6. R. N. Puri and A. Meister, *Proc. natn. Acad. Sci. U.S.A.* **80**, 5258 (1983).
7. M. E. Anderson, F. Powrie, R. N. Puri and A. Meister, *Archs Biochem. Biophys.* **239**, 538 (1985).
8. J. R. Dawson, K. Vahakangas, B. Jernstrom and P. Moldéus, *Eur. J. Biochem.* **138**, 439 (1984).
9. M. Berggren, J. Dawson and P. Moldéus, *Fedn Eur. Biochem. Soc. Lett.* **176**, 189 (1984).
10. D. Haberle, A. Wahllander and H. Sies, *Fedn. Eur. Biochem. Soc. Lett.* **108**, 335 (1979).
11. M. E. Anderson and A. Meister, *J. biol. Chem.* **255**, 9530 (1980).
12. Y. Ohmiya and H. M. Mehendale, *Pharmacology* **28**, 289 (1984).
13. R. M. Philpot and E. R. Wolf, in *Reviews in Biochemical Toxicology* (Eds. E. Hodgson, J. R. Bend and K. M. Philpot), Vol. 6, p. 51, Elsevier, New York (1981).
14. H. M. Mehendale, L. S. Angevine and Y. Ohmiya, *Toxicology* **21**, 1 (1981).
15. H. M. Mehendale, *Envir. Toxic. Chem.* **1**, 231 (1982).
16. D. J. Reed, J. R. Babson, P. W. Beatty, A. E. Brodic, W. W. Ellis and D. W. Potter, *Analyt. Biochem.* **106**, 55 (1980).
17. S. M. Deneke, B. A. Lynch and B. L. Fanburg, *J. appl. Physiol.* **58**, 571 (1985).
18. J. R. Dunbar, A. J. DeLucia and L. R. Bryant, *Res. Commun. Chem. Path. Pharmac.* **34**, 485 (1981).
19. J. R. Dunbar, A. J. DeLucia and L. R. Bryant, *Biochem. Pharmac.* **33**, 1343 (1984).

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Effects of serum-fractions from patients with Eaton–Lambert syndrome on rat cortical synaptosomal [^{3}H]acetylcholine release

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Eaton–Lambert syndrome is a rare disorder characterized by deficits in neuromuscular transmission that result from a presynaptic inhibition of acetylcholine (ACh) release [1, 2]. The electrophysiological characteristics of the disease include normal miniature end-plate potential (MEPP) amplitudes, reduced number of quanta at low levels of nerve stimulation, and marked increases in the amplitude of the compound muscle action potential evoked by repetitive, supramaximal nerve stimulation [1]. Biochemically, it is known that the number of nicotinic ACh receptors [3], the ACh levels [4], and choline acetyltransferase [4] are unaltered in biopsied muscles from Eaton–Lambert patients. The identity and mechanism of action of the circulating inhibitor are unknown. Some neoplastic forms of the syndrome may involve the synthesis and release of neuroactive tumor-peptides, since tumor-extracts from patients with the disease can inhibit neuromuscular transmission *in vitro* [5, 6]. Alternatively, some cases of Eaton–Lambert syndrome may involve an autoimmune disorder since IgG isolated from Eaton–Lambert patients and injected into mice for several weeks can attenuate neuromuscular transmission [7] and reduce the spontaneous and electrically stimulated release of ACh from skeletal tissue [8].

In this study we used rat cortical synaptosomes to ascertain whether a neurochemically heterogeneous, non-neuromuscular tissue was also sensitive to the cholinolytic actions of the circulating inhibitor in Eaton–Lambert syndrome. We also compared the effects of the circulating inhibitor on high affinity uptake and release for the first time. Our results indicate that this preparation will be useful for characterizing the neurochemical selectivity of the circulating release-inhibitory factor.

Methods

[*Methyl*-^{3}H]Choline (80 Ci/mmole) was obtained from the New England Nuclear Corp. (Boston, MA). Choline kinase was purchased from the Sigma Chemical Co. (St. Louis, MO). All solutions were made in Krebs–Ringer bicarbonate buffer (KR) as described previously [9].

Synaptosomal preparation. Synaptosomes were prepared from the cerebral cortices of adult male Sprague–Dawley rats (175–225 g) as described previously [10]. The synaptosomes were washed twice and resuspended in 2.5 ml of oxygenated KR buffer. To measure high affinity [^{3}H]choline uptake and [^{3}H]ACh synthesis, synaptosomes (0.5 to 1 mg protein/ml KR) were incubated for 3 min at 37° with 1 μM [^{3}H]choline plus or minus 5 μM hemicholinium-3, and then washed twice by centrifugation with cold KR. Some samples were treated with serum-fractions by substituting KR with equal volumes of serum-fractions. The synaptosomal pellet was then lysed in 0.5 ml of 5 mM sodium phosphate buffer (pH 7.4) containing 50 μM eserine, and the total hemicholinium-3 sensitive [^{3}H]choline uptake and [^{3}H]ACh synthesis were assayed as described previously [11].

Synaptosomes were incubated with 1 μM [^{3}H]choline for 10 min at 37° to load them with [^{3}H]ACh. They were then washed and resuspended in 20 ml of KR containing 50 μM eserine. Aliquots (0.3 ml, approximately 0.4 mg protein) of the synaptosomal suspension were added to 0.7 ml of the same buffer or equal volume substitution of serum-fraction. They were incubated for 3 min in the presence or absence of specified serum-fractions and depolarizing K^+ concentrations. Release-incubations were terminated by cooling the tissues in an ice bath for 5 min, followed by centrifugation at 10,000 *g* for 10 min. The inhibition of spontaneous and depolarization-induced release was normalized for daily variations in release by expressing values as a percentage of controls (untreated, 5.5 mM KCl). The average of duplicate values for each preparation of synaptosomes was obtained, multiplied by 100, and then averaged with other normalized values from other preparations. Labeled ACh in the synaptosomal supernatant fractions was separated from choline by liquid cation exchange as described previously [11]. Comparisons among several means were determined with one-way analysis of variance (F-test).

Protein determinations. Synaptosomal protein levels were determined using the Bio-rad procedure which involves Coomassie blue staining of the proteins.

Patient summaries. L. B. was a 61-year-old white woman who presented to the Medicine service with a 3-month history of weakness. On neurological examination, she was found to have normal cranial nerves. She had mild proximal muscle weakness in both upper and lower extremities without change with repetitive movements. Distal strength was normal. Sensory examination was normal, and deep tendon reflexes were equal bilaterally. Chest X-ray revealed a solitary pulmonary nodule in the right lung which on biopsy

proved to be a small cell carcinoma. An electromyogram revealed post-tetanic potentiation which was felt by the Neurology service to be consistent with an Eaton–Lambert syndrome and that this was the most likely basis for her muscular weakness. The only other pertinent finding was atrial fibrillation. She was begun on chemotherapy consisting of Cytoxan, methotrexate and CCNU and underwent a rapid response with complete resolution of the pulmonary nodule. However, the neurological syndrome did not improve, and she was subsequently placed on guanidine 125 mg q.i.d. She had a brief subjective response over the first 2 weeks but, without objective evidence of improvement, the medication was stopped 1 month later.

P. G. was a 52-year-old white man who presented to the Gainesville V.A. Hospital in 8/83 with a 6-month history of cough, weight loss, dysphagia and diffuse weakness. He was found to have a left peritracheal mass, left upper lobe collapse and a large pleural effusion, all of which proved to be secondary to his small cell undifferentiated carcinoma. He was seen by a neurologist because of his weakness. The neurologist noted diffuse muscle weakness, proximal greater than distal, as well as impotence and suggested a diagnosis of Eaton–Lambert syndrome which was confirmed by a nerve conduction study and electromyogram. He received six cycles of cyclophosphamide, Adriamycin, and vincristine which were followed by hemibody radiation. He had a partial tumor response as well as a modest improvement in his muscle weakness. He received no further chemotherapy because of persisting granulocytopenia and died in September 1984.

Serum-fractions. Sera were collected from L. B. and P. G. as well as from six normal controls. Neither of the patients was being treated with guanidine at the time of blood collection.

For other experiments, serum aliquots (5 ml) were filtered through a Diaflo membrane (Amicon Co., Ireland) with a 10,000 molecular weight cutoff at room temperature; the eluates and high molecular weight residue (approximately 0.5 ml) were lyophilized and resuspended in 2.5 ml of water and KR respectively. Aliquots of these fractions were then added directly to incubation tubes so that each percent serum value actually was twice as concentrated as the original serum fraction.

Results and discussion

Normal sera from six different volunteers had little effect on the deplorization-induced release of the transmitter (Table 1). In contrast, sera from L. B. and P. G. inhibited the spontaneous and depolarization-induced ACh release at similar concentrations when compared to control tissues (Table 1). No inhibition of [^{3}H]ACh synthesis was observed at any serum concentration (2, 5 or 10%) from either patient that reduced the release of the [^{3}H]ACh (Table 2).

Filtration of serum aliquots through a 10,000 molecular weight exclusion limit filter resulted in two fractions which, when tested for their effect on synaptosomal ACh release, gave different results (Fig. 1). The higher molecular weight fraction from the normal subjects had little effect on transmitter release at the concentration used, while the higher molecular weight fractions from L. B. and P. G. induced a significant reduction ($P < 0.05$) in both the spontaneous and depolarization-induced release of [^{3}H]ACh. The low molecular weight fractions from the normal and patient sera reduced spontaneous and K^+-induced transmitter release to a similar extent (Fig. 1).

Our synaptosomal results compare well with those using neuromuscular junctions to characterize the inhibitory actions of the circulating Eaton–Lambert inhibitor(s) [5, 6]. They suggest that a circulating inhibitory factor, molecular weight over 10,000, which acts at neuromuscular cholinergic terminals in neoplastic Eaton–Lambert syndrome, is active centrally when provided access *in vitro*. It should

Table 1. Effects of serum on the release of [^{3}H]ACh from synaptosomes

Serum treatment (% incubation volume)	[^{3}H]ACh release (% control) Spontaneous	K^+-induced
None	100 ± 4*	76 ± 8
Normal		
2%	92 ± 5	90 ± 11
5%	104 ± 7	66 ± 13
10%	118 ± 11	63 ± 8
Patient 1 (L. B.)		
2%	84 ± 9	81 ± 10
5%	83 ± 7*	55 ± 6
10%	71 ± 8*	35 ± 7*
Patient 2 (P. G.)		
2%	78 ± 6	74 ± 8
5%	78 ± 4*	64 ± 9
10%	78 ± 9*	38 ± 5*

Rat cortical synaptosomes were pre-loaded with [^{3}H]ACh and then incubated for 3 min at 37° with the specified volume of serum or equal volume of KR buffer, ±35 mM KCl (or equivalent NaCl addition). The labeled transmitter released into the medium was assayed and expressed here as percent of control, untreated value (control = 47,600 cpm/mg synaptosomal protein). Each serum fraction was tested at least six times (3 separate days in duplicate); the values for six volunteers were grouped (N = 36 total samples.)

* $P < 0.05$ compared to normal sera.

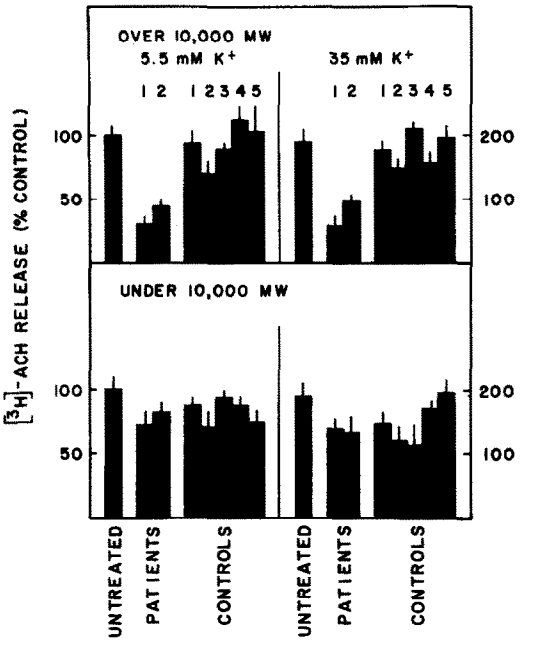

Fig. 1. Effects of filtered serum-fractions on the release of [^{3}H]ACh. Rat cortical synaptosomes that were preloaded with [^{3}H]ACh were incubated at 37° for 3 min in the presence or absence of 35 mM K^+ and serum-fractions from each of five control volunteers or patients 1 (L. B.) or 2 (P. G.). Ten percent of the final volume of release-incubation was serum in all cases. The release of labeled transmitter was measured and expressed here as means ±S.E.M. of triplicate observations (untreated control value = 47,890 cpm/mg protein in top and bottom panels).

Table 2. Effects of serum on the high affinity uptake and acetylation of [^{3}H]choline

Serum treatment (% incubation volume)	High affinity uptake (% control)	[^{3}H]ACh synthesis (% control)
None	100 ± 8	100 ± 9
Normal		
2%	105 ± 11	99 ± 3
5%	98 ± 2	98 ± 9
10%	92 ± 6	90 ± 4
Patient 1 (L. B.)		
2%	96 ± 12	109 ± 10
5%	94 ± 2	102 ± 7
10%	93 ± 5	100 ± 8
Patient 2 (P. G.)		
2%	103 ± 3	98 ± 4
5%	98 ± 5	118 ± 14
10%	96 ± 7	104 ± 6

Rat cortical synaptosomes were incubated for 5 min in the presence of 1 μM [^{3}H]choline with or without 5 μM hemicholinium-3 in KR buffer at 37°. They were washed with KR containing 50 μM eserine by centrifugation twice and assayed for total radioactivity and [^{3}H]ACh as described in the text. The difference in total choline uptake between hemicholinium-3-treated and untreated tissues was determined for each synaptosomal preparation and expressed as percent control for high affinity choline uptake. No [^{3}H]ACh synthesis was observed in hemicholinium-3-treated tissues. Untreated control values for high affinity choline uptake and [^{3}H]ACh synthesis were 1,087,000 cpm/mg protein and 530,000 cpm/mg protein respectively. Each serum-fraction was tested with at least three separate synaptosomal preparations; the values of six volunteers were grouped for normal values.

be noted that synaptosomes may be partially depolarized under basal conditions, so that the reduction in spontaneous (5.5 mM K^+) [^{3}H]ACh release also reflects a reduction in the depolarization-induced release process.

It is possible that the high affinity choline transporter that provides substrate for ACh synthesis [11] may be inhibited in Eaton–Lambert syndrome, reducing the levels of the newly synthesized transmitter pool. Newly synthesized pools of ACh are preferentially released by nerve stimulation, and these pools may be small enough so that changes in them are not observed when total neuromuscular ACh levels are assayed. Our results indicate that this is not the case, and that some process involved in ACh release apparently is selectively altered.

Taken together, our results are significant because: (1) they demonstrate that acute exposure of cholinergic terminals to a circulating factor in Eaton–Lambert's disease is sufficient to inhibit ACh release, as opposed to chronic exposures used in some models for the disease; (2) they indicate that ACh-release is affected without altering the rate-limiting step in ACh synthesis, high affinity choline transport; and (3) they suggest that the brain synaptosome preparation will be useful for determining the neurochemical specificity of this release-inhibition, as well as the potential roles of a variety of biochemical processes.

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REFERENCES

1. K. Elmquist and E. H. Lambert, *Med. Clins N. Am.* **43**, 689 (1968).
2. R. Sandyk, *S. Afr. med. J.* **63**, 323 (1983).
3. J. M. Lindstrom and E. H. Lambert, *Neurology* **28**, 130 (1978).
4. P. C. Molenaar, J. Newsom-Davis, R. L. Polak and A. Vincent, *Neurology* **32**, 1061 (1982).
5. K. Ishikawa, J. K. Engelhardt, T. Fujisawa, T. Okamoto and H. Katsuki, *Neurology* **27**, 140 (1977).
6. J. H. Kennedy, N. Coyne and P. Khairallah, *J. thorac. cardiovasc. Surg.* **57**, 276 (1969).
7. B. Lang, J. Newsom-Davis, D. Wray and A. Vincent, *Lancet* **2**, 224 (1981).
8. B. Lang, P. C. Molenaar, J. Newsom-Davis and A. Vincent, *J. Neurochem.* **42**, 658 (1984).
9. E. M. Meyer and J. R. Cooper, *J. Neurochem.* **36**, 467 (1981).
10. G. P. Sgaragli, I. Sen, A. Baba, R. A. Schulz and J. R. Cooper, *Proc natn. Acad. Sci. U.S.A.* **73**, 2664 (1977).
11. E. M. Meyer, D. A. Engel and J. R. Cooper, *Neurochem. Res.* **7**, 749 (1982).