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Transient Permeabilization Induced Osmotically in Membrane Vesicles from *Torpedo* Electroplax: A Mild Procedure for Trapping Small Molecules†

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ABSTRACT: During hypoosmotic stress, membrane vesicles enriched in acetylcholine receptors become more permeable to external tracer molecules. When vesicles are immersed in 3 volumes of water containing $^{22}Na^+$, 50-70% of $^{22}Na^+$ equilibration is attained within 90 s. On the other hand, the uptake of $^{22}Na^+$ is greatly diminished only 6-10 s after an osmotic shock, and vesicle resealing is completed within 15 s. Furthermore, 90 s after osmotic shock, efflux rates are comparable to those of native vesicles, which also indicates that the vesicles have resealed. During osmotic shock, the entry of molecules into the vesicles increases with the strength of the osmotic shock and also depends on the size of the permeant. With a given strength of osmotic shock, the large molecule [^{3}H]inulin (M_r 5000) is taken up less than the smaller molecules $^{22}Na^+$ and [^{3}H]sucrose. In addition, α-bungarotoxin binding latency of the vesicles is not affected by osmotic shock, indicating that the sideness of the vesicles remains unchanged. The acetylcholine receptors in the vesicles remain functional after osmotic shock. For example, 90 s after $^{22}Na^+$ and [^{3}H]sucrose are loaded into vesicles by osmotic shock, only $^{22}Na^+$ is released by dilution in a buffer containing carbamoylcholine (carbamylcholine). Also, the influx of $^{22}Na^+$ into previously shocked vesicles can be specifically stimulated by carbamoylcholine. Such stimulations in the shocked vesicles can be blocked by *d*-tubocurarine or α-bungarotoxin, and they can be desensitized by preincubation with carbamoylcholine. These results suggest the possibility of using osmotic shock to load molecular probes into these membrane vesicles, which could provide a powerful tool for studying inner surfaces of the intact vesicles.

Knowledge of neuromuscular transmission has been extended by studies on electric eels and rays, from which membrane vesicles (microsacs) enriched in nicotinic acetylcholine receptors can be obtained in relatively large quantities (Heidmann & Changeux, 1978). In general, cholinergic stimulation results in increased postsynaptic membrane permeability to alkali cations, and the in vitro counterpart of cholinergic excitability is a rapid release of tracer cations from preloaded membrane vesicles when they are exposed to agonists. The cholinergic responses of acetylcholine receptor containing vesicles isolated from eels and rays have been documented in a variety of studies (Heidmann & Changeux, 1978; Neubig et al., 1979; Eldefrawi et al., 1978; Bernhardt & Neumann, 1978; Hess et al., 1978; Lindstrom & Patrick, 1974; Miller et al., 1978; Moore et al., 1979), but, until recently (Hartig & Raftery, 1979), no isolated membrane preparations were available for studying the external and internal surfaces of the membranes independently. The preparation of Hartig & Raftery (1979) consists of right side out vesicles, an excellent material for studying the external vesicle surface. However, a method is needed to gain experimental access to the inner vesicle surface. We report here (1) a potential method to load molecular probes into acetylcholine receptor membrane vesicles by osmotic shock, (2) the early changes in membrane permeability that accompany exposure of the vesicles to osmotic stress, and (3) the effect of osmotic shock on the functional integrity of the acetylcholine receptor and on its orientation in the membrane.[1]

Materials and Methods

Preparation of Acetylcholine Receptor Enriched Membranes. Acetylcholine receptor enriched membranes were prepared isotonically from frozen electric organs of *Torpedo californica* (Pacific Biomarine, Venice, CA) essentially as described by Miller et al. (1978), with two exceptions: (1) 100 μM phenylmethanesulfonyl fluoride was included in

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[1] A preliminary account of this study has been reported previously (West & Huang, 1979). After this paper was submitted for publication, we learned that similar observations have been made by others (P. R. Hartig, H.-P. H. Moore, and M. A. Raftery, personal communication).

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homogenization and resuspension buffers and (2) membranes were resuspended with a Dounce homogenizer (40–50 strokes). The final membrane suspension contained 20–30 mg of protein/mL in 400 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), 0.1 mM phenylmethanesulfonyl fluoride, and 0.02% NaN_3, pH 7.4. The specific activity was 0.5–0.7 nmol of ^{125}I-labeled α-bungarotoxin (α-BT) binding sites/mg of protein. α-Bungarotoxin was purified from the lyophilized venom of *Bungarus multicinctus* (Miami Serpentarium, Miami, FL) as in Fambrough & Hartzell (1972), and ^{125}I-labeled α-bungarotoxin was prepared by using chloramine-T (Hunter & Greenwood, 1962). Protein was measured by the procedure of Lowry et al. (1951), with bovine serum albumin as the standard.

Radiotracer Uptake during Osmotic Stress. Radiotracer uptake was initiated by vortex mixing 5 μL of membrane suspension (100–150 μg of protein) with 3 volumes of "shock buffer" containing 0.5–3.0 μCi of radiotracer, 10 mM sodium phosphate, pH 7.4, and various concentrations of NaCl calculated to create the desired osmotic pressures. Radiotracers were obtained from New England Nuclear. ^{22}NaCl was carrier-free, while the specific activities of [*fructose*-1-^{3}H(N)]sucrose and [^{3}H(G)]inulin were 11.2 Ci/mmol and 198.4 mCi/g, respectively. At appropriate time intervals (5–90 s), uptake was terminated by dilution with 1 mL (50 volumes) of ice-cold "wash buffer" which contained 10 mM sodium phosphate, pH 7.4, and a NaCl concentration equal to the result of mixing 1 part of membrane vesicles with 3 parts of shock buffer. Exactly 10 s after dilution, a 1-mL aliquot was vacuum filtered through a wet Millipore filter (AAWP, 25-mm diameter, 0.8-μm pore size) at 20–25 psi and then washed once with 10 mL of cold wash buffer for ~15 s. The filter was always removed from the vacuum filtration manifold (Amicon, Model VFM-1) between 25 and 30 s following termination of uptake. Radioactivity was measured by γ or scintillation counting, and data were corrected for isotope cross-talk in double-label experiments and for radiotracer binding to filters. All time points were taken in triplicate and averaged.

Radiotracer binding to vesicle membranes was determined with salt concentrations matching the pertinent experimental conditions. This was accomplished by first applying the appropriate osmotic shock in the absence of radiotracer, resulting in the concomitant reduction in salt concentration. Then, radiotracers were added for the appropriate time intervals to permit their binding to the membranes. Since some tracers could enter the vesicles during extended incubations, three additional osmotic shocks with buffered H_2O (10 mM sodium phosphate, pH 7.4) were applied to remove any unbound, internal tracers. Then, the vesicles were filtered following restoration of the salt concentration, as described below.

Time Course of Vesicle Resealing. Membrane permeability was assessed at various times after administration of an osmotic shock by first shocking the vesicles, later adding $^{22}Na^+$, and then measuring the amount of $^{22}Na^+$ uptake that occurred during a 10-s interval. Membrane vesicles (5 μL) in buffered 400 mM NaCl were mixed with 3 volumes of buffered 400 mM NaCl (control) or buffered H_2O (harsh shock). After various time intervals, 1 μCi of $^{22}Na^+$ (5 μL) in buffered 400 mM or buffered 100 mM NaCl (for control or harsh shock treatments, respectively) was added for 10 s, and then the vesicles were filtered and washed as above. The zero time measurement was obtained by adding $^{22}Na^+$ to the shock buffer prior to adding the vesicles. Binding of $^{22}Na^+$ to the outside of vesicle membranes was estimated by (1) allowing time for shocked vesicles to reseal (30–60 s), (2) adding $^{22}Na^+$ for 10 s, (3) administering three successive osmotic shocks with buffered H_2O (75 μL, 265 μL, and 265 μL) to remove any $^{22}Na^+$ inside the vesicles, and (4) restoring the original salt concentration with 400 μL of concentrated buffered NaCl prior to filtering and washing the vesicles.

α-Bungarotoxin Binding Latency. Membrane vesicles were shocked osmotically by dilution in 3 volumes of water (shocked vesicles) or diluted with 3 volumes of 400 mM NaCl and 10 mM sodium phosphate, pH 7.4 (native vesicles). After 25–30 min at 25 °C, ^{125}I-labeled α-bungarotoxin binding latency was measured by using a combination of several published procedures (Lindstrom & Patrick, 1974; Harting & Raftery, 1979; Huang, 1979): 20–30 μg of membrane protein was suspended in 250 μL of 400 mM NaCl, 1 mM ethylenediaminetetraacetate, and 10 mM sodium phosphate, pH 7.4, with 2 μg of ^{125}I-labeled α-bungarotoxin/mL (a fivefold excess of toxin over available binding sites). With shocked vesicles, the result was the same when 100 mM instead of 400 mM NaCl was used in the buffer. After 2 h at 25 °C, 25-μL aliquots were mixed with 250 μL of solubilization buffer (50 mM NaCl, 2% Triton X-100, and 10 mM sodium phosphate, pH 7.4) with (outside sites) or without (total sites) 5 μg of native α-bungarotoxin/mL (a 25-fold excess of native toxin over iodinated toxin). After 1.5–2 h, 200-μL samples were centrifuged on sucrose gradients for 6 h at 190 000*g* in a Beckman SW 50.1 rotor and then fractionated into 200-μL fractions. Gradients consisted of, from the bottom up, 250 μL of 54% (w/v) sucrose cushion, 4.5 mL of 5–20% continuous sucrose gradient, 100 μL of 2% sucrose, 200-μL samples, and 200 μL of buffer. All sucrose solutions were made in solubilization buffer. By this procedure, the acetylcholine receptor–^{125}I-labeled α-bungarotoxin complex was separated from free ^{125}I-labeled α-bungarotoxin and determined quantitatively, after subtracting a blank in which native toxin was added 1–2 h prior to ^{125}I-labeled α-bungarotoxin. The assays on native and shocked vesicles were run simultaneously with duplicate or triplicate samples.

Efflux Experiments. Radiotracers were loaded into vesicles by osmotic shock in the same manner as described above. After appropriate loading times, radiotracer efflux was started by the addition of 50 volumes of wash buffer with or without pharmacological effectors or inhibitors. At desired time intervals, 1-mL samples were filtered, washed, and counted as described above.

Influx Experiments. To test the $^{22}Na^+$ influx following osmotic shock, we shocked membrane vesicles by mixing them with 3 volumes of buffered H_2O for 10–15 min and then centrifuged them for 25 min at 95000*g*. Native vesicles were diluted and centrifuged in buffered 400 mM NaCl. The native and shocked vesicle pellets were resuspended to half the original volumes in wash buffer containing 400 or 100 mM NaCl, respectively; then aliquots were mixed with appropriate volumes of wash buffer with or without various concentrations of carbamoylcholine (carbamylcholine), *d*-tubocurarine, or α-bungarotoxin. After a 10–30-min incubation at 25 °C, the vesicles were stored on ice until they were tested for $^{22}Na^+$ influx by mixing 5 μL of membrane suspension with 15 μL of the appropriate wash buffer containing 0.5–1 μCi of $^{22}Na^+$ with or without carbamoylcholine and other effectors. After a 10-s influx period, vesicles were diluted, filtered, and washed as described above. Carbamoylcholine and *d*-tubocurarine were obtained from Sigma Chemical Co.

Results

To study the effects of osmotic shock on acetylcholine receptor enriched membrane vesicles, we isolated the vesicles

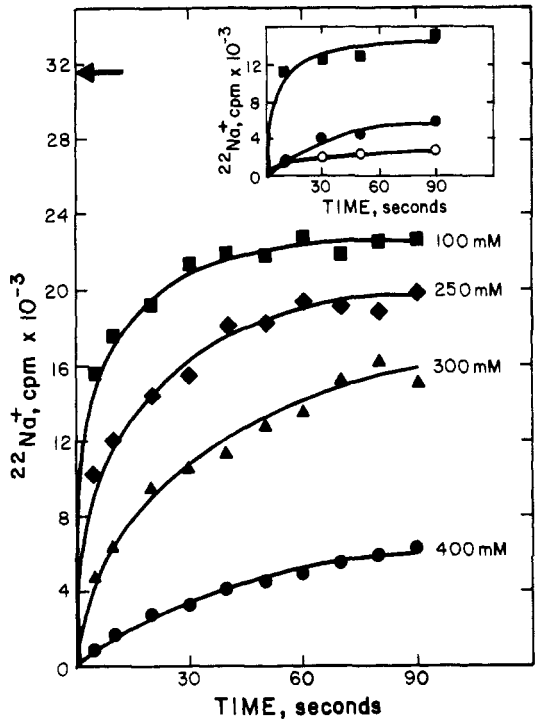

FIGURE 1: Uptake of $^{22}Na^+$ by *T. californica* membrane vesicles upon dilution into isotonic or hypotonic solution. Vesicle suspensions (5 μL) were diluted into 3 volumes of shock buffer containing 1 μCi of $^{22}Na^+$ and 400 mM (●), 267 mM (▲), 200 mM (♦), or no (■) NaCl, then further diluted with 50 volumes of the appropriate wash buffer (see Materials and Methods), filtered, and washed. (Abscissa) Time of exposure to $^{22}Na^+$-containing shock buffer before dilution with wash buffer. (Arrow) Vesicle-associated $^{22}Na^+$ after overnight equilibration. (Insert) Another experiment as above except that 800 mM sucrose (○) was included in the NaCl-free shock buffer, and 600 mM sucrose was added to the appropriate wash buffer. (Solid symbols) The same treatments as above, shown for comparison. Data are presented as averages of three triplicates. SD of three triplicates never exceeded 16% of the mean and averaged 6.4%.

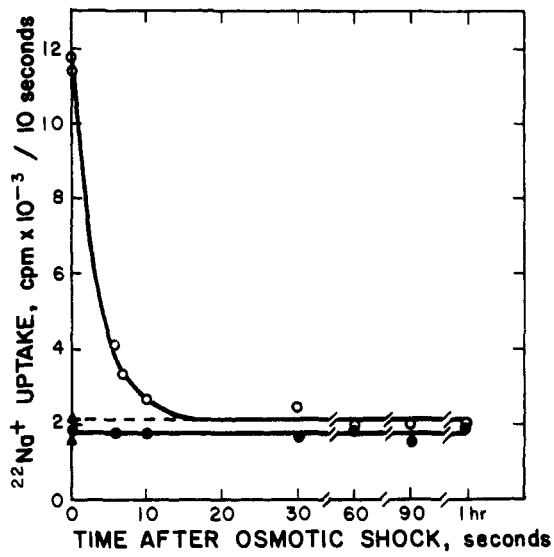

FIGURE 2: Time course of vesicle resealing. $^{22}Na^+$ was added to the vesicles at various times after osmotic shock. The amount taken up in a 10-s interval is plotted as a function of the time between the osmotic shock and the addition of $^{22}Na^+$. Osmotic shock with 3 volumes of buffered water (open circles); isotonic dilution with 3 volumes of buffered 400 mM NaCl (solid circles); respective measurements of binding of $^{22}Na^+$ to the vesicle membranes (triangles). Data are presented as averages of three triplicates.

by using buffered 400 mM NaCl to maintain isotonic conditions (Miller et al., 1978). In order to create transmembrane osmotic pressures, we diluted the vesicles into hypotonic buffers containing 0–300 mM NaCl. $^{22}Na^+$ was included in the hypotonic buffers (shock buffers) in order to follow its entry into the vesicles during osmotic stress. Figure 1 depicts the uptake of $^{22}Na^+$ during osmotic stresses of different magnitudes. "Uptake", as used here, refers to the amount of radiotracer retained by the vesicles following the designated influx period plus the 25-s washing period. The flux of the radiotracer is inward, opposite to the direction of the NaCl flux down its concentration gradient, and the $^{22}Na^+$ uptake increases when the concentration of NaCl in the solution outside the vesicles decreases. A harsh osmotic shock with 3 volumes of NaCl-free buffer results in a large uptake of $^{22}Na^+$, with an apparent steady state attained within 90 s. In this time interval, 50–70% of $^{22}Na^+$ equilibration is accomplished, and >1% of the total label added is taken up by the amount of vesicles used (100–150 μg of protein).

In order to verify that the $^{22}Na^+$ uptake in the experiment above is due to osmotic stress, we carried out another experiment in which vesicles were diluted with NaCl-free buffer with or without isoosmotic sucrose (800 mM). The rapid uptake of $^{22}Na^+$ induced with the NaCl-free shock buffer alone did not occur when an isoosmotic amount of sucrose was present in the shock buffer (Figure 1, insert). The uptake in the latter case was no greater than that with isotonic NaCl (400 mM). This demonstrates that the response to hypotonic NaCl is due to osmotic pressure and not due to other factors such as changes in ionic strength.

In order to determine how quickly the vesicles reseal, we added $^{22}Na^+$ to the incubation mixture at various times after the initiation of an osmotic shock and measured its uptake during a 10-s interval (Figure 2). A large increase of $^{22}Na^+$ uptake was observed only when it was included in the shocking solution. When $^{22}Na^+$ was added even as soon as 6–10 s after the shock, only a small increase due to the osmotic shock was observed, and the vesicle membranes resealed by 15–20 s after the shock was started. Furthermore, almost all of the $^{22}Na^+$ taken up by the nonshocked, control vesicles is bound to the membranes (triangles in Figure 2), as shown by an inability to remove it by additional osmotic shocks during dilution and washing. Likewise, the uptake of $^{22}Na^+$ by the shocked vesicles, at times longer than 20 s after shock, can be fully accounted for by binding to the membranes. Thus, resealed vesicles appear as tightly sealed as native, nonshocked vesicles, within the sensitivity limits of the 10-s uptake assay.

To examine the efficiency of loading larger molecules into these vesicles, we measured the uptake of three radiotracers of different molecular weights (sizes). In Figure 3, the amount of each radiotracer that has permeated across the membranes at the 90-s time point is expressed as the percentage of the total radiotracer added minus the amount of tracer bound to the vesicle membranes (see Materials and Methods). The strength of the osmotic shock is expressed as the initial difference in salt concentratin (Δ[NaCl]) inside and outside of the vesicles when the osmotic shock is administered. The data clearly show that osmotic shock enhances the permeation of all three tracers into the vesicles. In the absence of osmotic shock (Δ[NaCl] = 0 mM), the membranes are much less permeable to [^{3}H]inulin (M_r 5000) and [^{3}H]sucrose (M_r 342) than to $^{22}Na^+$. When an intermediate osmotic shock (Δ[NaCl] = 100 mM) is applied, the uptakes of [^{3}H]sucrose and [^{3}H]inulin increase to 62 and 17%, respectively, of the amount of $^{22}Na^+$ taken up. When a stronger osmotic shock (Δ[NaCl] = 300 mM) is applied, the uptakes of [^{3}H]sucrose and [^{3}H]inulin increase to 90 and 46%, respectively, of that of

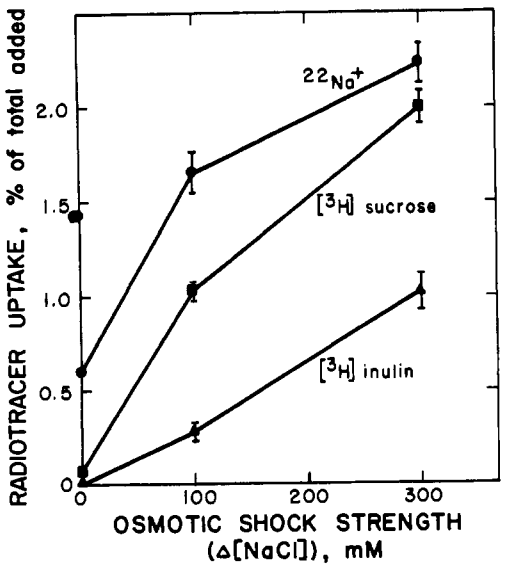

FIGURE 3: Relative uptake of $^{22}Na^+$ (●), [^{3}H]sucrose (■), and [^{3}H]inulin (▲) into vesicles in buffered 400 mM NaCl at the end of 90 s, after osmotic shock with 3 volumes of shock buffer containing appropriate radiotracers. Radiotracers bound to the external side of the membranes were estimated and subtracted (see Materials and Methods). The strength of the osmotic shock is expressed as the difference in salt concentration (Δ[NaCl]) inside and outside of the vesicles upon dilution with the appropriate shock buffer. Shock buffers contained 400 mM (Δ[NaCl]) = 0 mM), 267 mM (Δ[NaCl] = 100 mM), or no (Δ[NaCl] = 300 mM) NaCl. 100% of total radiotracers added = 1.80×10^6 cpm $^{22}Na^+$, 6.37×10^5 cpm [^{3}H]sucrose, and 4.72×10^5 cpm [^{3}H]inulin. Data are presented as averages of three triplicates ± SD.

Table I: Comparison of ^{125}I-Labeled α-Bungarotoxin (α-BT) Binding Latency of Vesicles before and after Osmotic Shock[a]

	acetylcholine receptor-^{125}I-labeled α-BT complex		
		outside sites	
vesicles	total sites (pmol/μL)	pmol/μL	% of total sites
native	14.15 ± 0.04	11.51 ± 0.26	81.3 ± 1.8
shocked	13.14 ± 0.32	10.19 ± 0.37	77.5 ± 2.8

[a] Vesicles were shocked osmotically by dilution in 3 volumes of water (shocked vesicles) or diluted with 3 volumes of isotonic buffer (native vesicles). After 15–30 min at 25 °C, ^{125}I-labeled α-bungarotoxin (α-BT) binding latency was measured by a sucrose gradient centrifugation technique (see Materials and Methods). Data are presented as averages of duplicate (native) or triplicate (shocked) determinations ± the range of replicates. Specific radioactivity = 2.42×10^5 cpm/pmol of ^{125}I-labeled α-BT. Blank (native toxin added first) = 0.289 pmol/μL.

$^{22}Na^+$. It is evident, then, that the uptakes of the test molecules depend on the strength of the osmotic shock. Furthermore, with a harsh osmotic shock the vesicles can be readily loaded with $^{22}Na^+$ and [^{3}H]sucrose, but the entry of a larger molecule, [^{3}H]inulin, is still limited.

To assess further the effects of osmotic shock on vesicle structure, we carried out measurments of toxin binding latency to detect any changes in membrane sideness that might have occurred during shock. The results of the ^{125}I-labeled α-bungarotoxin binding latency test (Table I) indicate that ~80% of the binding sites of native or shocked vesicles are accessible to ^{125}I-labeled α-bungarotoxin without solubilization of the membranes. The identical result for both native and osmotically shocked vesicle populations rules out the possibility of change in vesicle sideness induced by the shocking procedure. However, protein measurements reveal that the osmotic shock procedure removes ~20% of the nonreceptor protein from the

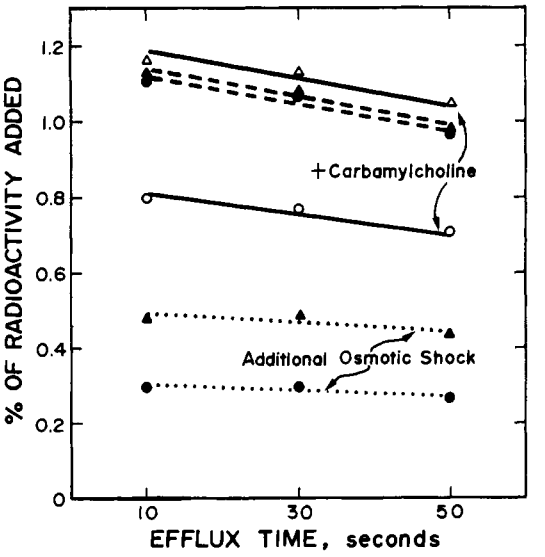

FIGURE 4: Agonist-induced efflux of $^{22}Na^+$ and [^{3}H]sucrose from vesicles loaded by osmotic shock. Immediately following a 90-s incubation of vesicles with radiotracers in NaCl-free shock buffer, efflux of $^{22}Na^+$ (circles) and [^{3}H]sucrose (triangles) was initiated by dilution (at time = 0 s) with 50 volumes of wash buffer (containing 10 mM sucrose and 100 mM NaCl) (dashed lines), wash buffer plus 0.5 mM carbamoylcholine (solid lines with open symbols), or 25 volumes of water for 3–5 s, followed by 25 volumes of double-strength wash buffer (dotted lines). At appropriate intervals, aliquots of the vesicle suspension were filtered and then washed. 100% of the total radioactivity added = 2.3×10^6 cpm $^{22}Na^+$ and 1.06×10^6 cpm [^{3}H]sucrose. Data are presented as averages of three triplicates. A lower maximum percentage of "total $^{22}Na^+$ added" was taken up here than was taken up in Figure 3, due to use of a smaller amount of vesicles in this experiment.

vesicles; hence, the specific α-bungarotoxin binding activity is ~25% higher in shocked vesicles.

To investigate the effects of osmotic shock on acetylcholine-receptor activity and to verify that radiotracers were actually being loaded into those vesicles in the population that contained functional acetylcholine receptors, we measured efflux rates of $^{22}Na^+$ and [^{3}H]sucrose in the presence or absence of carbamoylcholine (Figure 4). $^{22}Na^+$ and [^{3}H]sucrose were loaded into vesicles by harsh osmotic shock for exactly 90 s, and efflux measurements were taken immediately. In the absence of cholinergic effectors, the efflux of both tracers is linear and identical. However, upon addition of 0.5 mM carbamoylcholine, 28% of the loaded $^{22}Na^+$ is released within 10 s, while the [^{3}H]sucrose efflux remains unchanged. On the other hand, both radiotracers can be released by additional osmotic shock (lowest two lines in Figure 4). The magnitude of $^{22}Na^+$ release seen here is comparable to that of native (unshocked) vesicles loaded isotonically by overnight equilibration with $^{22}Na^+$, a widely used method to measure the ion channel activity of the acetylcholine receptor [e.g., Miller et al. (1978)]. These results show that vesicles containing acetylcholine receptors can definitely be loaded with radiotracers by the osmotic shock procedure and that the functional activity of the acetylcholine receptor in these membrane vesicles is still intact after loading.

As a rigorous test of acetylcholine-receptor integrity, we measured carbamoylcholine-induced stimulation of $^{22}Na^+$ influx into osmotically shocked vesicles. The vesicles were shocked with 3 volumes of buffered H_2O, centrifuged, and resuspended to ~12 μmol of α-bungarotoxin binding sites/L of buffered 100 mM NaCl with or without carbamoylcholine, *d*-tubocurarine, or α-bungarotoxin. Native vesicles were treated in an identical manner except that they were diluted

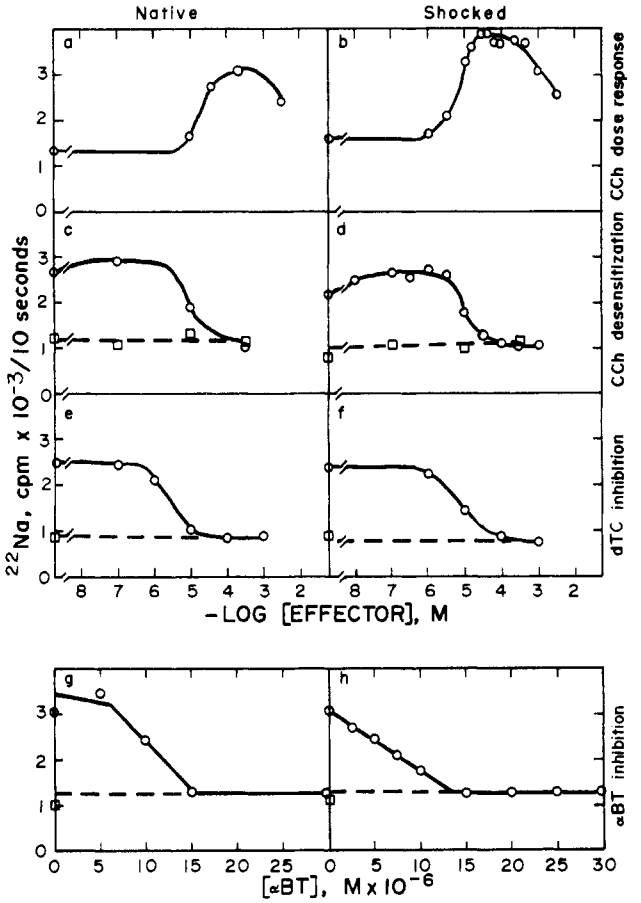

FIGURE 5: Pharmacological properties of vesicles after osmotic shock. Concentrations of effector shown on the abscissa represent those applied during the pretreatment (c–h). The concentration of effector in panels a and b represents that applied during the 10-s uptake interval. The concentration of carbamoylcholine (CCh) used to stimulate pretreated vesicles was chosen from the dose–response curves (a and b) to obtain a maximal response: 500 μM for native vesicles and 25–50 μM for shocked vesicles. Stimulated vesicles (○); controls without carbamoylcholine stimulation (□); no pretreatment (a and b); carbamoylcholine pretreatment (c and d); *d*-tubocurarine (dTC) pretreatment (e and f); α-bungarotoxin (α-BT) pretreatment (g and h). Data are presented as averages of three triplicates.

and resuspended in isotonic buffer (400 mM NaCl). As was shown in Figure 2, all of the $^{22}Na^+$ that associates with the vesicles in a 10-s interval can be accounted for by binding to the membranes, as long as osmotic shocks are avoided and cholinergic agonists are absent. When sufficient amounts of agonists are added, $^{22}Na^+$ influx into the vesicles occurs. This can be shown by using osmotic shock to remove the agonist-induced portion of the uptake. Furthermore, with optimal agonist concentrations the maximal stimulated influx occurs in <10 s; no further stimulation is seen beyond this time point. Such rapid desensitization of the receptor channel has been previously reported (Hess et al., 1979). Thus, using a 10-s $^{22}Na^+$ uptake assay as our measurement of acetylcholine receptor responsiveness, we obtained dose–response curves for the agonist, carbamoylcholine, which are presented in Figure 5a,b. Native and shocked vesicles both respond maximally at concentrations near 10^{-4} M, are unaffected by concentrations below 10^{-6} M, and are partially inhibited by concentrations above 10^{-3} M. The inhibition at high carbamoylcholine concentrations is probably due to rapid acetylcholine receptor desensitization as observed by Aoshima et al. (1980), using a quench-flow cation influx assay. Desensitization of the maximal response also results from the pretreatment of vesicles with carbamoylcholine concentrations >(2–3) × 10^{-6} M, with complete desensitization occurring at concentrations ≥10^{-4} M (Figure 5c,d). It is interesting to note that the carbamoylcholine concentration required to stimulate the receptor is approximately the same as that required to desensitize the receptor. Inhibition with *d*-tubocurarine occurs over the range 10^{-6}–10^{-4} M (Figure 5e,f), and inhibition with α-bungarotoxin occurs in a linear fashion over the narrow range from 0 to 15 μM when the vesicles are ~12 μM in toxin binding sites (Figure 5g,h). When [^{3}H]sucrose is included with $^{22}Na^+$ in double-label experiments, its uptake by the vesicles is small and unaffected by any of the treatments or pretreatments described above. In all of these experiments, results with native and shocked vesicles are essentially the same, which demonstrates that the functional integrity and pharmacological responsiveness of the acetylcholine receptor are completely intact after osmotic shock.

Discussion

In the present study, we have confirmed and extended the observation by Hartig & Raftery (1979) that acetylcholine receptor rich membrane vesicles are quite osmotically sensitive. During osmotic stress, the vesicles exhibit a transient increase in permeability (Figure 1). The vesicles reseal <15–20 s after shock (Figure 2). The transient permeability must be due to pores or fissures which form in the membrane, allowing small molecules to pass through. The amount of permeant that enters the vesicles from the surrounding solution depends on the molecular weight of the permeant and on the strength of the osmotic shock (Figure 3). Under a given shock strength, small molecules are taken up to a greater extent than larger ones. The restriction on large molecules could be due to the size of the pores in the membrane, in which case the pore size must be proportional to the shock strength. Alternatively, large fissures are formed, but since their lifetimes are short, the entry of the test molecules is limited by their diffusion rates. In any case, under the experimental conditions used, osmotic shock causes no gross rearrangement of the membrane structure of the vesicles, since the sideness of α-bungarotoxin binding sites remains unchanged after shock (Table I).

The uptake induced by osmotic stress is primarily due to transmembrane movement of radiotracer and not due to binding of radiotracers to the membrane. Such binding might be particularly enhanced by reductions in ionic strength. This possibility can be ruled out since the addition of isoosmotic sucrose to NaCl-free shock buffer prevented the low-salt induced uptake (Figure 1, insert). Therefore, enhanced uptake must have been caused by osmotic pressure, not by reduction in ionic strength. Also, most of the tracer taken up can be released by additional osmotic shock (Figure 4), and $^{22}Na^+$ taken up can be released specifically through the acetylcholine receptor channel by carbamoylcholine (Figure 4). These results show, therefore, that most of the uptake of radiotracer must represent transmembrane movement.

Following osmotic shock, the functional integrity of the acetylcholine receptor remains intact. This is evident since shocked vesicles still respond to the cholinergic agonist, carbamoylcholine, by momentarily increasing membrane permeability to $^{22}Na^+$. Our data indicate that this response is still specific, since a release of [^{3}H]sucrose loaded into the vesicles is not stimulated by carbamoylcholine (Figure 4). The response exhibits the pharmacological characteristics of the nicotinic acetylcholine receptor. It can be inhibited with the well-established cholinergic antagonists, α-bungarotoxin and *d*-tubocurarine, and it can be densensitized by preincubation with the agonist, carbamoylcholine (Figure 5). Furthermore, the dose–response curves for the osmotically shocked vesicles

are similar to, if not identical with, those of the native vesicles. These results show that the acetylcholine receptor retains all of the cholinergic characteristics after osmotic shock. The shock procedure is therefore a mild one.

The present studies bear resemblance to studies on red-cell ghost membranes after hemolysis. Red cells are usually hemolyzed with 20–40 volumes of ice-cold buffer of low osmolarity, and resealing of the lysed ghost membranes only occurs upon incubation above 15–20 °C and with the ionic strength reconstituted to >15 mM NaCl (Bjerrum, 1979; Johnson, 1975; Steck, 1974). We have observed a rapid resealing (within 15 s) of acetylcholine receptor rich membranes. In our experiments, osmotic shock was carried out at 20–25 °C, and ionic strengths of the solution never decreased below 100 mM NaCl. One would therefore expect a rapid resealing of the membranes after shocking. Indeed, instantaneous resealing was also observed with red cells by Bodemann & Passow (1972) when hemolysis was carried out at 37 °C.

The results of the present study suggest the possibility of loading molecular probes into the interior of these vesicles. This could open up new opportunities to study the cytoplasmic surfaces of electroplax membranes if used in conjunction with the right side out vesicles described by Hartig & Raftery (1979). We have shown that $^{22}Na^+$ and larger molecules can be readily loaded into the aqueous interior of these vesicles by a simple procedure: exposure of vesicles to hypotonic solutions containing the tracer molecules. This mild procedure does not alter vesicle sideness or acetylcholine-receptor function. Therefore, it could provide a powerful tool not only for studying inner surfaces of these vesicles but also for manipulating transmembrane ion distributions for studies of membrane functions.

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New Cleavable Photoreactive Heterobifunctional Cross-Linking Reagents for Studying Membrane Organization†

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ABSTRACT: The synthesis is described of four new cleavable, photosensitive, heterobifunctional cross-linking reagents for use in examining membrane organization: 4′-azidoazobenzene-4-oxysuccinimide ester (**1**), *N*-[4-(*p*-azidophenylazo)benzoyl]-3-aminopropyl-*N*′-oxysuccinimide ester (**2**), *N*-[4-(*p*-azidophenylazo)benzoyl]-6-aminohexyl-*N*′-oxysuccinimide ester (**3**), and *N*-[4-(*p*-azidophenylazo)benzoyl]-11-aminoundecyl-*N*′-oxysuccinimide ester (**4**). Two photoaffinity-directed cross-linking agents were prepared by attaching reagents **1** and **2** via their activated ester groups to soybean agglutinin (subunit M_r 30000). Irradiation of the lectin derivatives resulted in a decrease in their absorption spectra at 360 nm due to photolysis of the bound reagents. Cross-linking of soybean agglutinin subunits following irradiation of the soybean agglutinin derivative to which reagent **2** had been coupled was observed by the appearance of new Coomassie blue staining material (60000, 90000, and 120000 daltons) on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. On polyacrylamide gel electrophoresis in the absence of sodium dodecyl sulfate, soybean agglutinin oligomers were observed. Cleavage of the cross-linked soybean agglutinin with 0.1 M sodium dithionite for 25 min at room temperature resulted in the disappearance of the high molecular weight bands and an increase in the amount of uncross-linked material. The use of the photoaffinity-directed agents for probing membrane organization is discussed.

Cross-linking agents are becoming increasingly important tools for understanding membrane structure and organization [reviewed by Peters & Richards (1977), Friedman (1979), and Ji (1979)]. Homobifunctional cross-linking agents, while sufficient for examining the conformation and structure of protein molecules, when applied to cell membranes give, on polyacrylamide gel electrophoresis, complex patterns which

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