probe recovery efficiency is of critical importance in being able to measure basal ACh release when low levels of NEO are used.

Our analytical method is based on that of Potter *et al.* [11]. The use of normal-bore HPLC eliminated system maintenance issues often associated with microbore techniques. Additionally, the use of a 3-mm I.D. column offers the advantage that larger injection volumes can be tolerated. A polymeric stationary phase was chosen over a silica based one due to its greater stability at pH 8.0 (silica degrades at this pH) and due to better analyte peak-shape [41]. The analytical cell used a solid-state maintenance-free Pd reference electrode [42]. Of the two Pt working electrodes tested, the PEEK/Pt gave superior performance (diminished noise) which is probably due, in part, to the rigidity of the PEEK material when placed under pressure within the analytical cell. A simple electrochemical cleaning procedure eliminated the need for routine dismantling of the analytical cell for electrode polishing; typically mechanical polishing was necessary every 2–3 months. The SPR was stable for 2–3 months and the polymeric

analytical column was stable for at least 6 months of continuous use.

The flow-rate and temperature parameters of the analytical system used in this method were chosen carefully. The flow-rate of 0.35 ml/min represents the best compromise between analysis efficiency (enzymatic conversion and mass transport of H_2O_2 at the Pt surface) and analyte elution time. A temperature of 35°C represented optimal SPR efficiency for the acetylcholinesterase and choline oxidase enzymes used in this method and also produced consistent elution times.

To prevent bacterial contamination which would otherwise block the column, foul the electrode and diminish the apparent analytes' peak heights via bacterial production of catalase, a microbicide was included in the mobile phase. Microbicide was also included in the pump and autosampler wash solutions. Additionally, helium sparging also ensured that the mobile phase could be used for up to a week. The mobile phase was recycled when not running standards or samples.

High levels of Ch in microdialysis perfusates (typically 1000-fold in excess of ACh levels) may interfere with resolution of the two analytes if the chromatography is not adequately controlled. As with the majority of publications to date, we chose a method which had Ch eluting before ACh. Additionally, we designed the chromatography so that there was at least a 2–2.5 min period separating these analytes in order to minimize any effects of the large Ch peak on the resolution of the smaller ACh peak when running at high sensitivity. An alternative approach is to have ACh elute before Ch. However, due to inadequate separation between these analytes [16] and their close proximity to the solvent front, extra chromatographic steps may be required to resolve ACh from the large Ch peak when reduced AChEI levels are used. To date, two approaches have been used: (1) a second column placed in series to delay elution and improve resolution [43]; (2) the use of Ch oxidase/catalase SPR placed before the analytical column to prevent the Ch peak from overwhelming the ACh peak [43].

NEO was chosen as the AChEI for our experiments because of several advantages it presented over other AChEIs such as physostigmine (PHY). Not only is NEO a more potent inhibitor than PHY [44] but, unlike PHY, it is a reversible inhibitor and does not require a trapping column to remove it before it reaches the SPR [16]. Additionally, NEO's activity is independent of pH and is not light sensitive [19].

To date, several research groups have attempted to measure basal ACh release in the absence of AChEI. However, as can be seen from Table I, few have been able to do so using HPLC–ED with physiological (1.2 m*M*) Ca^{2+} levels in the perfusion medium and using more discrete probe sizes. Most researchers have attempted to mea-

TABLE I

MEASUREMENT OF ACh LEVELS IN ABSENCE OF INHIBITOR

Basal level (fmol/μl)	Flow-rate (μl/min)	Elution order	Probe type	Ca^{2+} levels (m*M*)	Region	Model	Ref.
12.5 ± 2.7	5.0	Ch, ACh	8 mm trans.	3.4	Striatum	Awake	20
12.9 ± 2.3	2.7	Ch, ACh	8 mm trans.	3.4	Striatum	Awake	28
<13.3–60	2.0	Ch, ACh	4 mm Loop	2.3	Hippocampus	Awake	29
<5	1.0	Ch, ACh	3 mm Concentric	2.3	Striatum	Awake	44
25 ± 5.0	1.0	ACh, Ch	4 mm Concentric	1.2	Hippocampus	Awake	46
5.3 ± 0.4	1.0	Ch, ACh	5.8 mm trans.	2.3	Cortex	Awake	47
5.6 ± 0.6	2.0	RIA[a]	3 mm Concentric	1.26	Striatum	Anesthetized	30
2.7 (fmol/sample)	–	–	–	–	Striatum	–	35
1.5 ± 0.2	1	Ch, ACh	4 mm Loop	1.2	Striatum	Anesthetized	This study

[a] Radioimmunoassay.

sure ACh release in the awake animal as anesthetics can decrease ACh release by more than 50% [45], therefore placing an additional burden on the analytical system. Our method, due to its enhanced sensitivity, allows for the measurement of ACh release even in the anesthetized animal. This method will also be suitable for ACh measurement in the awake animal model.

CONCLUSION

An HPLC–ED system was developed which can routinely detect both ACh and Ch < 20 fmol per 10-μl injection (S/N 3:1). As a direct result of the system's high sensitivity (coupled with high recovery microdialysis techniques), ACh levels can be monitored in anesthetized rat striatum using microdialysis with normal perfusate Ca^{2+} levels and low neostigmine concentrations. This analytical system routinely offers high sensitivity, selectivity, and stability without major maintenance.

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