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DIFFERENCES IN THE OSMOTIC FRAGILITY OF RECYCLING AND RESERVE SYNAPTIC VESICLES FROM THE CHOLINERGIC ELECTROMOTOR NERVE TERMINALS OF *TORPEDO* AND THEIR POSSIBLE SIGNIFICANCE FOR VESICLE RECYCLING

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In this study we demonstrate differences in the osmotic fragility of two metabolically and physically heterogeneous synaptic vesicle populations from stimulated electromotor nerve terminals. When synaptic vesicles isolated on sucrose density gradients are submitted to solutions of decreasing osmolarity 50% of VP_2-type vesicles lysed at (mean +S.E. (number of experiments)) 332 ± 14 (4) mosM and 50% of VP_1-type vesicles lysed at 573 ± 8 (3) mosM. These results indicate that recycling vesicles are more resistant to hypo-osmotic lysis and they are consistent with our earlier conclusion that changes in water content on recycling are secondary to changes in the content of the osmotically active small-molecular-mass constituents acetylcholine and ATP.

In previous work from this laboratory it has been established that the acetylcholine-rich synaptic vesicles from the cholinergic nerve terminals of the electric ray, *Torpedo marmorata*, are metabolically and physically heterogeneous and can be separated by density gradient centrifuging [1,2] or chromatography on columns of porous glass beads [3] into two populations: VP_1, consisting of lighter, larger vesicles whose acetylcholine stores do not significantly exchange with acetylcholine newly formed in the cytoplasm, and VP_2, comprising denser, smaller vesicles, which readily take up newly formed acetylcholine. In nerve terminals in resting tissue which has not been intentionally stimulated, the VP_2 population is small or absent, but it progressively increases at the expense of the VP_1 population as stimulation is applied and continued; on recovery the VP_2 population tends to reacquire the characteristics of the VP_1 population. Thus the two populations are interchangeable: prolonged stimulation increases VP_2 at the expense of VP_1; a subsequent period of rest allows the mean density and size of the VP_2 population to revert to that of VP_1.

Measurements of water space using dense, freely permeable solvents [4] have indicated [5] that the smaller size and increased density of recycling vesicles is secondary to a lower water content. This, in turn, could be a consequence of a lower content of the osmotically active small molecular mass vesicle constituents acetylcholine and ATP. The VP_1 vesicles in resting or recovered tissue may acquire a higher content of osmotically active solutes than is attained by the recycling VP_2 vesicles and this may induce an uptake of water, with consequent swelling [5].

A simple means exists whereby differences in the number of osmotically active solute molecules present in the core of vesicles or other storage particles (e.g., chromaffin granules) under differ-

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ent physical conditions can be detected; this is to compare their osmotic fragilities [6,7]. The internal solute content was reduced by partial lysis and resealing and the surviving granules were then shown to have an enhanced resistance to osmotic lysis consistent with a lower internal content of osmotically active solute molecules. We have now applied this approach to the two populations of synaptic vesicles from electric organ.

Previous work [2] has shown that radioactive acetylcholine is a valid marker for VP_2-type vesicles in vesicle preparations isolated from tissue stimulated during perfusion with a radioactive precursor and that endogenous acetylcholine is a valid marker for VP_1-type vesicles in preparations isolated from unstimulated tissue. It has thus been possible to follow the osmotic fragility of both types of vesicle in mixed preparations. A preliminary account of this work has been given [8].

Experimental procedures

Fish

Torpedo marmorata were supplied by the Institut Universitaire de Biologie Marine, Arcachon, France, and kept in tanks of circulating artificial sea-water at 16°C.

Isolation of synaptic vesicles

Synaptic vesicles from stimulated and unstimulated (control) electric organs of *Torpedo marmorata* were prepared as previously described (Ref. 5 and references cited therein) with the modifications mentioned in the following brief description. This method was preferred to the more complete separation obtainable with zonal rotors [2] because it diluted the radioactivity less and so permitted hypo-osmotic conditions to be established by simple dilution.

Entire electric organs, each with its four afferent nerve trunks and accompanying blood vessels, were removed by dissection from fish previously anaesthetized with Tricaine methane sulphonate, and perfused with [^{3}H]acetate (300 mCi/mmol, 5 μCi/ml) of perfusate) in a closed-circuit system. After 3 h of perfusion, one electric organ from each fish was stimulated via the nerves (0.17 Hz, 1800 pulses); 30 min after the end of stimulation, perfusion was discontinued, the electric organs were cut into blocks, frozen in liquid nitrogen and the vesicles extracted and separated on an 810 mosM (i.e. mosmol $\cdot$ l^{-1}) iso-osmotic glycine-sucrose gradient as previously described.

Acetylcholine was extracted from gradient fractions by liquid ion exchange and the two fractions from the middle of the gradient with maximum [^{3}H]acetylcholine content were used for the osmotic fragility experiments. The acetylcholine contents of the two preparations were (means $\pm$ S.E., number of experiments in parentheses): control, 17.2 ± 7 (3); stimulated, 2.84 ± 0.29 (4) nmol $\cdot$ (g of tissue)$^{-1}$. It should be noted that in this type of experiment the peak of [^{3}H]acetylcholine does not coincide with the peak of endogenous acetylcholine in gradients derived from stimulated tissue, whereas it does fairly well coincide in gradients derived from control tissue; the effect of stimulation is thus to increase the mean density of vesicles containing the newly synthesized, i.e. tritiated acetylcholine and this correlates well with the observed change in water content, assuming an unchanged membrane [3,5].

Measurement of osmotic fragility

Samples of the vesicle preparation (0.5 ml) were brought to the required osmotic pressure by the slow dropwise addition of 2.5 ml of glycine solutions of appropriate concentration during 1 min with gentle stirring. After 30 min duplicate 1 ml samples of the diluted vesicle suspensions were extracted by liquid ion exchange [9] for the determination of [^{3}H]acetylcholine; other samples were heated at pH 4.0 and 100°C for 10 min to release endogenous acetylcholine, which was then assayed on thin slips of the dorsal muscle of the leech [10]. The assumption that the results of both estimations refer to residual vesicular acetylcholine (released acetylcholine having been destroyed by acetylcholinesterase present in the vesicle suspensions) was checked by adding [^{14}C]acetylcholine (3μl containing 0.72 μCi of a preparation of specific radioactivity 52 mCi/mmol) to samples of vesicles diluted with either 810 or 150 mosM glycine. In all such controls with added free acetylcholine as an internal standard, the latter was 96 to 100% destroyed. All assays were performed on duplicate samples of vesicles. Osmotic fragility is expressed as the milliosmolality, ob-

tained by interpolation, causing 50% lysis (L_{50}); values are means of three (control) or four (stimulated) experiments and are given ± S.E.

Results

The effect of stimulation was, as expected, substantially to increase the amount and specific activity of the newly synthesized, thus radioactive acetylcholine in the region of the gradient in which VP_2-type vesicles congregate. As shown in Fig. 1, progressively reducing the osmolarity of the vesicle suspension medium from 810 to 150 mosM caused progressive release of both radioactive and endogenous vesicular acetylcholine from the two vesicle preparations. However, there are significant differences in the osmotic fragility depending on whether the vesicles have been isolated from stimulated or control tissue, and, to a lesser extent, on whether lysis was measured by the release of radioactive or endogenous acetylcholine. The L_{50} measured by the release of radioactivity from vesicles isolated from stimulated tissue (332 ± 14) and thus referring to VP_2-type vesicles, is considerably less than that measured by the release of endogenous acetylcholine from vesicles isolated from control tissue (513 ± 8), and thus referring to VP_1-type vesicles. We may thus conclude that VP_2-type vesicles are considerably more resistant to osmotic lysis than VP_1-type vesicles.

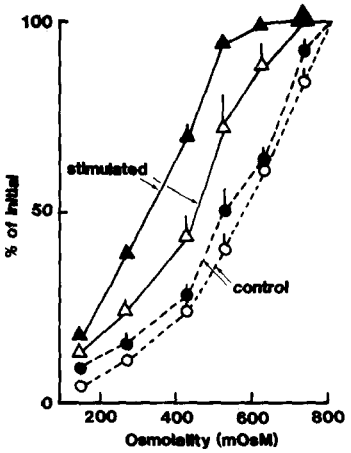

Fig. 1. Osmotic fragility curves for vesicles isolated from (continuous lines and triangles) stimulated or (broken lines and circles) control tissue blocks. Filled symbols refer to radioactive acetylcholine, open symbols to endogenous acetylcholine. Points are means of three (control) or four (stimulated) blocks from two fishes; bars represent S.E. Ordinates are remaining radioactive or endogenous acetylcholine expressed as a percentage of the vesicles suspended in 810 mosM glycine.

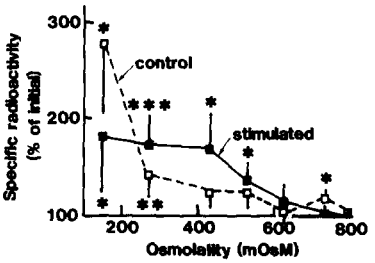

Fig. 2. Specific radioactivity of acetylcholine of vesicles from control (open symbols) or stimulated (filled symbols) tissue which survived varying amounts of osmotic lysis. Values for each experiment were expressed as a percentage of that at 810 mosM; points are mean values of three (control) or four (stimulated) experiments and bars represent S.E. The specific radioactivities of the vesicular acetylcholine of the preparations at 810 mosM were (means ± S.E. (number of experiments in parentheses)): from control tissue, 336 ± 23 (3); from stimulated, 2316 ± 145 (4) $dpm \cdot nmol^{-1}$. Paired *t*-tests showed that the specific activities of vesicles surviving hypo-osmotic conditions differed from those at 810 mosM with the following probabilities: ***$0.01 < P < 0.025$; **$P = 0.05$; *$0.05 < P < 0.1$; unasterisked $P \geq 0.1$ (null hypothesis).

It will be noted that the L_{50} for vesicles isolated from stimulated tissue when measured by the release of endogenous acetylcholine (468 ± 27) is intermediate between the two values just mentioned and thus not identical with the L_{50} for such vesicles measured by the release of radioactive acetylcholine. This is readily explained if the fragility measured in this way is a composite reflecting the behaviour of both VP_1- and VP_2-type vesicles. That this is likely to be so is shown by the filled symbols in Fig. 2, which plot the change in the specific radioactivity (as ordinates) of the vesicles surviving in media of the osmotic pressure plotted as abscissae. It will be seen that the specific radioactivity of the surviving vesicles does not remain constant but tends to rise. This suggests that the less highly labelled vesicles (i.e. those belonging to the VP_1 population) are osmotically more fragile, and thus have a higher internal content of osmotically active solutes, that the vesicles of the VP_2 population (exactly the same conclusion as is reached by comparing the vesicles from control and stimulated blocks).

In the case of the vesicles from control blocks, the difference between the L_{50} values measured by the release of radioactive (526 ± 15) or of endogenous acetylcholine (573 ± 8) is not so great and indeed is only just significant ($P \leq 0.05$, paired t-test). This can also be understood in terms of our model since the predominant vesicle type in this preparation is a VP_1 vesicle of low specific radioactivity contaminated with only a few more highly labelled VP_2-type vesicles. In Fig. 2 (open symbols) we see that once again, the specific radioactivity of vesicular acetylcholine does not remain constant during osmotic lysis but that the more highly labelled (VP_2-type) vesicles are more resistant to lysis than VP_1-type vesicles.

Discussion

Reserve and recycling vesicles are most efficiently separated on density gradients, using the high resolving power of a zonal rotor [1,2,11]. In experiments in which the vesicles are derived from tissue blocks that have been perfused with [^{3}H]acetate during stimulation the specific radioactivity of the acetylcholine of the VP_2 fraction may exceed by a factor of up to 16 that of the VP_1 fraction; thus when, for technical reasons, less efficient separations are carried out, as here, in iso-osmotic glycine-sucrose density gradients in a swing-out rotor [3,5], [^{3}H]acetylcholine may still be regarded as a valid marker for VP_2-type vesicles in preparations from stimulated tissue. Endogenous acetylcholine, by contrast, is distributed between the two types of vesicle. In vesicle preparations from control blocks, on the other hand, which contain few or no VP_2-type vesicles, endogenous acetylcholine is a valid marker for the VP_1-type vesicles: radioactive acetylcholine, by contrast, will label both VP_1- and (if present) VP_2-type vesicles. In the experiments that have just been described we have therefore felt justified in using the radioactive acetylcholine in vesicles derived from stimulated blocks as a valid marker for VP_2-type vesicles, and the endogenous acetylcholine in vesicles isolated from control blocks as a valid marker for VP_1-type vesicles.

The model previously put forward [5] to correlate the observed changes in water content, size and density of vesicles undergoing recovery after recycling proposed that the driving force for all these changes is a change in the internal osmotic pressure of the vesicle attendant on loss of the small molecular mass core constituents acetylcholine and ATP during exocytosis and their reuptake during subsequent recovery. The present results, by demonstrating a considerable difference in osmotic fragility in vesicles in the two states in a direction that implies that recycling vesicles have a lower internal content of osmotically active solutes than fully charged, reserve vesicles provides additional experimental evidence for this model.

The relative stability of the VP_2 population during stimulation and differences in the acetylcholine/ATP ratios of the two populations [12] suggest that there may well be two uptake processes with different kinetics: a rapid process involving an exchange of cytoplasmic acetylcholine and ATP for ions acquired from the extracellular medium during exo- and endocytosis and involving little change in the internal content of osmotically active molecules, and a much slower process involving osmotic work and an increase in the vesicular load of osmotically active molecules with attendant inward movement of water. The first of these processes favours ATP and the second, acetylcholine.

It is pertinent to consider whether phenomena similar to the bimodal behaviour of synaptic vesicles in stimulated electric organ are observed in mammalian cholinergic terminals. Bimodality and selective incorporation of newly synthesized acetylcholine into a denser vesicle fraction have recently been observed in the guinea-pig myenteric plexus [13]. From hypo-osmotically lysed guinea-pig brain synaptosomes, a denser fraction of highly labelled vesicles can be separated from a lighter, less labelled vesicle fraction when the synaptosomes are isolated from animals previously receiving an intracortical injection of labelled acetylcholine precursor [14]. The specific radioactivity of the highly labelled vesicles increases with decreasing endogenous acetylcholine levels, suggesting that the highly labelled vesicles are more resistant to hypo-osmotic lysis than the lighter vesicles. Thus there is reason to believe that vesicular metabolic and biophysical bimodality is not confined to electromotor terminals.

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