In favour of the vesicular hypothesis: neurochemical evidence that vesamicol (AH5183) inhibits stimulation-evoked release of acetylcholine from neuromuscular junction

¹E.S. Vizi

Dept. of Pharmacology, Institute of Experimental Medicine, Hungarian Academy of Sciences, 1450 Budapest, POB 67, Hungary and Department of Anesthesiology, Albert Einstein College of Medicine, Montefiore Medical Center, Bronx, NY, U.S.A.

1 The effects of optical isomers of vesamicol (2-(4-phenylpiperidino) cyclohexanol), an inhibitor of acetylcholine (ACh) storage, on stimulation-evoked release of [³H]-acetylcholine ([³H]-ACh) from the neuromuscular junction have been studied in the region of the mouse hemidiaphragm which contains the motor endplates, and which can easily be loaded with [³H]-choline. This method made it possible to detect exclusively the Ca_v-dependent release of [³H]-ACh in response to stimulation, and therefore to test the vesicular hypothesis.

2 (–)-Vesamicol was approximately 20 times more potent than (+)-vesamicol in reducing stimulation-evoked release of [³H]-ACh.

3 4-Aminopyridine, a potassium channel blocker, enhanced the release of ACh in response to stimulation, but failed to increase release from hemidiaphragm which had been pretreated with (–)-vesamicol.

4 The fact that (–)-vesamicol inhibited the release of [³H]-ACh in response to electrical stimulation only when it was administered prior to the loading of the tissue with [³H]-choline, and had no effect when the stores had already been filled with labelled [³H]-ACh indicates that the stimulation-evoked release of [³H]-ACh is of vesicular origin and (–)-vesamicol has no effect on the release process. This is the first neurochemical evidence for the vesicular origin of stimulation-evoked release of ACh from the neuromuscular junction.

Introduction

Recent evidence indicates that vesicles acquire acetylcholine (ACh) by a transport process (Anderson *et al.*, 1983; cf. Tucek, 1984) and vesamicol (2-(4-phenylpiperidino) cyclohexanol; AH5183) selectively inhibits the transport of ACh into synaptic vesicles (Marshall, 1970; cf. Marshall & Parsons, 1987). Whitton *et al.* (1986) provided evidence on the isolated unparalysed phrenic nerve-hemidiaphragm preparation of the mouse that vesamicol diminished the miniature endplate potential (m.e.p.p.) amplitude and that the reduction was not due to postjunctional block but was rather due

to a reduction in quantal size. Provided that ACh released in quanta at the neuromuscular junction (Del Castillo & Kata, 1954) emanates from terminal synaptic vesicles, vesamicol should inhibit the evoked release of freshly synthesized ACh.

It was of interest to determine whether vesamicol reduced ACh release by blocking the release process or by blocking the refilling of vesicular stores with newly synthesized ACh formed in the cytoplasm. If vesamicol blocks the transport of newly synthesized cytoplasmic ACh into the vesicular fraction, and this fraction is responsible for signal transmission at the neuromuscular junction, then vesamicol might be expected to block the refilling of vesicular stores with newly synthesized ACh. Vesamicol should then block the release of [³H]-ACh and the contraction

¹ Correspondence to: Institute of Experimental Medicine, Hungarian Academy of Sciences, 1450 Budapest, P.O. Box 67, Hungary.

of the muscle in response to electrical stimulation, but not block the uptake of [^3H]-choline and the subsequent transformation of choline into ACh.

To test these possibilities, a recently developed radioisotope method (Foldes *et al.*, 1984; Somogyi *et al.*, 1987) was used to measure the evoked, Ca^{2+} -dependent release of freshly synthesized ACh without the use of cholinesterase inhibition from a neuromuscular junction that had been loaded with [^3H]-choline.

Methods

Preparation of the innervated region of hemidiaphragm

Male Swiss-Webster mice, weighing 25–30 g were exposed to halothane and after the loss of the righting reflex they were decapitated. The middle portions of both hemidiaphragms (about 2–3 mm to the right and to the left of the insertions of the phrenic nerves), together with the attached rib segments, were suspended in organ baths of 1 ml. The rib end of the preparation was attached to the bottom of the bath and its tendon to an FT03 force displacement transducer. The organ bath was filled with Krebs solution (NaCl 113, KCl 4.7, CaCl_2 2.5, MgSO_4 0.9, NaHCO_3 25, KH_2PO_4 1.2 and glucose 11.5 mmol l^{-1}). The bath temperature was maintained at 37°C. The pH of the solution when aerated with 95% O_2 :5% CO_2 was 7.38–7.42.

Incubation

The release of ACh was determined in 1 ml organ baths. The preparations were loaded with [^3H]-choline ($4 \mu\text{Ci ml}^{-1}$) for 40 min. During this time the preparations were stimulated at 1 Hz to facilitate the incorporation of [^3H]-choline into the [^3H]-ACh pool of the motor nerve terminal. At the end of this period, the radioactivity of the muscles, consisting partly of [^3H]-choline and partly of [^3H]-ACh, was $3.61 \pm 0.4 \times 10^5 \text{ Bq g}^{-1}$ ($n = 10$). To remove excess [^3H]-choline the preparations were washed three times and then were transferred to another bath and superfused with modified Krebs solution at the rate of 2 ml min^{-1} for 60 min. Subsequently, hemicholinium-3 ($5 \times 10^{-5} \text{ mol l}^{-1}$) was added to the solution to prevent reuptake of [^3H]-choline and the perfusion rate was reduced to 0.5 ml min^{-1} .

Collection

During superfusion at the rate of 0.5 ml min^{-1} , 3 min (1.5 ml) effluents were collected continuously with a

fraction collector into scintillation vials throughout the experiment. The preparations were stimulated twice with 0.8 s trains of 50 Hz, rectangular wave, supramaximal stimuli of 0.2 ms duration every 10 s for 3 min, starting at the 7th (S_1) and 37th (S_2) min of the collection period.

Calculation

In the mouse phrenic nerve-hemidiaphragm preparation, as in many other preparations (Kilbinger & Wessler, 1980; Vizi *et al.*, 1984), electrical stimulation increases only the release of [^3H]-ACh while the output of [^3H]-choline remains unchanged (Wessler & Steinlein, 1987; Vizi *et al.*, 1987). Accordingly, the evoked release of [^3H]-ACh was calculated from the difference between the expected resting and the actual measured outflow of radioactivity caused by 3 min electrical stimulation. Resting release, assumed to follow first-order kinetics, was determined by 'least square' fitting to an exponential curve of the Bq g^{-1} concentrations of two 1.5 ml effluents collected before and after stimulation. The expected spontaneous outflow was obtained by interpolation between the fitted points.

The radioactivity of the samples was measured in a LKB Scintillation Beta Spectrometer. There was considerable variation from one preparation to the other in the amount of radioactivity (Bq g^{-1}) released during the stimulation periods. Therefore fractional release of radioactivity was calculated as a percentage of the total content of tritium in the tissue at the beginning of each collection period. The total content of tritium in the tissues was obtained by summing the radioactivity found in the tissues at the end of the experiments with the radioactivity released during the course of experiments. The stimulation-evoked release was expressed as a percentage of the tritium in the tissue after subtraction of the presumed basal release. A computer programme was used for calculation. The ratios of the radioactivity released by the two consecutive stimulation periods, S_2/S_1 , were constant.

Stimulation

Trains of 40 supramaximal rectangular wave stimuli were applied at 50 Hz with a Grass S-88 stimulator every 10 s. The stimulus duration was 0.2 ms. A pattern of short trains (0.8 s) of high frequency stimulation (50 Hz) was selected because, in mammals, voluntary muscle movements are elicited with short neuronal impulse bursts of 15–60 Hz (Zierler, 1974).

Statistical analysis

The statistical significance of the results was determined by one-way analysis of variance of the

Table 1 Effect of vesamicol on the release of radioactivity from hemidiaphragm loaded with [^3H]-choline

Present in the bath	Bq g^{-1}	Evoked release (S_1) of ACh FR (%)	S_2/S_1
Control	1784 ± 145 (5)	1.71 ± 0.13 (5)	0.91 ± 0.07 (5)
(-)-Vesamicol, $0.5 \mu\text{M}$ throughout the expt.	$1140 \pm 110^*$ (4)	$1.05 \pm 0.12^*$ (4)	0.84 ± 0.06 (4)
(-)-Vesamicol, $10 \mu\text{M}$ throughout the expts.	$371 \pm 50^*$ (5)	$0.26 \pm 0.03^*$ (5)	0.83 ± 0.10 (5)
(+)-Vesamicol, $10 \mu\text{M}$ throughout the expts.	$1193 \pm 110^*$ (4)	$1.10 \pm 0.11^*$ (4)	0.90 ± 0.07 (4)
(-)-Vesamicol, $10 \mu\text{M}$ during loading	$850 \pm 41^*$ (4)	$0.68 \pm 0.05^*$ (4)	0.84 ± 0.06 (4)

Phrenic nerve was stimulated twice (S_1 and S_2), 30 min elapsed between the two stimulations. Stimulation: 0.8 s train of 50 Hz, supramaximal stimuli of 0.2 ms every 10 s for 3 min.

*Significant difference ($P < 0.01$) from control.

Number of experiments is in parentheses. Note (-)-vesamicol has much greater effect than (+)-vesamicol.

logarithmically transformed data, followed by Dunn's test. $P < 0.05$ was considered significant. Results are presented as mean values $\pm$ s.e.mean.

Drugs

Vesamicol (2-(4-phenylpiperidino)cyclohexanol, AH5183) was a gift from Dr S. Parsons, (Univ. of California, Santa Barbara, U.S.A.) and the optical isomers were purchased from RBI (MA, U.S.A.). Hemicholinium-3 was purchased from Sigma Chemical Co.

Results

The content of radioactivity after 40 min incubation with [^3H]-choline followed by 90 min washout was $360750 \pm 44150 \text{ Bq g}^{-1}$ ($n = 10$). The resting release was $2044 \pm 180 \text{ Bq g}^{-1}$ in 3 min ($n = 10$). When the fractional release was calculated, this value proved to be $1.96 \pm 0.14\%$ of the radioactivity present in the tissue at the beginning of the consecutive stimulations of 3 min duration. Release evoked by the first stimulation was $1784 \pm 145 \text{ Bq g}^{-1}$ ($1.71 \pm 0.12\%$, $n = 5$), and the S_2/S_1 ratio was 0.91 ± 0.07 ($n = 5$).

When (-)-vesamicol was added to the bathing fluid between S_1 and S_2 in the concentration range $1\text{--}50 \mu\text{M}$, it did not affect the S_2/S_1 ratio, i.e. the stimulation evoked release. The resting release, however, was slightly reduced when $50 \mu\text{M}$ (-)-vesamicol was added to the organ bath; R_2/R_1 was reduced from 0.91 ± 0.08 to 0.65 ± 0.04 ($n = 5\text{--}5$, $P < 0.01$). At a concentration of $10 \mu\text{M}$ vesamicol had no effect.

When the tissue was exposed for 20 min to $10 \mu\text{M}$ (-)-vesamicol and subsequently loaded with [^3H]-choline in the presence of (-)-vesamicol, the uptake of radioactivity was $395500 \pm 46840 \text{ Bq g}^{-1}$ ($n = 4$). In further experiments (-)-vesamicol was kept in the perfusion fluid throughout the experiments. While

the resting release was unchanged ($1.80 \pm 0.21\%$ in 3 min), the stimulation evoked release was much smaller ($371 \pm 50 \text{ Bq g}^{-1}$, $0.26 \pm 0.03\%$, $n = 5$) from (-)-vesamicol treated hemidiaphragm, than from control tissue (Table 1). The effect of (-)-vesamicol was concentration-dependent. At $0.5 \mu\text{M}$ it was less effective, the release evoked by stimulation being $1.05 \pm 0.12\%$ of the total radioactivity ($n = 4$), but the reduction was still significant ($P < 0.01$). (+)-Vesamicol kept in the perfusion fluid throughout the experiment was much less effective in reducing the evoked release of ACh (Table 1). Racemic vesamicol ($10 \mu\text{M}$) also reduced the release of ACh ($920 \pm 160 \text{ Bq g}^{-1}$, $n = 3$) in response to stimulation (50 Hz, 18 trains of 40 stimuli). 4-Aminopyridine (4-AP) increased the release of ACh as indicated by the increase of S_2/S_1 from 0.88 ± 0.07 to 1.87 ± 0.11 . However, when (-)-vesamicol was kept in the perfusion fluid throughout the experiments, 4-AP did not enhance the release (Table 2).

Discussion

Shortly after Fatt & Katz (1952) first described m.e.p.s, vesicles were revealed within the nerve terminals by electronmicroscopy (de Robertis & Bennett, 1955) and neurochemical evidence was also obtained (Whittaker *et al.*, 1964) for ACh storage in vesicles. It was therefore natural to ascribe the quantal nature of ACh release to the vesicles. Since then many results have been published that are compatible with the idea that the ACh involved in signal transmission at many neuroeffector junctions is released from vesicles by a process of exocytosis. Nevertheless, there is some evidence that quantal release may originate from cytoplasmic stores of ACh (Tauc *et al.*, 1974; Israel *et al.*, 1979; Dunant, 1986).

Recently a sensitive radiochemical method was developed (Foldes *et al.*, 1984; Somogyi *et al.*, 1987)

Table 2 Effect of 4-aminopyridine on the release of radioactivity from mouse hemidiaphragm loaded with [^3H]-choline

Drugs	Release of ACh S_2/S_1
Control	0.88 ± 0.07 (7)
4-Aminopyridine, 50 μM	$1.87 \pm 0.11^*$ (4)
(-)-Vesamicol treated + 4-Aminopyridine, 50 μM	0.96 ± 0.12 (4)

Phrenic nerve was stimulated twice (S_1 and S_2), 30 min elapsed between the two stimulations. Drugs were added to the perfusion fluid 20 min prior to the second stimulation. (-)-Vesamicol (10 μM) was present in the perfusion fluid throughout the experiments.

*Significant difference from control ($P < 0.01$).

Note that (-)-vesamicol treatment prevents the effect of 4-aminopyridine.

that measures selectively the Ca-dependent, stimulation-evoked quantally released, freshly synthesized ACh from mouse phrenic nerve-hemidiaphragm *in vitro* without cholinesterase inhibition. In response to stimulation, ACh is released, therefore any radioactivity released above resting release represents the release of freshly synthesized ACh (Vizi & Somogyi, 1989). With this method the effect of vesamicol, a compound able to block the uptake of freshly formed ACh by synaptic vesicles (Anderson *et al.*, 1983; cf. Marshall & Parsons, 1987) was studied on stimulation-evoked release of ACh from mouse hemidiaphragm previously loaded with [^3H]-choline.

In the present study, (-)-vesamicol did not reduce the uptake of [^3H]-choline, but drastically reduced the release of radioactivity in response to stimulation only in those cases when the compound was loaded prior to and during [^3H]-choline loading, and was left in the organ bath throughout the experiment. When it was added after loading with [^3H]-choline, between S_1 and S_2 stimulations, the compound had no effect on the stimulation-evoked release of ACh, indicating that it has no effect on the release process. It also failed to affect resting release, which is Ca_o^{2+} -independent and mainly released in non-quantal form (Vizi & Vyskocil, 1979).

The finding that (-)-vesamicol was about 20 times more active than (+)-vesamicol suggests that the effect of the compound is stereoselective. This is in good agreement with the observation of Estrella *et al.* (1988), who studied the optical isomers of vesamicol on neuromuscular transmission in rat diaphragm and found that the (-)-form is more active. The inhibition of potassium channel conductances by 4-AP did not reverse the inhibition of ACh release when (-)-vesamicol was present in the fluid.

The results obtained in the present study indicate that vesamicol reduces the electrically evoked release of [^3H]-ACh from the neuromuscular junction, a result consistent with the earlier observation that it reduces the evoked release of ACh from CNS tissue (Ricny & Collier, 1986; Suszkiw & Toth, 1986), as it has been shown to do at peripheral autonomic synapses (Collier *et al.*, 1986; Cabeza & Collier, 1988). These data suggest that it blocks the uptake of [^3H]-ACh into synaptic vesicles.

The neurochemical evidence in the present study is in favour of the concept that ACh released by nerve stimulation originates from vesicles at least at this neuromuscular junction.

In the experiments of Whitton *et al.* (1986) using mouse hemidiaphragm preparations, vesamicol reduced the quantal size to a smaller extent than it reduced stimulation-evoked ACh release in our experiments. This point needs some explanation. The major difference between the two techniques is that in our experiments only the release of freshly formed ACh is measured, while in the experiments by Whitton *et al.* (1986) the release of endogenous ACh was recorded indirectly by an action on the post-junctional membrane. In the latter case the action of vesamicol, both on twitches (Marshall, 1970) and on electrophysiologically recorded events (Van der Kloot, 1986; Whitton *et al.*, 1986) is very slow, requires higher concentrations and is highly dependent on the amount of ACh depleted and not replaced. The reason for the quantitative difference in the neurochemical and electrophysiological studies may be the large margin of safety of ACh release at the neuromuscular junction (Paton & Waud, 1967): 6–10 times more ACh is released by stimulation than is required for signal transmission.

Thus, vesamicol only inhibited the release of ACh if the drug was administered prior to the loading of the [^3H]-ACh into the preparation. It had little or no effect on the release of ACh which was previously loaded into vesicles. In addition, it had no effect on the release mechanism, as indicated by its ineffectiveness on the S_2/S_1 ratio when it was administered just before the second stimulation period.

While in our experiments vesamicol treatment did not reduce resting release of ACh, Vyskocil (1985) observed a hyperpolarization in the mouse diaphragm following vesamicol administration, an effect attributed to the reduction of non-quantal release of ACh. This type of release is Ca_o^{2+} -independent, its rate depends on the activity of Na/K-activated ATPase (Vizi & Vyskocil, 1979; Vyskocil, 1985) and is of cytoplasmic origin. The finding that the presence of vesamicol was capable of inhibiting the release of Ca_o^{2+} -dependent, stimulation-evoked release of ACh, suggests that this type of release is of vesicular origin.

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