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## Acetylcholine Release and Choline Uptake by Cuttlefish (*Sepia officinalis*) Optic Lobe Synaptosomes

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Acetylcholine (ACh), which is synthesized from choline (Ch), is believed to hold a central place in signaling mechanisms within the central nervous system (CNS) of cuttlefish (*Sepia officinalis*) and other coleoid cephalopods. Although the main elements required for cholinergic function have been identified in cephalopods, the transmembrane translocation events promoting the release of ACh and the uptake of Ch remain largely unsolved. The ACh release and Ch uptake were quantitatively studied through the use of *in vitro* chemiluminescence and isotopic methods on a subcellular fraction enriched in synaptic nerve endings (synaptosomes) isolated from cuttlefish optic lobe. The ACh release evoked by K<sup>+</sup> depolarization was found to be very high (0.04 pmol ACh·s<sup>-1</sup>·mg<sup>-1</sup> protein). In response to stimulation by veratridine, a secretagogue (a substance that induces secretion) that targets voltage-gated Na<sup>+</sup> channels, the release rate and the total amount of ACh released were significantly lower, by 10-fold, than the response induced by KCl. The high-affinity uptake of choline was also very high (31 pmol Ch·min<sup>-1</sup>·mg<sup>-1</sup> protein). The observed ACh release and Ch uptake patterns are in good agreement with published data on preparations characterized by high levels of ACh metabolism, adding further evidence that ACh acts as a neurotransmitter in cuttlefish optic lobe.

Cuttlefish, like other coleoid cephalopods, has been the subject of many neurobiological studies. Although the func-

tional anatomy of the cuttlefish nervous system has been well illustrated, including investigation by electrical stimulation (1, 2), much less information is available on signaling mechanisms within the neural networks of cephalopods (3).

The CNS of cuttlefish consists of distinct but interconnected lobes, of which the paired optic lobes correspond to more than half (4). The cuttlefish optic lobe comprises an outer granule cell layer, a neurophil layer, an inner granule cell layer (the outer cortex), and numerous clusters of cell bodies separated by areas of nerve fibers and synaptic areas (the central medulla) (5). The optic lobes contain a repertoire of neuronal circuits, which integrate the central visual processing, visuomotor, learning and memory systems (6). ACh has been suggested to mediate neuronal signaling at the CNS of cuttlefish, given that the Koelle-Friedenwald stain revealed a neuropilar localization of the reaction of cholinesterases (7). Moreover, it has been shown that ACh catabolism increases in the optic lobes as a response to memory formation with a long-term retention delay (8). Additionally, the optic lobes, in which nicotinic-like receptors are widely present (9), are among the cuttlefish CNS regions with the highest levels of enzyme activity responsible for the synthesis (choline acetyltransferase) and degradation (acetylcholinesterase) of ACh (10). Furthermore, recent work on cuttlefish optic lobe slices (11) has shown that mecamylamine hydrochloride, a nicotinic receptor antagonist, blocks excitatory postsynaptic currents, which are due to spontaneously released ACh. As far as we know, our recent paper (12) was the first to provide a brief insight into the release of ACh and the uptake of its precursor (Ch) in cuttlefish optic lobe. The present study further characterizes

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Abbreviations: ACh, acetylcholine; Ch, choline; CNS, central nervous system; HC-3, hemicholinium-3.

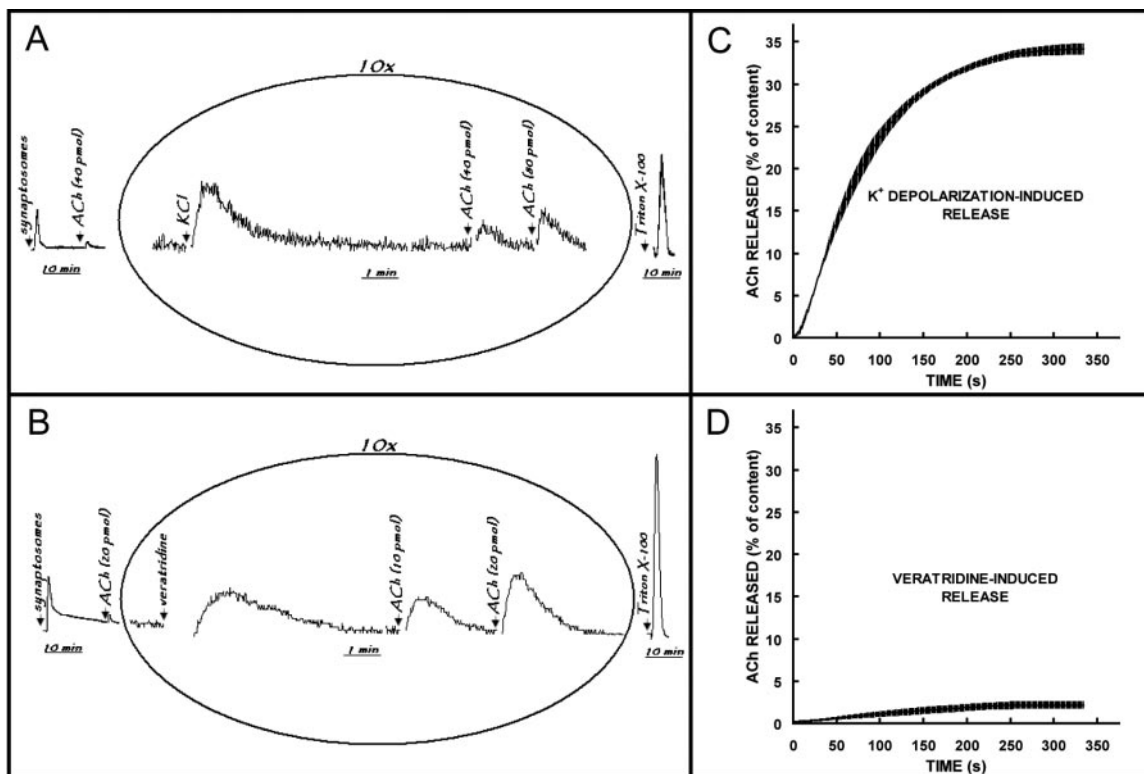
these major processes of cholinergic function, by analyzing the time-course of ACh release and Ch uptake and by comparing the effect of two secretagogues, KCl and veratridine, in pinched-off nerve terminals (synaptosomes) from cuttlefish optic lobes.

The synaptosomal fraction was isolated from the optical lobes of cuttlefish (*Sepia officinalis* L. 1758) captured from the Atlantic coast of Portugal (Algarve and Costa Nova). Isolation was by the method described by Crispino *et al.* (13), which involves tissue homogenization in ice-cold 0.7 mol l<sup>-1</sup> sucrose buffered with 20 mmol l<sup>-1</sup> Tris-Cl (pH 7.3) and sedimentation of nuclei and cell debris at low-speed centrifugation, before the final centrifugation step at 13,170 × *g* for 30 min. The resulting floating particulate layer (synaptosomal fraction) was collected by decantation, washed with the homogenizing medium, and gently resuspended in the same medium. After the protein content of the preparations was determined (14), freshly prepared synaptosomes were immediately used in either assays of acetylcholine release (15) or assays of choline uptake (16).

The procedure for the measurement of ACh released from

synaptosomes was as follows: Aliquots of synaptosomal suspension were transferred to continuously stirred luminometer tubes containing isosmotic Na<sup>+</sup>-enriched medium and various chemiluminescence agents: 460 mmol l<sup>-1</sup> NaCl, 10 mmol l<sup>-1</sup> KCl, 55 mmol l<sup>-1</sup> MgCl<sub>2</sub>, 11 mmol l<sup>-1</sup> CaCl<sub>2</sub>, 0.6 mmol l<sup>-1</sup> KHCO<sub>3</sub>, 10 mmol l<sup>-1</sup> Tris (pH 7.5); acetylcholinesterase (10 U·ml<sup>-1</sup>); choline oxidase (5 U·ml<sup>-1</sup>); peroxidase (6 U·ml<sup>-1</sup>); and 33 μmol l<sup>-1</sup> luminol. After incubation for several minutes at room temperature, the depolarization stimulus was applied by addition of KCl (final concentration 40 mmol l<sup>-1</sup>) or veratridine (final concentration 40 μmol l<sup>-1</sup>). Finally, the amount of ACh occluded in synaptosomes was estimated after detergent permeabilization with Triton X-100 (final concentration 0.01%). All responses were calibrated individually by injecting a known amount of standard acetylcholine perchlorate.

An initial strong light emission fell to a constant resting emission (Fig. 1A, B). Then, when KCl was added to the assay tube in the presence of external Ca<sup>2+</sup>, the light emission once again showed a strong increase (Fig. 1A). In contrast, addition of veratridine produced, after a short



**Figure 1.** Chemiluminescent detection of endogenous ACh released from synaptosomes isolated from cuttlefish optic lobe. The synaptosomal fractions were prepared from optic lobes (8 to 12 per preparative procedure) rapidly dissected from decapitated heads. Aliquots (30 μl) of the synaptosomal suspension were transferred to continuously stirred luminometer tubes containing 300 μl of reaction mixture consisting of isosmotic Na<sup>+</sup>-enriched medium and the chemiluminescence agents. Representative records of chemiluminescent response to 40 mmol l<sup>-1</sup> KCl (A) and 40 μmol l<sup>-1</sup> veratridine (B). Arrows denote time point at which synaptosomal suspension, ACh, KCl/veratridine, and Triton X-100 were added. Profiles for cumulative ACh release triggered by K<sup>+</sup> depolarization (C) and veratridine (D). Values are the mean ± SE of three experiments.

latency, a moderate and slow enhancement of light emission (Fig. 1B). Accordingly, the final ACh content of synaptosomes, detected as light emission after permeabilization with Triton X-100, was higher after treatment with veratridine than after KCl.

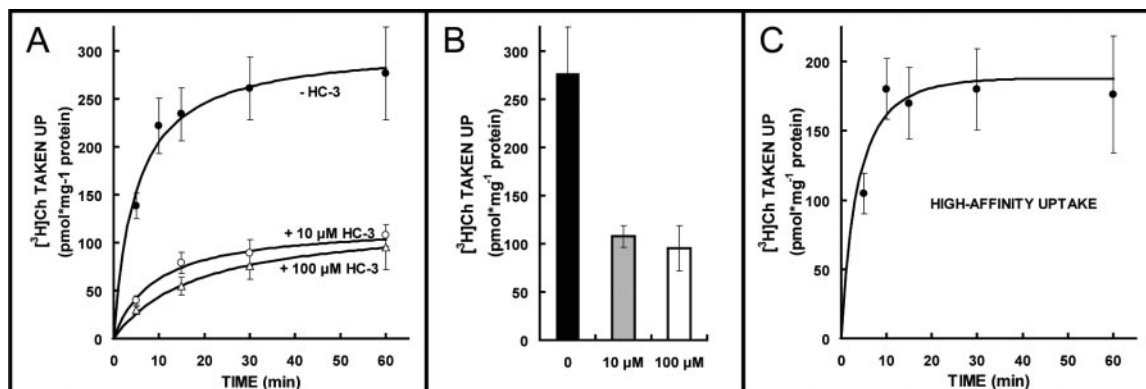
To further evaluate the effects of high potassium concentration and of veratridine on the neurotransmitter release, we analyzed the cumulative release profiles. The cumulative release was calculated as the area under the release curve and above baseline at a given time, divided by the area of the peaks corresponding to the total amount of released ACh during secretion and liberation after the addition of Triton X-100, which was considered to correspond to the total ACh content of synaptosomes (100%). The cumulative profile of the release induced by  $K^+$  depolarization (Fig. 1C) rose rapidly between 15 and 45 s ( $0.04 \text{ pmol ACh}\cdot\text{s}^{-1}\cdot\text{mg}^{-1}$  protein), then slowly decreased, approaching zero in about 300 s. Hence the light emission returned to baseline in about 300 s (Fig. 1A). Benech *et al.* (17) found that changing the external  $K^+$  concentration from 10 to 30  $\text{mmol l}^{-1}$  KCl evoked a transient increase in cytoplasmic  $\text{Ca}^{2+}$  in synaptosomes isolated from squid optic lobe. The  $\text{Ca}^{2+}$  transient in that study was calculated to be around 340  $\text{nmol l}^{-1}$  and reached its maximal level in less than 10 s, which seems to be consistent with the typical behavior of voltage-gated  $\text{Ca}^{2+}$  channels that inactivate rapidly. The increase we recorded in the chemiluminescent response to KCl addition thus seems to represent the  $\text{Ca}^{2+}$ -dependent release of ACh by cuttlefish synaptosomes. We estimated the total amount of ACh released to be  $0.9 \pm 0.3$  (mean  $\pm$  SE)  $\text{nmol}\cdot\text{mg}^{-1}$  protein, which corresponds to  $34\% \pm 1\%$  of the total content of the synaptosomal fraction. When veratridine, a hydrophilic toxin that targets the type 2 receptor site of voltage-gated  $\text{Na}^+$  channels (18), was applied to synaptosomes, the release rate and the total amount of ACh released were significantly lower, by 10-fold, than the response induced by  $K^+$  depolarization (Fig. 1D). Voltage-gated  $\text{Na}^+$  channels are responsible for the generation of action potentials in nerve cells. Veratridine causes  $\text{Na}^+$  channels to stay open, producing a sustained membrane depolarization that leads to many secondary effects, including exocytosis. Consequently, veratridine is frequently used as a secretagogue (19).

As shown in Figure 1, the veratridine-induced release was much lower than the KCl-induced release of ACh by synaptosomes isolated from cuttlefish optic lobe. Putative voltage-gated  $\text{Na}^+$  channels were found to be expressed in the squid optic lobe, mostly at the second-order visual giant neurons (20, 21), suggesting an unequal distribution of  $\text{Na}^+$  channels among different cell types in the cuttlefish optic lobe. Although the chemiluminescent method has often been applied to follow ACh release from tissue slices and synaptosomal fractions, one can argue that it detects Ch in addition to ACh. In the present study, we evaluated this

possibility by measuring light emission upon stimulation with KCl or veratridine of synaptosomes pretreated with 47  $\mu\text{mol l}^{-1}$  of echothiophate iodide, an acetylcholinesterase inhibitor. When the acetylcholinesterase inhibitor was present, we observed no significant enhancement of light emission after the addition of the secretagogues (data not shown); therefore we had neglected Ch release under our experimental conditions. Synaptosomal fractions contain acetylcholinesterase that hydrolyses ACh to Ch; and Ch, which is actively transported back to the intrasynaptosomal space, is used as a substrate for ACh synthesis by choline acetyltransferase. Both enzymatic activities have been evaluated in cuttlefish optic lobe by Bellanger *et al.* (22). According to the authors, up to 16 pmol of ACh can be formed and more than 20 nmol of ACh can be degraded per milligram of protein within the time-scale resolution (1 s) of ACh release experiments. In the present work we explored the ability of synaptosomes to take up Ch. High-affinity Ch uptake is considered the primary mechanism by which cholinergic nerve endings accumulate Ch for ACh synthesis (23, 24).

Figure 2 depicts the time course of [ $^3\text{H}$ ]Ch uptake by synaptosomes isolated from cuttlefish optic lobe. The suspension of synaptosomes was first allowed to recover for 15 min in isosmotic  $\text{Na}^+$ -enriched medium at room temperature, in the absence or in the presence of hemicholinium-3 (HC-3), a potent and selective inhibitor of the high-affinity uptake system for Ch at presynaptic nerve terminals (25). Then, choline + [ $^3\text{H}$ ]choline ([ $^3\text{H}$ ]Ch) was added to obtain a final Ch concentration of 5  $\mu\text{mol l}^{-1}$  and a specific radioactivity of 50  $\mu\text{Ci}\cdot\mu\text{mol}^{-1}$ . At designated reaction times, the reactions were stopped by rapid filtration of aliquots, and the values for [ $^3\text{H}$ ]Ch uptake were calculated after subtraction of blank values obtained by filtering aliquots of reaction media containing [ $^3\text{H}$ ]Ch (0.25  $\mu\text{Ci}\cdot\text{ml}^{-1}$ ).

The synaptosomal fraction isolated from cuttlefish optic lobe was capable of taking up Ch when incubated in a  $\text{Na}^+$ -enriched medium containing 460  $\text{mmol l}^{-1}$  NaCl, 10  $\text{mmol l}^{-1}$  KCl, 55  $\text{mmol l}^{-1}$   $\text{MgCl}_2$ , 11  $\text{mmol l}^{-1}$   $\text{CaCl}_2$ , 0.6  $\text{mmol l}^{-1}$   $\text{KHCO}_3$ , 10  $\text{mmol l}^{-1}$  Tris at pH 7.5 (Fig. 2A). Exposure to micromolar concentrations of HC-3 significantly decreased the amount of [ $^3\text{H}$ ]Ch retained by synaptosomes throughout the assay (60 min). The results suggest that cuttlefish optic lobes contain a high-affinity Ch uptake system localized at nerve terminals, given that the degree of inhibition of [ $^3\text{H}$ ]Ch uptake remained constant when the HC-3 concentration was increased from 10 to 100  $\mu\text{mol l}^{-1}$  (Fig. 2A, B). The time course of the high-affinity Ch uptake by cuttlefish optic lobe synaptosomes displayed a hyperbolic trend (Fig. 2C), approaching steady-state in less than 10 min. It is evident from our results that [ $^3\text{H}$ ]Ch uptake by the high-affinity transporter can proceed at rates higher than 20 pmol  $\text{Ch}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$  protein, promoting the accumulation



**Figure 2.** Uptake of exogenous Ch by synaptosomes isolated from cuttlefish optic lobe. The synaptosomal fractions were prepared from optic lobes (12 to 30 per preparative procedure) rapidly dissected from decapitated heads. Synaptosomal suspensions were incubated in isosmotic  $\text{Na}^+$ -enriched medium, in the absence and in the presence of  $10 \mu\text{mol l}^{-1}$  or  $100 \mu\text{mol l}^{-1}$  HC-3. After 15 min, the reactions were started by the addition of  $[^3\text{H}]\text{Ch}$  ( $5 \mu\text{mol l}^{-1}$  and  $0.25 \mu\text{Ci/ml}$ ), and were stopped at designated times by rapid filtration of aliquots through glass-fiber filters (Whatman GF/B) prewashed with ice-cold isosmotic media without Ch. The filters were then washed with the same ice-cold media and plunged into scintillation cocktail (Universol<sup>TM</sup> ES) for further radioactivity measurement by liquid scintillation. The points are the mean  $\pm$  SE of two to five experiments for each time. (A) The time-course of  $[^3\text{H}]\text{Ch}$  uptake in the absence and in the presence of HC-3. Data from panel A are replotted in panels B and C to depict the inhibitory effect of HC-3 at 60 min (B) and the time-course of the HC-3 sensitive uptake of  $[^3\text{H}]\text{Ch}$  (C).

of  $[^3\text{H}]\text{Ch}$  up to  $176 \pm 42$  (mean  $\pm$  SE)  $\text{pmol}\cdot\text{mg}^{-1}$  protein when the synaptosomes were exposed to  $5 \mu\text{mol l}^{-1}$   $[^3\text{H}]\text{Ch}$ .

We know of no published articles describing ACh release and Ch uptake in the CNS of cuttlefish. Dowdall and Simon (26) and Matsumura (27) showed that Ch is transported by synaptosomes isolated from squid optic lobes. The uptake of Ch by synaptosomes isolated from cuttlefish optic lobe exhibited similar characteristics, including a high initial rate of accumulation and sensitivity to HC-3.

In this study, we characterized the depolarization-triggered release of ACh and the high-affinity uptake of Ch (ACh precursor) in cuttlefish optic lobe synaptosomes. Once again, we wish to draw attention to the evidence, provided in our recent paper (12), that synaptosomal fractions isolated from cuttlefish optic lobe are suitable *in vitro* models to study neurotransmission. In particular, we have demonstrated that the uptake of Ch is inhibited by the selective inhibitor of the  $(\text{Na}^+/\text{K}^+)\text{ATPase}$ , ouabain, suggesting that the high-affinity Ch transporter of cuttlefish optic lobe also requires a transmembrane  $\text{Na}^+$  gradient to mediate active transport of Ch. In fact, dependence on  $\text{Na}^+$  and high sensitivity to HC-3 represent the main functional characteristics of the high-affinity choline transporter 1 (CHT1), which is specifically expressed in cholinergic neurons (23).

In conclusion, the results described here, together with preliminary data from earlier studies (2, 5, 7–12), provide further strong evidence that ACh performs an important physiological role in mediating neuronal signaling at synapses of the cuttlefish optic lobe.

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