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Vesamicol blocks the recovery, by recycling cholinergic electromotor synaptic vesicles, of the biophysical characteristics of the reserve population

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The effect of vesamicol on the ability of recycling cholinergic synaptic vesicles to recover, during a period of post-stimulation rest, the biophysical properties of the reserve pool was studied in prestimulated perfused blocks of the electric organ of the electric ray, *Torpedo marmorata*, a tissue rich in cholinergic synapses. The effect of the drug was analysed by high-resolution centrifugal density-gradient fractionation in a zonal rotor of the extracted vesicles. The two vesicle fractions were identified by their ATP and acetylcholine content and the recycled vesicles by their acquisition of [^{3}H]acetylcholine derived from [^{3}H]acetate in the perfusate. Vesamicol (10 μM) blocked the uptake of tritiated acetylcholine by recycled vesicles and also prevented them from rejoining the reserve pool. This is consistent with a previously formulated model of the recovery process, whereby the increase in the acetylcholine and ATP content of the recycled vesicles which takes place during a post-stimulus period of rest increases their osmotic load and thus their content of free water. Vesamicol, by blocking acetylcholine uptake, also blocks rehydration of the recycled vesicles and thus the accompanying decrease in their density to the value characteristic of fully charged vesicles.

Introduction

Synaptic vesicles are the characteristic organelles, approx. 50 nm in diameter (but 90 nm in electromotor terminals), of presynaptic axonal varicosities and terminals. Their function is to store synaptic transmitters and, on stimulation, to release them by exocytosis, thereby initiating and sustaining synaptic transmission [1,2]. The vesicles undergoing exocytosis are retrieved, reloaded and again exocytosed through an unknown number of cycles before being finally discarded. Work with the electric organ of the electric ray, *Torpedo marmorata* [3] and with the guinea-pig myenteric plexus [4] has shown that these recycling vesicles are smaller and denser than those of the reserve pool and can be separated from the latter by high-resolution centrifugal density-gradient fractionation in a zonal rotor [3,4] or by particle-exclusion chromatography [5,6]. The proportion of vesicles in the two pools is a function of the intensity and duration of the stimulus and the duration of a post-stimulus period of rest. When the rest period is sufficiently long (up to 18 h in electromotor terminals [7]) the recycled vesicles recover the size and density of those in the reserve pool from which they were originally derived.

Other work [8,9] has shown that synaptic vesicles are osmotically sensitive structures and that the difference in biophysical properties between reserve and recycling vesicles is brought about by an osmotically driven loss of water from reserve vesicles when they enter the recycling pool. Refilling of vesicles with transmitter during recycling is only partial; thus the osmotic load is less and this results in a loss of free water from the vesicle in order to re-establish osmotic equilibrium with the cytoplasm. A reduction in diameter and an increase in density are the consequences.

The compound 2-(4-phenylpiperidino)cyclohexanol, also known as AH 5183 and vesamicol [10], is able, in low concentrations, to block the release of newly synthesized and/or endogenous acetylcholine from brain tissue in vivo, intact tissue preparations and synaptosomes from a variety of sources (Refs. 11–18 and

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references therein), including electric organ [11,14]. Combined with the results of uptake studies with isolated vesicles [19], these observations strongly suggest that vesamicol exerts its effect by blocking the refilling of recycling synaptic vesicles with acetylcholine.

We have now analysed the effect of vesamicol on the dynamics of the vesicle population of electromotor terminals by making use of perfused blocks of electric tissue, in which the proportion of vesicles in the recycling pool had been augmented by prior stimulation. The effect of vesamicol was analysed by extracting the vesicles and separating them into reserve and recycled pools by high-resolution density-gradient centrifugation in a zonal rotor [1,3–5,7,9,20]. The following predictions were tested: vesamicol in low concentrations should block the uptake of newly synthesized acetylcholine into the recycling pool, and, if the osmotic model for the biophysical differences between recycling and reserve vesicles is correct, it should also at such concentrations block the reacquisition by the recycling vesicles of the biophysical properties of the reserve population during the recovery of the tissue from stimulation.

Methods

Fish

Adult female specimens of *Torpedo marmorata* were provided by the Institut Universitaire de Biologie Marine, Arcachon, and were maintained in a sea-water aquarium until required.

Stimulation and perfusion of tissue

The preparation and perfusion of blocks of electric organ with their accompanying nerves were as fully described previously (Appendix A in Ref. 1, and Refs. 7 and 9). In a first set of experiments, designed to check that vesamicol, when perfused through blocks of electric tissue, did indeed block the uptake of newly synthesized acetylcholine into recycled vesicles, stimulation (1800 pulses of 1 ms duration at a frequency of 0.15 Hz) was applied to the electric lobes of anaesthetized fish through a hole in the skull. Blocks of electric tissue [$62 \pm 10(12)$ g] were then removed by dissection, weighed and prepared for closed-circuit perfusion at 1.5 ml/min and 20°C for 2 or 4 h with a modified Ringer's solution containing (in mM): NaCl, 280; KCl, 3.5; $MgCl_2$, 1.2; $CaCl_2$, 3.4; glucose, 10; urea, 300; sucrose, 100; $NaHCO_3$, 5.0; choline, 0.01; NaH_2PO_4, 5.0; adjusted to pH 7.3. To allow identification of the recycled pool of vesicles [^{3}H]acetate (2 μCi/ml; specific radioactivity, 100 μCi/mmol; NEN, Dreieich, Germany) was added to the perfusion medium; in addition vesamicol (10 μM; racemate) (kindly supplied by Dr. S.M. Parsons) was added as required. At the end of perfusion the blocks were placed on open-circuit perfusion for 30 min to wash out radioactivity and any vesamicol present; they were then reweighed, frozen in liquid nitrogen and kept there until fractionated as described below.

In another series of experiments, designed to test the effect of vesamicol on the recovery, by recycled vesicles, of the biophysical properties of the reserve population during a post-stimulus period of rest, the tissue blocks [$30 \pm 3(13)$ g] were prepared without prior stimulation, washed out by a 20-min period of open-circuit perfusion and then, during closed-circuit perfusion with a perfusate containing [^{3}H]acetate, were submitted to combined nerve and field stimulation using the same stimulus parameters as before. Control blocks (no recovery) were frozen immediately after stimulation: other blocks were allowed to recover before being frozen by perfusing them for 1 h on open circuit and then on closed circuit for a further 11 h; vesamicol in a final concentration of 10 μM was added to the perfusate at the beginning of the recovery period as required. It had been previously found that prolonged open-circuit perfusion causes deterioration of the tissue. The flow rate in this series of perfusions was ~ 1.5 ml/min at a temperature of 23–25°C.

The efficiency of perfusion was checked by calculating the amount of swelling of the blocks from their increases in weight. In the first series this was $38 \pm 4(12)$% w/w and in the second, $52 \pm 5(13)$% w/w. The various treatments made no significant difference to the mean amounts of swelling.

Separation of vesicles

A cytoplasmic extract was prepared from crushed and frozen tissue, was sampled and the remainder ($33 \pm 1(12)$ ml corresponding to $46 \pm 7(12)$ g of original tissue (first series) or $25 \pm 1(13)$ ml corresponding to $19 \pm 1(13)$ g of original tissue (second series)) was fractionated by centrifugal density-gradient centrifuging in a small (Beckman Z60, 330 ml capacity) zonal rotor as previously described [1,7,9]. To ensure good separation of reserve (VP_1) and recycled (VP_2) vesicles with the gradient profile previously used, it was found essential to maintain the rotor temperature as close to 0–4°C as possible and certainly not to allow it to exceed 8–10°C.

Assays

Vesicle fractions were located in the density gradients by the rapid and convenient luciferin-luciferase ATP assay (Ref. 1; modified from Ref. 21). Endogenous acetylcholine was measured by bioassay [1,22] using a small slip of the dorsal muscle of the leech suspended in a 0.05-ml organ bath or by a fluorimetric method [23] based on the production of H_2O_2 after stepwise hydrolysis of acetylcholine by immobilized acetylcholinesterase and the oxidation of the choline so

TABLE I

Effect of vesamicol on the incorporation of radioactive acetylcholine into synaptic vesicles and on vesicular acetylcholine and ATP

In column 3 results are calculated per volume of gradient fractions equivalent to 1 g of original tissue. Values are means ± S.E. of 3 blocks; the results with vesamicol-treated blocks are expressed as a percentage of those of contralateral control blocks from the same fish of approximately the same weight. Although the effect of vesamicol appears to be greater at 4 h than at 2 h, due to the rather large variance the means in column 5 were not significantly different from those in column 4 ($P > 0.1$). However, in paired t-tests, the vesamicol values were significantly lower ($P < 0.01$) than those of the controls.

Component	Units	Control, no vesamicol (units/ g of tissue)	10 μM vesamicol (as % of control)	
			2 h	4 h
[^{3}H]Acetylcholine	dpm × 10^{-3}	11 ± 2	15 ± 6	9 ± 1
Acetylcholine	nmol	138 ± 9	72 ± 9	63 ± 19
ATP	nmol	30 ± 8	71 ± 13	48 ± 10

released by immobilized choline oxidase. The sensitivities of the two methods were comparable.

Radioactive acetylcholine was separated from unesterified acetate by liquid ion-exchange [24] and counted in a liquid scintillation counter. About 0.5% of the counts in the vesicle peaks was estimated to be attributable to unesterified [^{3}H]acetate.

Recoveries were 92 ± 2(14)% (radioactivity), 83 ± 4(11)% (endogenous acetylcholine) and 68 ± 5(11)% (ATP). The lower recovery of ATP may have resulted from the presence of cytoplasmic ATP, as this, unlike cytoplasmic acetylcholine which, in the absence of an anti-cholinesterase (as here), is rapidly destroyed, sometimes survives extraction but is unstable under the conditions of fractionation.

The refractive index (RI) of density-gradient fractions was measured in an Abbé refractometer. RIs were converted to densities by the equation [1]

$$\text{Density (in g/ml)} = (\text{RI} \times 3.182) - 3.260$$

Expression of results

To facilitate comparisons between runs, density-gradient parameters were expressed per amount of fraction equivalent to 1 g of original tissue (Table I, Fig. 1).

The degree of recovery, by recycled vesicles, of the density of the reserve pool was measured by the differences in RI (converted to density) between the VP_1 and VP_2 peaks and the volume of gradient separating them.

Unless otherwise stated values (including those already given) are means ± S.E. with the number of experiments in parentheses. Tests of significance included Student's test and, where appropriate, the paired t-test and Walsh's and Lord's tests (giving the probability that the mean of differences in paired results are different from zero).

Results

RIs and densities of the vesicle peaks

The RI of the VP_1 peak (pooled data) was 1.3552 ± 0.0033(17), close to that previously reported (1.3552 ± 0.0002(16) [7]). This corresponds to a mean density of 1.054 g/ml, close to that measured in an iso-osmotic glycine-sucrose gradient (1.055 ± 0.001(13) g/ml [25]). The mean RI of the VP_2 peak, 1.3618 ± 0.0017(10), corresponds to a mean density of 1.073 g/ml, a little higher than values (1.065 to 1.070 g/ml) reported for vesicles isolated in iso-osmotic gradients [9,26]. The increasing hyperosmolarity of the zonal gradient after RI 1.3552 may have enhanced the density of the VP_2 vesicles by contributing to their osmotic dehydration.

Vesamicol blocks the uptake of newly synthesized acetylcholine into recycled vesicles

Fig. 1 shows, superimposed, two representative zonal separations of extracts of matched blocks from tissue

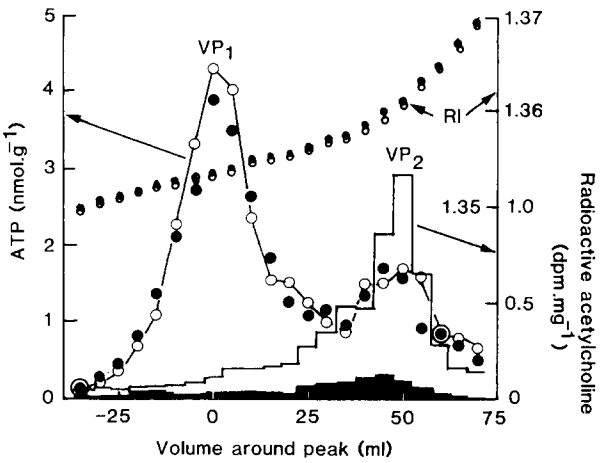

Fig. 1. The graph shows the superimposed distributions, in zonal density gradients, of (circles) vesicular ATP and (blocks) [^{3}H]acetylcholine after centrifugal fractionation of cytoplasmic extracts of perfused blocks of electric organs which had been previously stimulated in vivo. The blocks were allowed to recover before extraction for 2 h in the presence of [^{3}H]acetate and in the absence (empty circles and blocks) or presence (filled circles and blocks) of 10 μM vesamicol. Note that [^{3}H]acetate, in the form of [^{3}H]acetylcholine (right ordinate, lower scale), is selectively incorporated into the denser (VP_2) of the two vesicle fractions, that comprising the recycled population, and that vesamicol blocks this incorporation while having relatively little effect on vesicular ATP (left ordinate). The left ordinate and right ordinate, lower scale, are expressed as the amounts of the components in that volume of each fraction equivalent to 1 g (ATP) or 1 mg (dpm) of original tissue. The small circles and right ordinate, upper scale, give the refractive index (RI) of each fraction, empty and filled symbols referring to experiments without and with vesamicol respectively. The 5-ml fractions are plotted as the volumes of gradient denser (positive values) or lighter (negative values) than the VP_1 vesicle peak.

TABLE II

Poststimulation reacquisition, by VP_2 vesicles, of the density of VP_1 vesicles and the effect of vesamicol thereon

Tests of significance: [a], [a]; [b], [b]; [c], [c]; [d], [d]: significantly different ($P < 0.01$); [e], [e]; [f], [f]; [g], [g]: not significantly different ($P > 0.1$). Measurements of [^{3}H]acetylcholine indicated a blocking effect of vesamicol comparable to that recorded in Table I.

No. of blocks perfused	Recovery time (h)	Vesamicol (μM)	RI		Difference in density (g/ml)	Separation of peaks (ml)
			VP_1	VP_2		
6	0	–	1.3551 ± 0.0004 [d]	1.3630 ± 0.0028 [a,d]	0.026 ± 0.009 [b,f]	48 ± 5 [c,g]
3	12	–	1.3563 ± 0.0013 [e]	1.3573 ± 0.0014 [a,e]	0.003 ± 0.002 [b]	10 ± 6 [c]
4	12	10	1.3562 ± 0.0005	1.3611 ± 0.0010	0.016 ± 0.003 [f]	40 ± 4 [g]

stimulated in vivo, one (open circles and blocks) showing the distribution of (circles) vesicular ATP and (blocks) [^{3}H]acetylcholine after 2 h perfusion in the absence of vesamicol, the other (filled circles and blocks) after 2 h perfusion in its presence.

It will be seen that, as expected, vesicular ATP is bimodally distributed and that [^{3}H]acetylcholine is taken up only into the denser of the two vesicle fractions i.e. into VP_2, the fraction of recycled vesicles. Table I gives the mean values ± S.E. of six such experiments. Vesamicol reduced both the acetylcholine and ATP content of the total ($VP_1 + VP_2$) vesicle population to a small, albeit significant extent and markedly reduced the incorporation of [^{3}H]acetylcholine; this effect appeared to be progressive with time. The differences between the 2- and 4-h results were not, in fact, significant but the means of the pooled results were significantly ($P < 0.01$) lower than those of the control blocks (paired t-test) for all parameters measured.

Vesamicol blocks the recovery, by recycled vesicles, of the biophysical properties of the reserve population

The second series of experiments were designed to test whether vesamicol, in a concentration shown to be effective in blocking the uptake of newly formed acetylcholine into recycled (VP_2) vesicles, would also block the recovery, by such vesicles, of the density of the reserve (VP_1) vesicles. The pooled results are presented in Table II.

The RIs of the VP_1 peaks obtained under the various conditions (column 4) were not significantly different but the fall in the RI of the VP_2 peak resulting from the 12-h period of recovery (column 5, compare lines 1 and 2) and the corresponding falls in density differences (column 6) and volume separation (column 7) between VP_1 and VP_2 were significant ($P < 0.01$). Further, while the mean RI of VP_2 separated immediately after stimulation differed significantly from that of VP_1 (compare line 1, columns 4 and 5), that of VP_2 after 12 h post-stimulation recovery (line 2, columns 4 and 5) did not.

When 10 μM vesamicol was present during the post-stimulation recovery period, little if any change took place in the RI, density and volume separation of the VP_2 vesicle pool relative to blocks allowed no time for recovery. Although the mean density difference and peak separation between VP_1 and VP_2 derived from the vesamicol-treated blocks were somewhat lower than these parameters of the zero-time control, the implied small degree of recovery was not, in fact, significant.

Discussion

The analysis, by extraction and high-resolution centrifugal density-gradient fractionation in a zonal rotor, of the metabolic and biophysical properties of the synaptic vesicle populations in perfused blocks of electric tissue has provided a useful way of studying the intracellular dynamics of the vesicle population in situ under conditions of stimulus and subsequent rest [1,3–5,7,9,20]. We have now extended this to the study of the action of a drug, vesamicol, which powerfully blocks the incorporation of newly synthesized transmitter into recycling vesicles, a mode of action the evidence for which has previously mainly been derived from studies of isolated vesicles [19].

Under our conditions vesamicol reduced vesicular ATP to approximately the same extent as endogenous acetylcholine (Table I). This is in contrast to results with hemicholinium-3, which has been reported selectively to deplete the acetylcholine content of vesicles [20]. Unlike hemicholinium-3, which depletes acetylcholine by blocking the cytoplasmic uptake and reutilization of choline, vesamicol acts directly on the vesicular acetylcholine carrier probably via a vesamicol receptor which is linked allosterically to it [27]. The action of vesamicol on ATP uptake may be indirect in that ATP uptake is likely to be linked to the uptake of a cation such as acetylcholine (for discussion see Ref.1).

Vesamicol, perfused through the blocks at a final concentration of 10 μM, not only blocked the uptake of radioactive acetylcholine into the VP_2 vesicles, it also prevented, during a subsequent 12 h period of recovery, their reacquiring the density of the VP_1 vesicles from which they were originally derived. This is fully consistent with the osmotic model of the vesicle originally formulated [1,9,27] to account for the greater density and smaller size of recycling vesicles relative to

the reserve pool. In this model, when vesicles enter the recycling pool, the rate at which they take up transmitter from the cytoplasm is too slow to allow them to become fully charged in the intervals between retrieval and renewed exocytosis. The reduced loading (about 30% of maximum [28]) induces an osmotically driven loss of water which results in a reduction of size and an increase in density in this osmotically sensitive structure. Only during a subsequent period of rest are the recycled vesicles able to acquire their full load of transmitter (and ATP) and thereby rehydrate and reacquire the lower density and larger size of reserve vesicles. Vesamicol, applied after the formation of a recycling pool by the application of an appropriate stimulus, presumably prevents this rehydration by blocking the post-stimulus uptake of the vesicle's full complement of transmitter.

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