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Acetylcholine and ATP are coreleased from the electromotor nerve terminals of *Narcine brasiliensis* by an exocytotic mechanism

(synaptosome/electric organ/AH5183/ecto-ATPase)

C. D. UNSWORTH* AND R. G. JOHNSON

Howard Hughes Medical Institute and Departments of Medicine, Physiology, and Biochemistry and Biophysics, University of Pennsylvania, School of Medicine, Philadelphia, PA 19104

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ABSTRACT **Although the exocytotic mechanism for quantal acetylcholine (ACh) release has been widely accepted for many years, it has repeatedly been challenged by reports that ACh released upon stimulation originates from the cytosol rather than synaptic vesicles. In this report, two independent experimental approaches were taken to establish the source of ACh released from the electromotor system of *Narcine brasiliensis*. Since ATP is colocalized with ACh in the cholinergic vesicle, the exocytotic theory predicts the corelease of these two components with a stoichiometry identical to that of the vesicle contents. The stimulated release of ATP from isolated synaptosomes could be accurately quantitated in the presence of the ATPase inhibitor adenosine 5′-[α,β-methylene]triphosphate (500 μM), which prevented degradation of the released ATP. Various concentrations of elevated extracellular potassium (25–75 mM), veratridine (100 μM), and the calcium ionophore ionomycin (5 μM) all induced the corelease of ACh and ATP in a constant molar ratio of 5–6:1 (ACh/ATP), a stoichiometry consistent with that established for the vesicle content. In parallel to these stoichiometry studies, the compound 2-(4-phenylpiperidino)cyclohexanol (AH5183) was used to inhibit specifically the vesicular accumulation of newly synthesized (radiolabeled) ACh without affecting cytosolic levels of newly synthesized ACh in cholinergic nerve terminals. Treatment with AH5183 (10 μM) was shown to inhibit the release of newly synthesized ACh without markedly affecting total ACh release; thus, the entry of newly synthesized ACh into the synaptic vesicle is essential for its release. We conclude that ACh released upon stimulation originates exclusively from the vesicular pool and is coreleased stoichiometrically with other soluble vesicle contents.**

The highly specialized electromotor system found in a variety of marine species has been recognized for many years as an excellent system for the study of acetylcholine (ACh) neurotransmission (1). Despite extensive study, one fundamental aspect still remains controversial—namely, the cellular mechanism by which ACh is released from the presynaptic nerve terminal of the elasmobranch electromotor system. There is considerable evidence in support of a classical exocytotic process for ACh release (2) [for example, the uptake of extracellular markers in recycling vesicles (3) and the release of false transmitters in proportion to vesicular content (4)]. In contrast, other observations, such as the depletion of cytosolic but not vesicular ACh following stimulated release (5), a lack of stoichiometry of the released components compared to the vesicular content (6, 7), and calcium-dependent ACh release from ACh-loaded synaptosome ghosts, devoid of vesicles (8), are cited as evidence that released ACh originates directly from the cytosol. This alternative proposal has received further support following the isolation of a nerve terminal membrane protein reported to mediate this release of ACh (9–11). In the present study, two independent approaches were used to elucidate the mechanism of ACh release from the presynaptic nerve terminals of the electromotor system of *Narcine brasiliensis*. It is widely accepted that the cholinergic synaptic vesicle contains high levels of ATP in addition to ACh (12–15), and the exocytotic theory predicts that ACh and ATP would be coreleased with the same stoichiometry as the vesicle content. By using inhibitors of acetylcholinesterase (AChE) and ATP degradation, it was possible to quantitate the ACh and ATP coreleased from synaptosomes in response to a variety of stimuli.

A second approach utilized the compound AH5183 [2-(4-phenylpiperidino)cyclohexanol], an inhibitor of vesicular ACh uptake (16). Incubation of cholinergic nerve endings with radiolabeled choline resulted in the rapid appearance of radiolabeled ACh in the cytosol followed by a gradual accumulation into the vesicular pool. In the presence of AH5183, radiolabeled ACh still appeared in the cytosol, but its vesicular accumulation was significantly reduced. The pharmacological distinction of these two subcellular pools of ACh was therefore possible, and a comparison of the release of newly synthesized (radiolabeled) ACh with that of total ACh was used to identify the subcellular origin of the ACh released upon stimulation.

The data obtained from this series of experiments support the exocytotic theory of ACh release in this electromotor system.

MATERIALS AND METHODS

Materials. Electric rays, of the species *N. brasiliensis*, were obtained from the Panacea Institute of Marine Science (Panacea, FL). Following transport to the laboratory, the specimens were maintained in marine tanks for at least 1 week prior to use.

Luminol and ATP-monitoring reagent were obtained from LKB; choline kinase was from Boehringer Mannheim; phospholine iodide as an ophthalmic solution was supplied by Ayerst Laboratories; AH5183 was from Research Biochemicals (Natick, MA); 3-heptanone was obtained from Eastman Kodak; A23187 and ionomycin were from Calbiochem; [*methyl*-^{3}H]choline (80 Ci/mmol; 1 Ci = 37 GBq) was from New England Nuclear; and the [^{3}H]hexadecane standard was obtained from Amersham. All other reagents were obtained from Sigma. Stainless steel wire mesh was obtained from Newark Wire Cloth (Newark, NJ); filter screen mesh (spectramesh) was supplied by Fisher.

Preparation of Synaptosomes. The method used was similar to that previously described (17, 18), with additional Percoll

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Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; α,β-methylene ATP, adenosine 5′-[α,β-methylene]triphosphate.
*To whom reprint requests should be addressed.

and Ficoll density gradient centrifugation steps included in the purification procedure.

Preparation of the Electric Organ Slices. The preliminary steps of this procedure were identical to those for the synaptosome preparation except sucrose was omitted from the physiological medium (17, 18). After passage through the first wire sceen (1000-μm mesh), the homogenate was allowed to settle and the supernatant was decanted. The sedimented material was washed five times with physiological medium, resuspended in 500 ml, and centrifuged at 1000 $\times$ g for 10 min. The upper region of the pellet, which is comparatively free of connective tissue, was selected, washed, and resuspended in 50 ml of physiological medium. Any remaining connective tissue was manually removed.

Release Experiments. Samples of the synaptosome preparation (50–100 μl), generally at a concentration of 0.5–1.0 mg of protein per ml, were pelleted by centrifugation at 10,000 $\times$ g for 4 min and resuspended in the desired volume (50–150 μl) of physiological medium. Phospholine and adenosine 5'-[α,β-methylene]triphosphate (α,β-methylene ATP) were added to the resuspended synaptosomes to give final concentrations of 50 μM and 500 μM, respectively. The samples were then preincubated for 10–15 min at room temperature to allow inactivation of the endogenous AChE. For potassium stimulation, a modified physiological medium was prepared by increasing the potassium chloride concentration to 100 mM and decreasing the urea concentration to 100 mM. Addition of the desired amount of this high potassium medium to the resuspended synaptosomes gave the required final potassium concentration, and release was allowed to proceed for 4 min at room temperature. The samples were centrifuged at 10,000 $\times$ g for 4 min at room temperature and placed on ice, and aliquots of the supernatant were assayed for ACh and ATP. Parallel control samples were processed in which the high potassium medium was replaced by physiological medium for quantitation of basal release. It was established that phospholine did not affect either basal or stimulated release of ACh and ATP and that α,β-methylene ATP did not alter basal or stimulated ACh release. The final volume for the synaptosomal release experiments was 200 μl. Recovery of ACh and ATP was calculated from synaptosome fractions to which known amounts of ACh and ATP standards had been added. Recoveries were generally >80%, and the values for released ACh and ATP were corrected accordingly.

For the electric organ tissue slices, the same procedure was employed, using a 400-μl sample of the tissue suspension and stimulating with an equivalent volume of high potassium physiological medium. Preincubation with phospholine (120 μM) for at least 2 hr was necessary to inhibit endogenous AChE in this preparation.

Radiolabeling Experiments. A 250-μl sample ($\approx$200 μg of protein) of the synaptosome suspension was taken, and 5–10 μCi of [*methyl*-^{3}H]choline (1 μCi/μl) added. The samples were incubated for 5 hr at room temperature after which 750 μl of ice-cold physiological medium was added to each tube. These were centrifuged at 10,000 $\times$ g for 10 min at 5°C, and the pellet was resuspended in 150 μl of physiological medium. A 50-μl sample of resuspended synaptosomes was centrifuged at 10,000 $\times$ g for 10 min at 5°C, the supernatant was aspirated, and the pellet was resuspended in 200 μl of 2 M acetic acid, a procedure that, in conjunction with a freeze/thaw cycle, efficiently extracted and stabilized synaptosomal ACh. Two additional 50-μl aliquots were taken, treated with phospholine, and used to assess basal and stimulated release of ACh as described above.

For the electric organ tissue slices, phospholine was added at a final concentration of 120 μM to a 400-μl sample of the suspension. Following addition of 5 μCi of [*methyl*-^{3}H]-choline (1 μCi/μl) and incubation at room temperature for 2 hr, the tissues were washed twice with 750 μl of ice-cold physiological medium before resuspension in 400 μl of physiological medium. The radiolabeled tissue slices were stimulated as previously described, and the supernatant was retained for assay of ACh and radiolabeled ACh. The pellet of electric organ slices obtained following release was extracted with 1 ml of 10% (vol/vol) acetic acid/0.1 M hydrochloric acid for quantitation of tissue ACh content. Resuspension of the slices in acid, coupled with a freeze/thaw cycle, efficiently extracted and stabilized tissue ACh.

ACh Assay. The luminometric method (5) was used to detect choline in the samples after treatment with AChE. The reaction was measured in a LKB luminometer model 1250.

ATP and ATPase Assays. The luminometric method using luciferin/luciferase was used to quantitate ATP.

This sensitive ATP detection system was used to study ATPase activity associated with the synaptosomes (19). A 50-μl aliquot of ATP-monitoring reagent (prepared in 5 ml of physiological medium) was added to 350 μl of physiological medium in a cuvette. A known amount of ATP was added, and the luminescence was recorded. Addition of the synaptosome suspension (50–100 μl), containing ATPase activity, started the reaction, which was monitored as a steady decline in the luminescence as the ATP was hydrolyzed. The rate of ATP hydrolysis could be calculated from the linear relationship between the ATP concentration and the photomultiplier output.

Quantitation of Radiolabeled Acetylcholine. The tetraphenylboron extraction procedure was used to quantitate radiolabeled ACh (20).

Protein Assay. Tissue samples were dissolved in a 0.6 M sodium hydroxide, and the protein content was determined against bovine serum albumin standards by using the Lowry method (21).

RESULTS

Synaptosomal Preparation. The specific activities of ACh and ATP in these synaptosomes were 248.49 $\pm$ 21.63 (n = 8) and 27.92 $\pm$ 3.44 (n = 4) nmol/mg of protein, respectively (values are means $\pm$ SEM). These values indicate a high degree of synaptosomal purity, comparable with other reports (17, 22, 23).

Synaptosomal ATPase Activity. Initial studies indicated that, although the synaptosomes released ACh in a calcium-dependent manner following potassium-induced depolarization, no ATP release could be detected. The association of an ecto-ATPase with synaptosomes from the electric organ and the central nervous system has been reported (24–26); it was confirmed that this synaptosome preparation did possess an ATPase activity with a K_m of 0.88 μM and a V_{max} of 3.00 nmol/min per mg of protein. Three analogs of adenine nucleotides, α,β-methylene ATP, adenosine 5'-[α,β-methylene]diphosphate, and adenosine 5'-[β,γ-methylene]-triphosphate, were found to inhibit this synaptosomal ATPase activity. The most effective compound was α,β-methylene ATP with an IC_{50} of $\approx$25 μM. The other two analogs were significantly less potent, with IC_{50} values >500 μM. At a concentration of 500 μM, α,β-methylene ATP inhibited ATP degradation by >85% without interfering in the ATP assay and therefore permitted accurate measurement of ACh/ATP ratios.

Stoichiometry of ACh and ATP Release. Table 1 shows the amounts of ACh and ATP released from synaptosomes following various stimuli. While the amount of released ACh and ATP increased with the degree of potassium depolarization, the ratio of ACh/ATP remained constant at $\approx$6:1. This indicates that isolated synaptosomes not only release ACh upon potassium-induced depolarization but also corelease ATP in approximately the same proportions as determined

Table 1. Stoichiometry of ACh and ATP release

Experiment	Secretagogue	ACh, pmol	ATP, pmol	ACh/ATP
1	25 mM K^+	7.27 ± 2.50	1.31 ± 0.45	5.91 ± 1.86
	50 mM K^+	22.05 ± 1.47	3.84 ± 0.26	5.76 ± 0.27
	75 mM K^+	26.93 ± 0.48	4.42 ± 0.50	6.17 ± 0.61
2	50 mM K^+	18.75 ± 0.31	3.44 ± 0.40	5.55 ± 0.60
	100 μM veratridine	10.97 ± 1.17	1.79 ± 0.12	6.17 ± 0.80
3	50 mM K^+	10.95 ± 0.51	2.12 ± 0.25	5.22 ± 0.45
	10 μM A23187	29.77 ± 1.74	3.51 ± 0.28	8.52 ± 0.45*
4	75 mM K^+	9.38 ± 0.88	1.88 ± 0.30	5.11 ± 0.91
	2.5 μM ionomycin	45.65 ± 3.61	7.21 ± 0.31	6.33 ± 0.27
	2.5 μM A23187	6.69 ± 1.32	0.75 ± 0.30	9.57 ± 1.49*

Synaptosomal release experiments were performed as described in experimental procedures. Values are expressed per 10 μl of release medium and are the means ± SD (n = 4).
*$P < 0.005$ compared to corresponding potassium stimulation (Student's t test).

for the vesicular content (2). Veratridine (100 μM) evoked release of both ACh and ATP to a lesser degree than potassium stimulation, but the ratio of these coreleased substances was constant. A calcium ionophore, ionomycin, demonstrated no significant difference in the ratio of ACh/ATP released compared to potassium depolarization. In contrast to these consistent ratios obtained with potassium-, veratridine-, and ionomycin-induced release, the calcium ionophore A23187 demonstrated a significant deviation in the ratio of released ACh/ATP. Over a range of concentrations, A23187 consistently induced corelease of ACh and ATP in a higher ratio than potassium depolarization.

Radiolabeling Studies in Synaptosomes. The synaptosomes demonstrated a considerable capacity to take up and acetylate radiolabeled choline. Radiolabeled ACh accumulated rapidly in the cytosol but showed a slower entry into the vesicular pool (Table 2). This marked differential distribution of ACh and radiolabeled ACh may result in an overestimation of the vesicular radiolabeled ACh content, should a small percentage of cytosolic ACh survive a freeze/thaw cycle. Despite this qualification, AH5183 had no significant effect on the cytosolic ACh content or level of radiolabeled ACh in the cytosol, indicating that this compound did not affect choline uptake or its acetylation.

Since AH5183 specifically inhibits the entry of radiolabeled ACh into the vesicular pool without affecting levels of radiolabeled ACh in the cytosol, quantitation of radiolabeled ACh release upon stimulation was used to indicate the subcellular origin of the released ACh (Fig. 1). Following potassium-induced depolarization of these synaptosomes, there was no significant difference in the amount of ACh released, but there was a highly significant inhibition of the release of newly synthesized ACh from synaptosomes treated with AH5183 during the radiolabeling period. This inhibition of release cannot simply be attributed to the decrease in synaptosomal content, as the inhibition of release is of a much greater magnitude (100%) than the decrease in content (20%). In contrast, synaptosomes that received AH5183 after the radiolabeling period showed no such inhibition of radiolabeled ACh release compared to controls, confirming that AH5183 was not inhibiting a transporter-mediated release of radiolabeled ACh directly from the cytosol. These data indicate that the release of ACh following potassium-induced depolarization is inhibited if the entry of ACh into the storage vesicle is blocked.

Radiolabeling Studies in Electric Organ Tissue Slices. In contrast to synaptosomes, which generally release <5% of their ACh content, electric organ slices are capable of releasing up to 50% of their ACh content upon potassium depolarization. This preparation was also found to accumulate and acetylate radiolabeled choline more rapidly than synaptosomes and thus represents a more viable system for the study of ACh release. The data presented in Fig. 2 illustrate a radiolabeling experiment in electric organ slices similar to that described above for synaptosomes. When depolarized with an elevated potassium concentration, the AH5183-treated slices showed only a slight reduction in the amount of ACh released but a very significant inhibition in the release of radiolabeled ACh. Thus, in this preparation, where a much higher degree of evoked ACh release can be obtained, the effect of AH5183 on the release of ACh compared to newly synthesized ACh is qualitatively identical to that found in synaptosomes and corroborates a vesicular source of released ACh.

DISCUSSION

Although the electromotor system has been widely employed in the study of cholinergic neurotransmission, the mechanism by which ACh is released from the presynaptic nerve terminal into the synaptic cleft is still the subject of active debate. The conflicting opinions have been presented in a series of

Table 2. Effect of 10 μM AH5183 on the subcellular distribution of acetylcholine and newly synthesized acetylcholine in synaptosomes

Subcellular pool	Treatment	ACh, nmol	Labeled ACh, dpm	Specific activity, dpm/pmol
Total	Control	6.70 ± 0.22	868,432 ± 26,347	129.82 ± 5.09
	AH5183	5.92 ± 0.20*	739,202 ± 25,523*	125.03 ± 3.91
Vesicular	Control	4.88 ± 0.13	116,631 ± 10,845	23.88 ± 1.89
	AH5183	3.94 ± 0.28*	44,405 ± 5,846*	11.44 ± 2.14*
Cystosolic	Control	1.82 ± 0.22	751,801 ± 26,347	420.12 ± 51.84
	AH5183	1.98 ± 0.20	694,797 ± 25,523	355.05 ± 33.59

Radiolabeling experiments with [*methyl*-^{3}H]choline were performed as detailed in experimental procedures. Vesicular ACh was determined as that which was resistant to a freeze/thaw cycle consisting of 15 min at −80°C followed by incubation at 20°C until thawed. Values are expressed per 50 μl of synaptosome suspension and are the means ± SD (n =4).
*$P < 0.005$ compared to control (Student's t test).

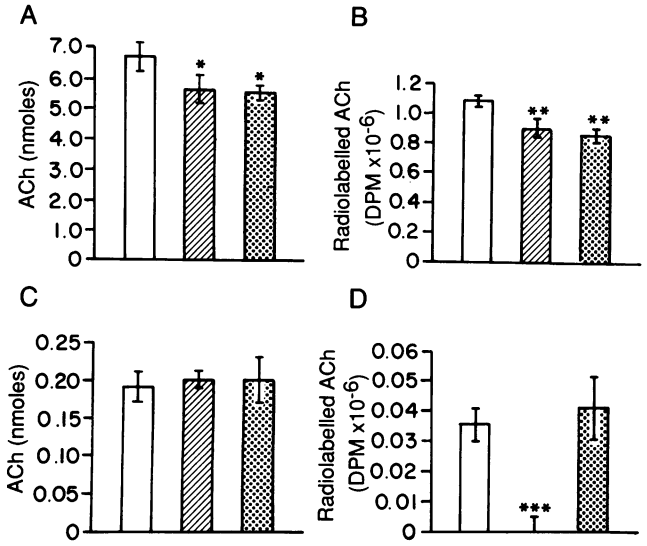

FIG. 1. Effect of AH5183 on synaptosomal content and potassium-stimulated release of ACh and newly synthesized (radiolabeled) ACh. Synaptosomes were labeled for 5 hr at room temperature with [*methyl*-^{3}H]choline and processed for the determination of ACh and radiolabeled ACh content and the quantity of ACh and radiolabeled ACh released following depolarization with 75 mM potassium. Synaptosomal content of ACh (*A*) and radiolabeled ACh (*B*). Amount of released ACh (*C*) and released radiolabeled ACh (*D*). The three groups shown are controls (open bar), 10 μM AH5183 present during the period of radiolabeling and potassium-stimulated release (hatched bar), and 10 μM AH5183 present for 15 min following the labeling period and during the potassium-stimulated release (stippled bar). Values given are per 50-μl synaptosome suspension and are the means ± SD (n = 4). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.01$ compared to corresponding control (one-way analysis of variance).

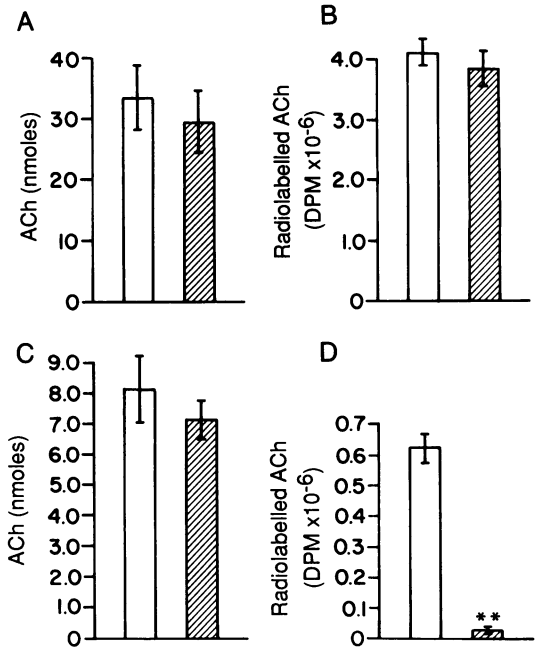

FIG. 2. Effect of AH5183 on the content and potassium-stimulated release of ACh and newly synthesized (radiolabeled) ACh in electric organ slices. A suspension of electric organ slices was radiolabeled for 2 hr at room temperature with [*methyl*-^{3}H]choline and processed for the determination of ACh and radiolabeled ACh content and the quantity of ACh and radiolabeled ACh released following depolarization with 50 mM potassium. Tissue content of ACh (*A*) and radiolabeled ACh (*B*). Amount of released ACh (*C*) and released radiolabeled ACh (*D*). The two groups shown are controls (open bar) and 10 μM AH5183 present during the labeling period (hatched bar). Values given are per 400 μl of electric organ slice suspension and are the means ± SD (n = 4). **, $P < 0.001$ compared to control (Student's t test).

review articles (27–31). In other systems, most notably the adrenal medulla, it was possible to confirm the exocytotic theory of release since the chromaffin granules costore several high molecular weight proteins, which are coreleased with catecholamines upon stimulation (32). In contrast, ACh storage vesicles are not richly endowed with soluble matrix proteins. An alternative soluble biochemical marker for the cholinergic vesicle is ATP, which is colocalized with ACh throughout the vesicle population (12–14). Previous reports regarding the corelease of ACh and ATP in the electromotor system have been in conflict with an exocytotic mechanism (6, 7). Initial observations in the present study also indicated that, although ACh could be found in the extracellular medium following potassium-induced depolarization of synaptosomes, no corelease of ATP could be demonstrated. However, an assessment of ATP recovery indicated the association of an ATPase activity with this synaptosomal preparation, an observation that has also been reported by others (24–26). Characterization of this ATPase revealed a low K_m (0.88 μM), suggesting that this enzyme would be active in hydrolyzing ATP at the concentration it would theoretically reach in the release medium, based on a 6:1 ACh/ATP stoichiometry. While this ATPase activity may be localized to the synaptosomes or to a contaminating fraction copurifying with the synaptosomes, it is unlikely to be unique to our preparation. The specific activities of ACh and ATP in this synaptosome fraction compare very favorably with values reported by others (17, 22, 23), indicating a comparable degree of purity. An inhibitor of this ATPase, α,β-methylene ATP, allowed the quantitative recovery of released ATP without affecting ACh release or interfering in the ATP assay.

To interpret studies on the stoichiometry of released ACh and ATP, with reference to a vesicular or nonvesicular origin for the released material, it is necessary to compare the ratios of released ACh/ATP with values reported for cholinergic vesicles isolated from electric organ (13, 14, 33–38). These reported ratios range between 2.3:1 and 10.8:1 and have a mean value of 5.6:1 (ACh/ATP).

From the data presented in Table 1, it is clear that all the secretagogues tested (whether depolarizing agents or calcium ionophore), except A23187, induced the corelease of ACh and ATP in a ratio generally accepted for the vesicle content. The exception, the calcium ionophore A23187, produced ratios of released ACh/ATP consistently higher than the other secretogogues. At present, the reasons for this difference in response to two calcium ionophores are obscure; it is possible that a portion of the A23187-induced release of ACh may be due to a secondary pharmacological effect of this compound unrelated to the increase in cytosolic calcium concentration. Interestingly, many of the studies in support of the proposal that ACh is released from the cytosol have employed A23187 as the secretagogue (11, 15, 18).

The ratios of released ACh/ATP reported in the present study and the ratios from isolated vesicles reviewed above are in contrast to previous findings from *Torpedo* synaptosomes, which have indicated either no detectable release of ATP (6) or release of ACh and ATP in a ratio of 45:1 with potassium depolarization or in a ratio of 10:1 with *Glycera convoluta* venom (17). These higher ratios have been frequently referenced as evidence against an exocytotic mechanism of ACh and ATP corelease (27, 29, 30). Other reports indicate that ACh release from synaptosomes treated with either botulinum or tetanus toxin is inhibited, whereas ATP release is unaffected (39). A study using synaptosomes from the electric organ of *Ommata discopyge* established a ratio of ACh/ATP release of 2.3:1 and was taken as evidence for

exocytosis (40). Although the constant 5–6:1 molar ratio of released ACh/ATP is certainly in agreement with an exocytotic mechanism of ACh release, this observation alone did not exclude the possibility that both ACh and ATP are coreleased in this ratio from the cytosol by an alternative process. As a parallel to this study of ACh:ATP stoichiometry, AH5183 was used to obtain clear biochemical distinction between the cytosolic and vesicular pools of radiolabeled ACh (Table 2). In agreement with previous reports (41–44), AH5183 did not markedly affect the tissue ACh content, rate of ACh synthesis, or amount of ACh released by potassium depolarization, but it virtually abolished the release of newly synthesized ACh. Since AH5183 inhibits the entry of the radiolabeled ACh into the vesicle without markedly affecting the cytosolic level of radiolabeled ACh, this result further supports the conclusion that the release of ACh from this system occurs from the vesicular pool and not from the cytosolic ACh pool. The possibility that a fraction of the released ACh may originate from the cytosol by an alternative mechanism cannot be excluded. However, from the studies with AH5183 in electric organ tissue slices and synaptosomes, assuming that any residual radiolabeled ACh release is appearing from the cytosol, it can be calculated that a maximum of 5% of the released ACh could originate from this pool. Therefore, in these preparations, at least 95% of the ACh released by potassium depolarization originates directly from the vesicle.

The data from these two series of experiments represent an appreciable body of evidence in support of the vesicular mechanism of ACh release in the electromotor system of *N. brasiliensis*. Any mechanism by which ACh is proposed to be released from the cytosol in this system must not only incorporate a capability to corelease ATP with the stoichiometry established in this study but must also address the effect of AH5183 whereby inhibition of vesicular accumulation of newly synthesized ACh leads to selective inhibition of its release.

Having identified the vesicular content as the chemical mediator of synaptic transmission in this system, the physiological role of the coreleased vesicle contents, their regulation within the presynaptic nerve terminal, and potential interactions following release can be more fully investigated to further characterize the mechanism of cholinergic neurotransmission in this highly specialized electromotor system.

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1. Feldberg, W. & Fessard, A. (1942) *J. Physiol. (London)* **101,** 200–216.
2. Whittaker, V. P. (1987) *Ann. N.Y. Acad. Sci.* **493,** 77–91.
3. Zimmerman, H. & Denston, C. R. (1977) *Neuroscience* **2,** 695–714.
4. Luqmani, Y. A., Sudlow, G. & Whittaker, V. P. (1980) *Neuroscience* **5,** 153–160.
5. Israel, M. & Lesbats, B. (1981) *J. Neurochem.* **37,** 1475–1483.
6. Michaelson, D. M. (1978) *FEBS Lett.* **89,** 51–53.
7. Morel, N. & Meunier, F.-M. (1981) *J. Neurochem.* **36,** 1766–1773.
8. Israel, M., Lesbats, B. & Manaranche, R. (1981) *Nature (London)* **294,** 474–475.
9. Israel, M., Lesbats, B., Morel, N., Manaranche, R., Gulik-Krzywicki, T. & Dedieu, J. C. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 277–281.
10. Birman, S., Israel, M., Lesbats, B. & Morel, N. (1986) *J. Neurochem.* **47,** 433–444.
11. Israel, M., Morel, N., Lesbats, B., Birman, S. & Manaranche, R. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 9226–9230.
12. Dowdall, M. J., Boyne, A. F. & Whittaker, V. P. (1974) *Biochem. J.* **140,** 1–12.
13. Carlson, S. S., Wagner, J. A. & Kelly, R. B. (1978) *Biochemistry* **17,** 1188–1199.
14. Tashiro, T. & Stadler, H. (1978) *Eur. J. Biochem.* **90,** 470–487.
15. Wagner, J. A., Carlson, S. S. & Kelly, R. B. (1978) *Biochemistry* **17,** 1199–1206.
16. Anderson, D. C., King, S. C. & Parsons, S. M. (1983) *Mol. Pharmacol.* **24,** 48–54.
17. Israel, M., Manaranche, R., Mastour-Frachon, P. & Morel, N. (1976) *Biochem. J.* **160,** 113–115.
18. Morel, N., Israel, M., Manaranche, R. & Mastour-Frachon, P. (1977) *J. Cell Biol.* **75,** 43–55.
19. Lundin, A., Rickardsson, A. & Thore, A. (1976) *Anal. Biochem.* **75,** 611–620.
20. Fonnum, F. (1969) *Biochem. J.* **113,** 291–298.
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
22. Richardson, P. J. & Whittaker, V. P. (1981) *J. Neurochem.* **36,** 1536–1542.
23. Israel, M., Lazereg, S., Lesbats, B., Manaranche, R. & Morel, N. (1985) *J. Neurochem.* **44,** 1107–1110.
24. Keller, F. & Zimmermann, H. (1983) *Life Sci.* **33,** 2635–2641.
25. Grondal, E. J. M. & Zimmermann, H. (1986) *J. Neurochem.* **47,** 871–881.
26. Nagy, A. K., Shuster, T. A. & Delgado-Escueta, A. V. (1986) *J. Neurochem.* **47,** 976–986.
27. Israel, M., Dunant, Y. & Manaranche, R. (1979) *Prog. Neurobiol.* **13,** 237–275.
28. Ceccarelli, B. & Hurlbut, W. P. (1980) *Physiol. Rev.* **60,** 396–441.
29. Tauc, L. (1982) *Physiol. Rev.* **62,** 857–893.
30. Dunant, Y. (1986) *Prog. Neurobiol.* **26,** 55–92.
31. Van der Kloot, W. (1988) *Neuroscience* **24,** 1–7.
32. Viveros, O. H., Arqueros, L. & Kirshner, N. (1969) *Science* **165,** 911–913.
33. Boyne, A. F. (1976) *Brain Res.* **114,** 481–491.
34. Israel, M., Manaranche, R., Marsal, J., Meunier, F. M., Morel, N., Frachon, P. & Lesbats, B. (1980) *J. Membr. Biol.* **54,** 115–126.
35. Ohsawa, K., Dowe, G. H. C., Morris, S. J. & Whittaker, V. P. (1979) *Brain Res.* **161,** 447–457.
36. Zimmermann, H. & Denston, C. R. (1976) *Brain Res.* **111,** 365–376.
37. Zimmermann, H. & Denston, C. R. (1977) *Neuroscience* **2,** 715–730.
38. Michaelson, D. M. & Ophir, I. (1980) *Monogr. Neural Sci.* **7,** 19–29.
39. Rabasseda, X., Solsona, C., Marsal, J., Gustau, E. & Bizzini, B. (1987) *FEBS Lett.* **213,** 337–340.
40. Schweitzer, E. (1987) *J. Neurosci.* **7,** 2948–2956.
41. Carroll, P. T. (1985) *Brain Res.* **358,** 200–209.
42. Jope, R. S. & Johnson, G. V. W. (1985) *Mol. Pharmacol.* **29,** 45–51.
43. Michaelson, D. M. & Burstein, M. (1985) *FEBS Lett.* **188,** 389–393.
44. Collier, B., Welner, S. A., Ricny, J. & Araujo, D. M. (1986) *J. Neurochem.* **46,** 822–830.