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+ depolarization was found to be very high (0.04 pmol ACh s⁻¹ mg⁻¹ protein). In response to stimulation by veratridine, a secretagogue (a substance that induces secretion) that targets voltage-gated Na⁺ 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⁻¹ mg⁻¹ 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.

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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\(^{-1}\) sucrose buffered with 20 mmol l\(^{-1}\) Tris-Cl (pH 7.3) and sedimentation of nuclei and cell debris at low-speed centrifugation, before the final centrifugation step at 13,170 \(\times\) \(g\) 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\(^{+}\)-enriched medium and various chemiluminescence agents: 460 mmol l\(^{-1}\) NaCl, 10 mmol l\(^{-1}\) KCl, 55 mmol l\(^{-1}\) MgCl\(_2\), 11 mmol l\(^{-1}\) CaCl\(_2\), 0.6 mmol l\(^{-1}\) KHCO\(_3\), 10 mmol l\(^{-1}\) Tris (pH 7.5); acetylcholinesterase (10 U ml\(^{-1}\)); choline oxidase (5 U ml\(^{-1}\)); peroxidase (6 U ml\(^{-1}\)); and 33 \(\mu\)mol l\(^{-1}\) luminol. After incubation for several minutes at room temperature, the depolarization stimulus was applied by addition of KCl (final concentration 40 mmol l\(^{-1}\)) or veratridine (final concentration 40 \(\mu\)mol l\(^{-1}\)). 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\(^{2+}\), the light emission once again showed a strong increase (Fig. 1A). In contrast, addition of veratridine produced, after a short

![Figure 1](image-url)

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 \(\mu\)l) of the synaptosomal suspension were transferred to continuously stirred luminometer tubes containing 300 \(\mu\)l of reaction mixture consisting of isosmotic Na\(^{+}\)-enriched medium and the chemiluminescence agents. Representative records of chemiluminescent response to 40 mmol l\(^{-1}\) KCl (A) and 40 \(\mu\)mol l\(^{-1}\) 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\(^{+}\) 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 pmol AChs-1mg-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 mmol l-1 KCl evoked a transient increase in cytoplasmic Ca2+ in synaptosomes isolated from squid optic lobe. The Ca2+ transient in that study was calculated to be around 340 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 Ca2+ channels that inactivate rapidly. The increase we recorded in the chemiluminescent response to KCl addition thus seems to represent the Ca2+-dependent release of ACh by cuttlefish synaptosomes. We estimated the total amount of ACh released to be 0.9 ± 0.3 (mean ± SE) nmol-mg-1 protein, which corresponds to 34% ± 1% of the total content of the synaptosomal fraction. When veratridine, a hydrophilic toxin that targets the type 2 receptor site of voltage-gated 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 Na+ channels are responsible for the generation of action potentials in nerve cells. Veratridine causes 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 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 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 μmol 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 [3H]Ch uptake by synaptosomes isolated from cuttlefish optic lobe. The suspension of synaptosomes was first allowed to recover for 15 min in isosmotic 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+ [3H]choline ([3H]Ch) was added to obtain a final Ch concentration of 5 μmol l-1 and a specific radioactivity of 50 μCi μmol-1. At designated reaction times, the reactions were stopped by rapid filtration of aliquots, and the values for [3H]Ch uptake were calculated after subtraction of blank values obtained by filtering aliquots of reaction media containing [3H]Ch (0.25 μCi/ml-1).

The synaptosomal fraction isolated from cuttlefish optic lobe was capable of taking up Ch when incubated in a Na+-enriched medium containing 460 mmol l-1 NaCl, 10 mmol l-1 KCl, 55 mmol l-1 MgCl2, 11 mmol l-1 CaCl2, 0.6 mmol l-1 KHCO3, 10 mmol l-1 Tris at pH 7.5 (Fig. 2A). Exposure to micromolar concentrations of HC-3 significantly decreased the amount of [3H]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 [3H]Ch uptake remained constant when the HC-3 concentration was increased from 10 to 100 μmol 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 [3H]Ch uptake by the high-affinity transporter can proceed at rates higher than 20 pmol Ch min-1 mg-1 protein, promoting the accumulation
of $[^3]$HCh up to 176 ± 42 (mean ± SE) pmol·mg$^{-1}$ protein when the synaptosomes were exposed to 5 μmol l$^{-1}$ $[^3]$HCh.

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 (Na$^+$)/K$^+$)ATPase, ouabain, suggesting that the high-affinity Ch transporter of cuttlefish optic lobe also requires a transmembrane Na$^+$ gradient to mediate active transport of Ch. In fact, dependence on 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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