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Aplysia Synaptosomes. II. Release of Transmitters

Gilbert J. Chin, Eli Shapiro, and James H. Schwartz

Howard Hughes Medical Institute, Center for Neurobiology and Behavior, Columbia University, New York, New York 10032

A subcellular fraction (P3) from *Aplysia* is enriched in synaptosomes (Chin et al., 1988) and is capable of accumulating 5-HT and choline. At an external ^{3}H-5-HT concentration of 1.8 μM, the P3 fraction took up 0.12 nmol/mg protein in 30 min. Uptake was dependent on external Na^+. Electron microscopic autoradiography showed that much of the accumulated ^{3}H-5-HT is localized to synaptosomes. At 0.5 μM ^{3}H-choline, P3 took up 0.11 nmol/mg protein in 30 min and converted 40% to ^{3}H-ACh. This synaptosomal fraction was also capable of releasing transmitter. After ^{3}H-5-HT or ^{3}H-choline was taken up, P3 released about 5% of the total radioactive transmitter in a Ca^{2+}-dependent manner during a 30 sec exposure to a depolarizing concentration of K^+ (100 mM). Identified, prelabeled synaptosomes were prepared by injecting ^{3}H-choline into the large cholinergic neuron L10. The abdominal ganglia containing the injected cells were then fractionated, yielding synaptosomes containing radioactivity derived from L10. After this synaptosomal fraction was exposed to high K^+, 2% of the radioactivity was released in a Ca^{2+}-dependent manner. This release was completely blocked by 0.1 mM histamine, a modulatory transmitter that has previously been shown to cause presynaptic inhibition in L10.

Synaptosomes from both vertebrate and invertebrate nervous systems (Blaustein, 1975; Morel et al., 1977; Breer and Jeserich, 1984) can retain the ability to release transmitters when exposed to depolarizing concentrations of K^+ in the presence of Ca^{2+} (reviewed by Bradford, 1986). Isolated synaptic endings also have been used to study the modulatory effects of exogenous agents on release (see, for example, DeBelleroche and Bradford, 1978; Nichols et al., 1987). The preceding paper presented the biochemical and morphological characterization of synaptosomal fractions prepared from the CNS of *Aplysia*. Here we describe the uptake and release of 5-HT and ACh using *Aplysia* synaptosomes. The abdominal ganglion contains a large, cholinergic interneuron, L10, which has been characterized neurochemically (Giller and Schwartz, 1971), biophysically (Shapiro et al., 1980a, b), physiologically (Kandel et al., 1967), and behaviorally (Koester et al., 1974; Koester and Koch, 1987). L10 displays several forms of synaptic plasticity. Of particular interest is the presynaptic inhibition produced by stimulating L32 cells that is mimicked by application of histamine (Kretz et al., 1986a, b). We were able to observe release of transmitter from identified L10 synaptosomes and modulation of this release by histamine.

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Correspondence should be addressed to James H. Schwartz, M.D., Ph.D., Howard Hughes Medical Institute, Research Annex/Room 708, 722 West 168th Street, New York, NY 10032.

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Materials and Methods

Preparation of *Aplysia* synaptosomes using 2-step and 5-step discontinuous sucrose gradients is described in the accompanying paper (Chin et al., 1989). Ca^{2+}-free normal seawater (NSW-Ca^{2+}) contained 460 mM NaCl, 10 mM KCl, 55 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.4), and 10 mM glucose. Ca^{2+}-free high-K^+ seawater (KSW-Ca^{2+}) contained 370 mM NaCl, 100 mM KCl, 55 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.4), and 10 mM glucose. Normal seawater (NSW) and high-K^+ seawater (KSW), in addition, contained 11 mM $CaCl_2$. Radiochemicals were from New England Nuclear (Boston); other chemicals were from Sigma (St. Louis).

Data are presented as means ± SEM (n = number of independent experiments). Significance was assessed using analysis of variance and 2-tailed t tests.

Uptake and release of 5-HT. P3 and P2 fractions containing synaptosomes (see Fig. 1, Chin et al., 1989) were incubated with ^{3}H-5-HT (24 Ci/mmol) in 0.2 ml NSW-Ca^{2+} at an external concentration of 0.8–2.1 μM. After 30 min at 23°C, samples were diluted with 1.0 ml cold NSW-Ca^{2+} and centrifuged in an Eppendorf microfuge for 5 min at 4°C. The pellets were washed twice and resuspended in cold NSW-Ca^{2+}. The specific activity of uptake was calculated using the average value of protein in the fraction (Chin et al., 1989, Table 1). To examine the Na^+ dependence of 5-HT uptake, we compared uptake by synaptosomal fractions P2 and P3 in NSW-Ca^{2+} with uptake in 1.1 M sucrose containing 25 mM Tris-HCl (pH 7.4). Release was assayed by diluting 1 vol of ^{3}H-5-HT-loaded synaptosomes into 9 vol of NSW, NSW-Ca^{2+}, KSW, or KSW-Ca^{2+} maintained at 23°C. After 30 sec, the samples were cooled on ice and then centrifuged in a microfuge for 10 min at 4°C. Release was expressed as the percentage of the total radioactivity (supernatant plus pellet) found in the supernatant.

Autoradiography. Fractions P3 and P2 were loaded with ^{3}H-5-HT and washed as described above. In one experiment, the pellets were fixed directly and processed for electron microscopy and autoradiography (Schwartz et al., 1986). In a second experiment, the pellets were resuspended in cold NSW-Ca^{2+} and exposed to 9 vol of KSW for 30 sec at 23°C as in a release assay. These samples were then centrifuged, and the pellets fixed and processed. No significant differences in morphology were observed between the 2 treatments.

Choline uptake and release of ACh and choline. P3 and P2, each in 0.1–0.2 ml NSW-Ca^{2+}, were added to tubes containing 0.24–0.83 μM ^{3}H-choline (80 Ci/mmol, dried down from ethanol) and incubated for 30 min at 23°C. The samples were diluted with cold NSW-Ca^{2+} and centrifuged, and the pellets washed twice. Uptake was calculated as for 5-HT. The release assay was carried out by centrifugation. The pellets were resuspended in NSW-Ca^{2+} to the same volume as the supernatants; both were then assayed for ^{3}H-ACh and ^{3}H-choline. Release of ACh or choline was expressed as for 5-HT. To inhibit endogenous acetylcholinesterase activity, eserine salicylate (20 μM) was added to all of the seawaters (Giller and Schwartz, 1971). Release and assay of ACh were unaffected by varying the concentration of eserine (10–50 μM).

Assay of ACh and choline. ^{3}H-ACh and ^{3}H-choline were measured following Goldberg and McCaman (1974) with modifications to remove eserine and to eliminate differential effects of NSW and KSW on ion pairing with the tetraphenylboron anion. Samples (0.05–0.2 ml) were

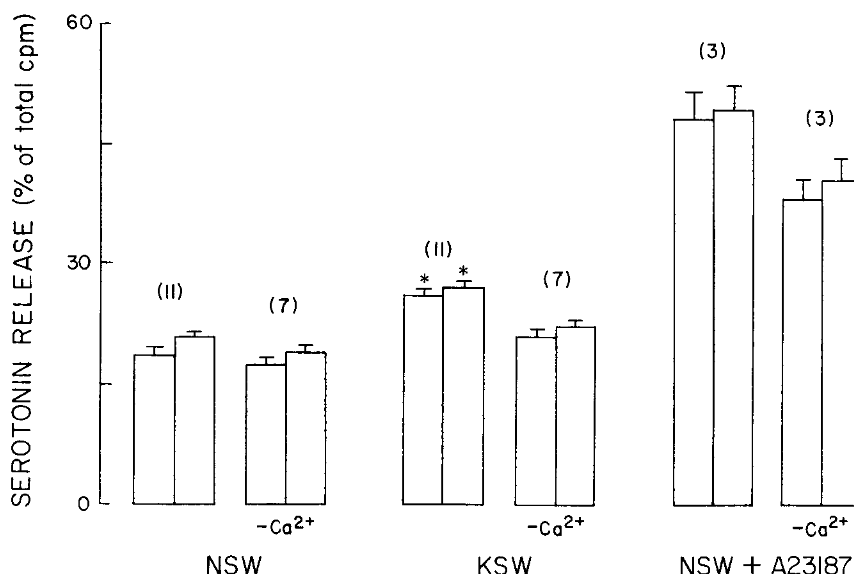

Figure 1. Release of ^{3}H-5-HT from synaptosomes. P3 and P2, prepared from *Aplysia* nervous tissue using a 2-step sucrose gradient, were assayed for release of ^{3}H-5-HT by centrifugation as described in Materials and Methods. Release was expressed as the percentage of total cpm recovered in the supernatants. A23187 diluted from a 10 mM solution in dimethylsulfoxide was used at a final concentration of 25 μM. The *left-hand bar* of each pair represents P3, the *right-hand bar*, P2. The number of experiments is given in parentheses. *, significantly different from NSW ($p < 0.001$) and KSW-Ca^{2+} ($p < 0.002$).

mixed with 5 vol of chloroform in order to extract eserine, to denature any endogenous acetylcholinesterase, and to solubilize choline and ACh. The aqueous phase was divided into 3 equal portions (A, B, C), each of which was diluted 10-fold with 50 mM NaH_2PO_4 (pH 7.0), 5 mM $MgCl_2$, 5 mM ATP. To A, no additions were made. To B, 25 mU choline kinase (yeast) was added. To C, 25 mU choline kinase and 2.0 U acetylcholinesterase (electric eel) were added. After they were incubated for 45 min at 23°C, the samples were extracted with sodium tetraphenylboron in 3-heptanone (10 mg/ml), and half of the organic layer was counted by scintillation. In control assays, the extent of reaction and efficiencies of extraction were determined using radioactive standards of choline, ACh, and phosphorylcholine.

Intracellular injection of ^{3}H-choline. Cell L10 was identified electrophysiologically (Kandel et al., 1967), concentrated ^{3}H-choline was pressure-injected (Koike et al., 1974), and the ganglia maintained overnight at 14°C in supplemented artificial seawater (Eisenstadt et al., 1973). The ganglia were then examined visually for the presence and integrity of L10. About 10% of the injected radioactivity escaped into the bath (Eisenstadt et al., 1973). In control experiments, isolated abdominal ganglia were pinned, the connective tissue sheath was removed as for injection of L10, and ^{3}H-choline was added directly to the bath. These ganglia were also maintained overnight.

Preparation of synaptosomal fractions from injected cells and assay of release. Abdominal ganglia containing ^{3}H-choline, labeled either by intracellular injection or by uptake, were mixed with freshly dissected ganglia from the CNS of 4 animals and homogenized to prepare synaptosomes using a 2-step discontinuous sucrose gradient (Chin et al., 1989). Release was assayed by filtration instead of centrifugation in order to eliminate sample losses due to transfer. The P3 pellet was washed with NSW-Ca^{2+} and resuspended in cold NSW-Ca^{2+}. In the top chambers of a 12-port filtration unit (Amicon, Danvers, MA) were placed 2 ml of NSW, NSW-Ca^{2+}, KSW, KSW-Ca^{2+}, NSW + 0.1 mM histamine, and KSW + 0.1 mM histamine, all prewarmed to 23°C, and then 20–30 μl of P3 was added with mixing. The reaction mixtures were kept for 30 sec at 23°C and then were suctioned through glass fiber filters (Whatman GF/A, Hillsboro, OR). Filtrates were collected directly in scintillation vials. The filters were air-dried and then soaked in 2 ml of 1% Triton X-100 in vials. Release was expressed as the percentage of total radioactivity (filter and filtrate) found in the filtrate.

Results

Uptake and release of 5-HT

The synaptosomal fractions P3 and P2 (Chin et al., 1989) were capable of accumulating 5-HT. At external ^{3}H-5-HT concentrations of 1.5–2.1 μM, P3 took up 119 ± 35 pmol/mg ($n = 6$) and P2 101 ± 16 pmol/mg ($n = 6$) during 30 min incubations. In a separate series of experiments, we examined the dependence of ^{3}H-5-HT uptake on external Na^+. In NSW-Ca^{2+}, at external ^{3}H-5-HT concentrations of 0.8–1.6 μM, P3 took up 58.4 ± 8.5 pmol/mg ($n = 4$). In a solution in which all salts were replaced with 1.1 M sucrose, P3 accumulated only 35.1 ± 8.0 pmol/mg ($n = 4$), an inhibition of 40%. Uptake into P2 was also dependent on external Na^+ (data not shown). Removal of Na^+ has previously been shown to inhibit the uptake of choline in the intact ganglion (Eisenstadt et al., 1975).

Metabolic conversion of ^{3}H-5-HT to glycoconjugates, observed when ^{3}H-5-HT was injected intracellularly (Goldman and Schwartz, 1977), did not occur in fractions P3 or P2. Thin-layer chromatography (*n*-butanol/acetic acid/H_2O, 25:4:10) on cellulose (Baker, Phillipsburg, NJ) revealed that 87–92% of the radioactivity in the washed P3 and P2 pellets and in the stock solution of ^{3}H-5-HT migrated with a 5-HT standard. Furthermore, the same percentage was found when the entire P3 and P2 incubations were analyzed at the end of the 30 min loading period. If diffusible products of 5-HT metabolism had been formed (Goldman and Schwartz, 1977), they should have been present in these samples. Their absence in the synaptosomal fractions suggests that the enzymes that catalyze the conjugation of 5-HT either are localized to the cell body or are inactivated during homogenization.

The synaptosomal fractions were capable of releasing transmitter when exposed to depolarizing concentrations of K^+ in the presence of Ca^{2+}. Exposure to 100 mM K^+ for 30 sec in the presence of 11 mM Ca^{2+} (KSW) significantly increased the release of ^{3}H-5-HT from P3 and P2 by 30–40% as compared with the release seen in normal seawater (NSW) (Fig. 1). These increases were largely Ca^{2+}-dependent (69–77%), as shown by the reduction in release when the fractions were exposed to the same depolarizing K^+ seawater without Ca^{2+} (KSW-Ca^{2+}).

In these release assays, almost 20% of the radioactivity appeared in the supernatant after a 30 sec exposure at 23°C to NSW-Ca^{2+}. Some of the factors that possibly contribute to this basal efflux are (1) ^{3}H-5-HT that is not completely removed by washing of the pellets; (2) release from synaptosomes that are unable to maintain a membrane potential or are leaky to Ca^{2+}; and (3) efflux through the 5-HT-uptake system. The contribution of the first factor is important because, in wash-out experiments, up to 30% of the final radioactivity in loaded P3 and

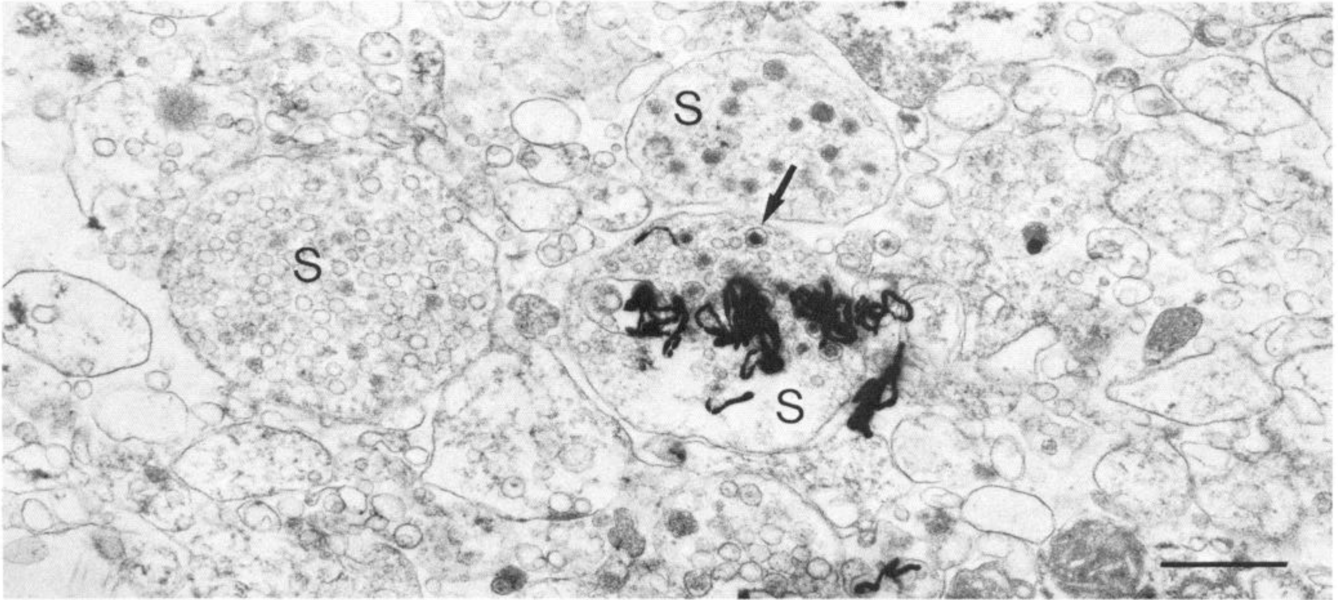

Figure 2. EM autoradiography of the gradient fraction P2 after ^{3}H-5-HT uptake. A field of 3 synaptosomes (S) enclosing vesicles of different type. The synaptosome that contains vesicles with a sharply defined dense core (*arrow*), typically found in serotonergic neurons (see Fig. 7 of Goldman et al., 1976), is heavily labeled with silver grains. The autoradiograph was exposed for 9 d. Scale bar, 0.5 μm.

P2 fractions was removed by 4 additional washes. The difficulty in assessing the contribution of this factor precisely lies in the concomitant loss of pellet material with each subsequent centrifugation and resuspension. The second factor, leakiness to Ca^{2+}, would appear negligible because addition of EGTA decreased the efflux seen in NSW-Ca^{2+} from 92% of the NSW value only to 86%. The third factor also contributes in a minor way because extending the time of incubation in NSW-Ca^{2+} increased the proportion of total radioactivity recovered in the supernatant by less than 1%/min.

The Ca^{2+} ionophore A23187, present only during the 30 sec exposure, increased the proportion of ^{3}H-5-HT released in NSW to 50% of the total radioactivity for both P3 and P2 (Fig. 1). Dimethylsulfoxide alone (0.25%) did not affect release. About 33% of the A23187 stimulation was eliminated when no Ca^{2+} was added (NSW-Ca^{2+}); this could be increased to 45% by adding 0.5 mM EGTA (not shown). (A possible explanation for the $[Ca^{2+}]_{ext}$-independent stimulation by A23187 is that intrasynaptosomal stores of Ca^{2+} are liberated by the ionophore.)

It has not yet been possible consistently to observe K^{+}-stimulated uptake of $^{45}Ca^{2+}$ in these *Aplysia* synaptosomal fractions. In some preparations, we did observe Ca^{2+} uptake activities of 5–9 nmol/mg during 30 sec exposures to high K^{+} at 23°C. This uptake compares well with the results of Blaustein (1975) and with the value of 5.4 ± 0.9 (n = 5) nmol/mg in 30 sec at 23°C that we obtained with a rat brain synaptosomal fraction. But in other *Aplysia* preparations, stimulation was either not seen or not inhibited by Co^{2+}, a known blocker of voltage-activated Ca^{2+} channels in *Aplysia*. We think that the inconsistency results from the small amounts of protein in the P3 and P2 fractions. Using a filtration assay and wash solutions containing EGTA, we found an average of 1 nmol of Ca^{2+} adsorbed to the filter and an equal amount taken up on exposure to NSW. Adsorption of $^{45}Ca^{2+}$ to filters was variable, even within experiments. In 7 experiments, up to 1 nmol of variation in adsorption was observed between duplicate measurements. In order to measure a stimulation above this highly variable background reliably, at least 40 μg protein is required for each point; this is equivalent to the yield of P3 from one animal (see Table 1, Chin et al., 1989).

Autoradiography with ^{3}H-5-HT

We attempted to localize the sites of ^{3}H-5-HT uptake in P3 and P2 with electron microscopic autoradiography (Fig. 2). High-affinity uptake of ^{3}H-5-HT has previously been localized to neuropil structures (Pentreath and Cottrell, 1972; Turner and Cottrell, 1978; Bailey et al., 1983). From a survey of 299 fields containing 204 silver grain clusters (3 or more), we found that about 70% of the grain clusters were associated with synaptosomes. Fewer than 5% of the synaptosomes were labeled. In addition, 53% of the labeled synaptosomes contained dense-core vesicles characteristic of serotonergic neurons (Fig. 2) (Goldman et al., 1976; Shkolnik and Schwartz, 1980; Bailey et al., 1983; Cleary and Schwartz, 1987). These results suggest that synaptosomes are the predominant site of ^{3}H-5-HT accumulation. A relatively minor contribution to uptake (about 10% of the grain clusters) comes from lysosomes, which have been shown previously to be labeled after intracellular injection of ^{3}H-5-HT (Schwartz et al., 1979). The remaining 20% of the grain clusters could not be assigned to identifiable membrane structures.

Uptake of choline and ACh release

As do cholinergic terminals in intact ganglia, the synaptosomal fractions accumulated choline and converted it to ACh (Table 1). At an external choline concentration of 0.24–0.83 μM, the specific activity of uptake for P3, 110 pmol/mg, was 3 times that of P2 (39 pmol/mg). This is consistent with the greater enrichment of synaptosomes in P3 (see Table 2, Chin et al., 1989), as well as earlier work in which the high-affinity choline uptake system (K_m = 5 μM) was localized to nerve terminals (Schwartz et al., 1975). P3 was also twice as efficient as P2 in synthesizing ACh (Table 1).

The fate of ^{3}H-choline taken up was restricted to ACh syn-

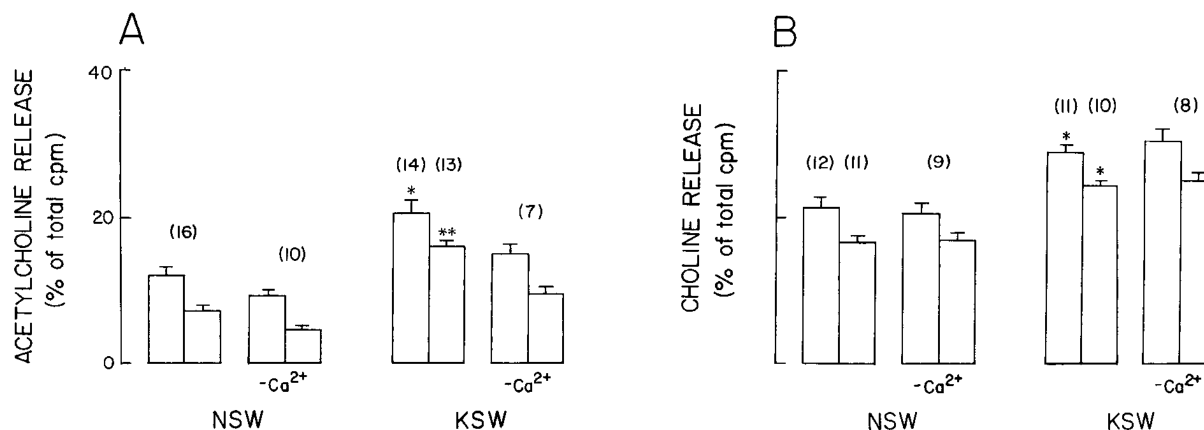

Figure 3. Release of ^{3}H-ACh and ^{3}H-choline from synaptosome fractions. P3 and P2, prepared from *Aplysia* nervous tissue using a 2-step sucrose gradient, were assayed for release of ^{3}H-ACh and ^{3}H-choline by centrifugation. Release was expressed as the percentage of total ^{3}H-ACh or ^{3}H-choline recovered in the supernatants. The *left-hand bar* of each pair represents P3, the *right-hand bar,* P2. *A,* ^{3}H-ACh release. *, significantly different from NSW ($p < 0.001$) and KSW-Ca^{2+} ($p < 0.02$); **, significantly different from NSW and KSW-Ca^{2+} ($p < 0.001$). *B,* ^{3}H-choline release. *, significantly different from NSW ($p < 0.001$).

thesis and packaging. Greater than 95% of the total radioactivity was either ACh or choline. Choline was not metabolized to betaine, a known oxidation product in *Aplysia* (Schwartz et al., 1975), or phosphorylcholine in significant amounts during the 30 min incubation.

Approximately twice as much ^{3}H-ACh was released from P3 and P2 fractions during a 30 sec exposure to KSW as compared with NSW (Fig. 3*A*). This stimulation was 70% Ca^{2+}-dependent (compare values in KSW to those in KSW-Ca^{2+}). In NSW-Ca^{2+}, approximately 20% of the loaded ^{3}H-choline taken up was recovered in the supernatant after the 30 sec exposure at 23°C. As discussed above for 5-HT, some of this release of ^{3}H-choline is likely to be apparent, and result from contaminating radioactivity. This explanation also would account for the much lower release (about 7%) in NSW-Ca^{2+} of the internally synthesized ^{3}H-ACh. On the other hand, the Ca^{2+}-independent stimulation of choline release by KSW and KSW-Ca^{2+} (Fig. 3*B*) is consistent with efflux through the Na^+-dependent choline uptake system (Eisenstadt et al., 1975).

In the experiments reported in the companion paper (Chin et al., 1989), we used a 5-step sucrose gradient to prepare synaptosomes from *Aplysia* nervous tissue; the microfuge pellet derived from the 0.35 M/0.75 M interface was estimated to contain 31% synaptosomes and yielded 43 μg protein per animal. In one experiment, this fraction was found to be more active than P3 in taking up choline (210 pmol/mg/30 min) and converted 42% of the ^{3}H-choline taken up to ACh. The lysosomal (0.9 M/1.1 M interface) and mitochondrial (1.1 M/1.5 M interface) fractions were much less active (85 and 42 pmol/mg/30 min), although the choline that was taken up was also converted (33 and 26%). Furthermore, the 0.35 M/0.75 M interface fraction released twice as much ACh when exposed to KSW as compared with NSW, and this stimulation was 87% Ca^{2+} dependent. In contrast, release from the mitochondrial fraction was stimulated only 27% by KSW.

Table 1. Uptake of ^{3}H-choline and conversion to ^{3}H-ACh in 2-step gradient fractions

Fraction	Choline uptake (pmol/mg)	Acetylcholine synthesis (% of total cpm taken up)
P2	39 ± 5 (10)	19.5 ± 2.3 (12)
P3	110 ± 17 (14)	39.8 ± 2.5 (16)

Choline uptake and ACh synthesis were measured in gradient fractions as described in Materials and Methods. The number of experiments is given in parentheses.

Release of transmitter from presumptive identified synaptosomes

After intracellular injection of ^{3}H-choline, L10 synthesizes ^{3}H-ACh and releases radioactivity from its synapses when depolarized (Eisenstadt et al., 1973; Koike et al., 1974). The L10 cells of 4 ganglia were each injected with 10–100 pmol ^{3}H-choline. The ganglia were maintained overnight to allow conversion of ^{3}H-choline into ^{3}H-ACh and transport to synaptic terminals. These labeled ganglia were mixed with carrier nervous tissue, and synaptosomes were prepared using a 2-step gradient.

We first examined the fate of the injected ^{3}H-choline (Table 2). Approximately 2% of the radioactivity in the homogenate was recovered in P3. Most was soluble. In experiments in which S2 and P2 were separated, the distribution of radioactivity was similar to that of S3 and P3: 90% appeared in the supernatant. Eisenstadt and Schwartz (1975) showed that after large ^{3}H-choline injections, most of the ^{3}H-ACh synthesized exceeds the capacity of the vesicles and remains unpackaged in the cytoplasm.

In P3, ^{3}H-ACh accounted for 29.4 ± 5.0% ($n = 5$) of the total radioactivity recovered in choline and ACh. In P2, this percentage was 24.8 ± 4.7 ($n = 5$). In these experiments, since the fractionation of nervous tissue was carried out after the synthesis of ACh had occurred, the efficiencies of conversion do not reflect the greater content of synaptosomes in P3 (compare with experiments in Table 1). These values are in agreement with earlier results on ACh synthesis as a function of amount of choline injected *in vivo* (Eisenstadt et al., 1973; Eisenstadt and Schwartz, 1975).

Release of the radioactivity injected into L10 from P3 was stimulated 3% by exposure to elevated K^+ in the presence of Ca^{2+} (Fig. 4). In experiments with identified terminals, we only monitored release of total radioactivity (which consists of ACh

Table 2. Distribution of radioactivity in gradient fractions prepared from injected L10 cells

Fraction	Distribution (% of total cpm)
P1	14.2 ± 1.3
S2 + P2	63.2 ± 2.7
P3	2.1 ± 0.3
S3	20.5 ± 2.4

Abdominal ganglia in which L10 had been injected with ^{3}H-choline were homogenized and fractionated on a 2-step sucrose gradient as described in Materials and Methods. The range of total choline injected in each preparation was 13–196 pmol (n = 11).

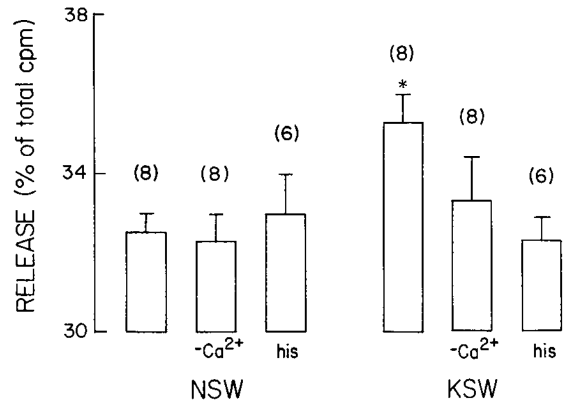

Figure 4. Release of radioactivity from synaptosome fractions containing prelabeled identified neurons. P3, prepared from *Aplysia* nervous tissue containing L10 cells injected with ^{3}H-choline, was assayed for release by filtration. Release was expressed as the percentage of total cpm recovered in the filtrates. *his,* 0.1 mM histamine included in the 30 sec exposure to NSW or to KSW. *, significantly different from NSW and KSW + his ($p < 0.01$).

and choline) because we were unable to inject enough choline to assay subsequently the release of ^{3}H-ACh from P3. (This difficulty might be overcome by injecting a larger number of L10 cells for each synaptosome preparation.) Although small, the stimulation observed is significant, and was about 70% Ca^{2+} dependent by comparison with the stimulation seen in KSW-Ca^{2+}. Furthermore, the presence of 0.1 mM histamine, a ligand that effectively inhibits synaptic release from L10 in the intact ganglion (Kretz et al., 1986a, b), inhibited the high-K^+-induced release of radioactivity from the fractions containing prelabeled L10 terminals (Fig. 4). In 2 other experiments we tested the specificity of this modulation by comparing the effect of 0.1 mM histamine and 0.1 mM 5-HT on K^+-evoked release from L10 terminals in the P3 fraction. In both experiments histamine inhibited K^+-evoked release, while 5-HT, which does not alter release of transmitter from L10 in the ganglion, had no effect (data not shown).

We also tested the ability of Ba^{2+} ions to support release from fractions containing prelabeled L10 terminals. [In the abdominal ganglion extracellular Ba^{2+} can substitute for Ca^{2+}; release with Ba^{2+} lasts longer than with Ca^{2+} and is more prominent at resting membrane potentials (Shapiro et al., 1980a).] In these experiments, Ba^{2+}SW (22 mM $BaCl_2$ substituted for $CaCl_2$) stimulated the release of 4.4% of the total radioactivity compared with NSW-Ca^{2+}, while the stimulation in 22 mM Ca^{2+} SW was only 2.9%. In comparison, Ba^{2+}KSW and 22 mM Ca^{2+}KSW both evoked the release of an additional 6.6% of the total radioactivity compared with KSW-Ca^{2+}.

Because a high-affinity choline uptake system is present in the neuropil and because P3 contained only 2% of the injected radioactivity, it seemed possible that we were actually observing leakage of injected ^{3}H-choline from the L10 cell body into the bath, uptake into neuronal processes with subsequent ACh synthesis. This would obviously render the origin of any labeled synaptosomes ambiguous because other cholinergic neurons have terminals in the abdominal ganglion. We attempted to measure the contribution of leakage to the results obtained with injected L10s by incubating ganglia with ^{3}H-choline (8.4–92 pmol) directly and then preparing synaptosomes. In these experiments, the homogenate contained only 3.3 ± 0.4% (n = 3) of the total radioactivity in the bath; of this, 2.9 ± 0.8% (n = 3) was recovered in P3. Since in the cell injection experiments the leakage from L10 into the bath was about 10% of the injected radioactivity, only 0.01% ($0.1 \times 0.033 \times 0.029 = 0.0001$) of the total ^{3}H-choline would be expected to appear in P3. Since P3 actually contained 2% of the total injected ^{3}H-choline, 99.5% of the radioactivity in P3 must be derived directly from L10.

Discussion

Synaptosomal fractions can be prepared from *Aplysia* nervous tissue that take up ^{3}H-5-HT and ^{3}H-choline and release 5-HT and ACh in response to depolarization in the presence of Ca^{2+}. The K^+-stimulated release of 5–10% of the 5-HT or ACh content of the *Aplysia* fractions is comparable to that observed in other synaptosome preparations (Blaustein, 1975; Suszkiw and O'Leary, 1982).

Uptake and release

The conclusion that synaptosomes take up and release the transmitters rests upon 3 observations. First, autoradiography with ^{3}H-5-HT demonstrated that more than half of the labeled structures are identified as synaptosomes that contain vesicles of a type associated with serotonergic neurons in *Aplysia*. Second, P3 is more active than P2 in taking up ^{3}H-choline and synthesizing ACh. These activities are characteristic of cholinergic nerve terminals in intact nervous tissue and correlate well with the higher enrichment of synaptosomes in P3. Third, the better resolution of components obtained on the 5-step gradient indicates that the uptake and release activities are much greater in synaptosomal fractions than in the lysosomal and mitochondrial fractions.

The release protocols (centrifugation or filtration) used in this work reveal a small but reproducible Ca^{2+}-dependent stimulation by elevated K^+, and they were simple to design and perform, even though limited by the small amounts of material and the rapid decay of activity.

We have not yet found modulation of the K^+-stimulated release of ^{3}H-5-HT and ^{3}H-ACh with exogenously added transmitters (5-HT, ACh, histamine, $FMRF_{NH2}$) or with reagents that affect second-messenger mechanisms (cAMP, phorbol ester). This is in keeping with the expectation that a heterogeneous population of synaptosomes participate in uptake and release of radioactivity and that no particular class of synaptic terminal in which, for example, ACh release is inhibited by histamine, holds a plurality. This experimental limitation is common to most heterogeneous CNS preparations of synaptosomes, given the diversity of transmitters and neuromodulatory pathways.

On the other hand, the lack of modulation suggests that indirect effects on release during the 30 sec incubation either are not taking place or are affecting only a small subset of synaptosomes.

Release of transmitter from identified synaptosomes

The large, identifiable nerve cells of molluscans have been used to examine the electrophysiological and biophysical mechanisms of modulation of transmitter release. A critical and controversial assumption in most of these studies is that electrical recordings from neuronal somata faithfully monitor release events occurring in the neuropil region (see Koester and Byrne, 1980). Our observation of histamine modulation of high-K^+-evoked release from the identified synaptosomes of L10 provides important evidence supporting this assumption. Previous electrophysiological and voltage-clamp analysis of transmitter release from L10 and inhibition of release by histamine revealed that histamine both reduces the Ca^{2+} current and increases a K^+ current (Kretz et al., 1986a). These analyses were complicated by the electrical distance between the soma recording site and the neuropil, where transmitter release occurs, making it difficult to determine the contribution of each conductance mechanism (see Kretz et al., 1986b). The ability of Ba^{2+} to support, and of histamine to inhibit, K^+-evoked release from L10 terminals in a synaptosomal fraction further implicates the role of Ca^{2+} current modulation in presynaptic inhibition.

Recent results suggest that a pertussis toxin-sensitive G protein (Vogel et al., 1989) and arachidonic acid metabolites are involved in the mechanism of L10's response to histamine (Piomelli et al., 1987). *Aplysia* synaptosomes, which retain the ability to metabolize arachidonic acid (Piomelli et al., 1987), provide a useful preparation to examine this complex modulatory pathway further. It may also be possible to introduce other molecules, such as fluorescent indicators of Ca^{2+} concentration, into synaptosomes by injection prior to homogenization. This would allow study of both Ca^{2+} flux and transmitter release in isolated synaptic terminals of identified *Aplysia* neurons.

References

Bailey, C. H., R. D. Hawkins, and M. C. Chen (1983) Uptake of [^{3}H] serotonin in the abdominal ganglion of *Aplysia californica.* Further studies on the morphological and biochemical basis of presynaptic facilitation. Brain Res. *272:* 71–81.

Blaustein, M. P. (1975) Effects of potassium, veratridine, and scorpion venom on calcium accumulation and transmitter release by nerve terminals *in vitro.* J. Physiol. (Lond.) *247:* 617–655.

Bradford, H. F. (1986) *Chemical Neurobiology,* W. H. Freeman, New York.

Breer, H., and G. Jeserich (1984) Invertebrate synaptosomes—Implications for comparative neurochemistry. Curr. Top. Res. Synapses *1:* 165–210.

Chin, G. J., E. Shapiro, S. S. Vogel, and J. H. Schwartz (1989) *Aplysia* synaptosomes. I. Preparation and biochemical and morphological characterization of subcellular membrane fractions. J. Neurosci. *9:* 38–48.

Cleary, L. J., and J. H. Schwartz (1987) Movement of newly synthesized membrane by fast transport along the axon of an identified *Aplysia* neuron. J. Comp. Neurol. *263:* 92–105.

DeBelleroche, J., and H. F. Bradford (1978) Biochemical evidence for the presence of presynaptic receptors on dopaminergic nerve terminals. Brain Res. *142:* 53–68.

Eisenstadt, M. L., and J. H. Schwartz (1975) Metabolism of acetylcholine in the nervous system of *Aplysia californica.* III. Studies of an identified cholinergic neuron. J. Gen. Physiol. *65:* 293–313.

Eisenstadt, M., J. E. Goldman, E. R. Kandel, H. Koike, J. Koester, and J. H. Schwartz (1973) Intrasomatic injection of radioactive precursors for studying transmitter synthesis in identified neurons of *Aplysia californica.* Proc. Natl. Acad. Sci. USA *70:* 3371–3375.

Eisenstadt, M. L., S. N. Treistman, and J. H. Schwartz (1975) Metabolism of acetylcholine in the nervous system of *Aplysia californica.* II. Regional localization and characterization of choline uptake. J. Gen. Physiol. *65:* 275–291.

Giller, E., and J. H. Schwartz (1971) Choline acetyltransferase in identified neurons of abdominal ganglion of *Aplysia californica.* J. Neurophysiol. *34:* 93–107.

Goldberg, A. M., and R. E. McCaman (1974) An enzymatic method for the determination of picomole amounts of choline and acetylcholine. In *Choline and Acetylcholine: Handbook of Chemical Assay Methods,* I. Hanin, ed., pp. 47–61, Raven, New York.

Goldman, J. E., and J. H. Schwartz (1977) Metabolism of [^{3}H]serotonin in the marine mollusc, *Aplysia californica.* Brain Res. *136:* 77–88.

Goldman, J. E., K. W. Kim, and J. H. Schwartz (1976) Axonal transport of [^{3}H]serotonin in an identified neuron of *Aplysia californica.* J. Cell Biol. *70:* 304–318.

Kandel, E. R., W. T. Frazier, R. Waziri, and R. E. Coggeshall (1967) Direct and common connections among identified neurons in *Aplysia.* J. Neurophysiol. *30:* 1352–1376.

Koester, J. H., and J. H. Byrne, eds. (1980) *Molluscan Nerve Cells: From Biophysics to Behavior,* Cold Spring Harbor Laboratory, New York.

Koester, J., and U. T. Koch (1987) Neural control of the circulatory system of *Aplysia.* Experientia *43:* 972–980.

Koester, J., E. Mayeri, G. Liebeswar, and E. R. Kandel (1974) Neural control of circulation in *Aplysia.* II. Interneurons. J. Neurophysiol. *37:* 476–496.

Koike, H., E. R. Kandel, and J. H. Schwartz (1974) Synaptic release of radioactivity after intrasomatic injection of choline-^{3}H into an identified cholinergic interneuron in abdominal ganglion of *Aplysia californica.* J. Neurophysiol. *37:* 815.

Kretz, R., E. Shapiro, C. H. Bailey, M. Chen, and E. R. Kandel (1986a) Presynaptic inhibition produced by an identified presynaptic inhibitory neuron. II. Presynaptic conductance changes caused by histamine. J. Neurophysiol. *55:* 131–146.

Kretz, R., E. Shapiro, and E. R. Kandel (1986b) Presynaptic inhibition produced by an identified presynaptic inhibitory neuron. I. Physiological mechanisms. J. Neurophysiol. *55:* 113–130.

Morel, N., M. Israel, R. Manaranche, and P. Mastour-Frachon (1977) Isolation of pure cholinergic nerve endings from *Torpedo* electric organ. J. Cell. Biol. *75:* 43–55.

Nichols, R. A., J. W. Haycock, J. K. T. Wang, and P. Greengard (1987) Phorbol ester enhancement of neurotransmitter release from rat brain synaptosomes. J. Neurochem. *48:* 615–621.

Pentreath, V. W., and G. Cottrell (1972) Selective uptake of 5-HT by axonal processes in *Helix pomatia.* Nature [New Biol.] *239:* 213–214.

Piomelli, D., E. Shapiro, S. J. Feinmark, and J. H. Schwartz (1987) Metabolites of arachidonic acid in the nervous system of *Aplysia*: Possible mediators of synaptic modulation. J. Neurosci. *7:* 3675–3686.

Schwartz, J. H., M. L. Eisenstadt, and H. Cedar (1975) Metabolism of acetylcholine in the nervous system of *Aplysia californica.* I. Source of choline and its uptake by intact nervous tissue. J. Gen. Physiol. *65:* 255–273.

Schwartz, J. H., L. J. Shkolnik, and D. J. Goldberg (1979) Specific association of neurotransmitter with somatic lysosomes in an identified serotonergic neuron of *Aplysia californica.* Proc. Natl. Acad. Sci. USA *76:* 5967–5971.

Schwartz, J. H., A. Elste, E. Shapiro, and H. Gotoh (1986) Biochemical and morphological correlates of transmitter type in C2, an identified histaminergic neuron in *Aplysia.* J. Comp. Neurol. *245:* 401–421.

Shapiro, E., V. F. Castellucci, and E. R. Kandel (1980a) Presynaptic membrane potential affects transmitter release in an identified neuron of *Aplysia* by modulating the Ca^{2+} and K^+ channels. Proc. Natl. Acad. Sci. USA *77:* 629–633.

Shapiro, E., V. F. Castellucci, and E. R. Kandel (1980b) Presynaptic inhibition in *Aplysia* involves a decrease in the Ca^{2+} current of the presynaptic neuron. Proc. Natl. Acad. Sci. USA *77:* 1185–1189.

Shkolnik, L. J., and J. H. Schwartz (1980) Genesis and maturation of serotonergic vesicles in identified giant cerebral neurons of *Aplysia.* J. Neurophysiol. *43:* 945–967.

Suszkiw, J. B., and M. E. O'Leary (1982) Differential labeling of depot

and active acetylcholine pools in nondepolarized and potassium-depolarized rat brain synaptosomes. J. Neurochem. *38:* 1668–1675.

Turner, J. D., and G. A. Cottrell (1978) Cellular accumulation of amines and amino acids in the central ganglia of a gastropod mollusc, *Planorbis corneus*: An autoradiographic study. J. Neurocytol. *7:* 759–776.

Vogel, S. S., G. J. Chin, S. M. Mumby, M. Schonberg, and J. H. Schwartz (1989) G proteins in *Aplysia*: Biochemical characterization and regional and subcellular distribution. Brain Res. (in press).