Communication

Electrogenic Behavior of Synaptic Vesicles from *Torpedo californica* *

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SUMMARY

Electrical potential changes in pure synaptic vesicles from *Torpedo californica* were monitored with the fluorescent dye 3,3'-dipropylthiadicarbocyanine iodide. Vesicles resuspended in variable external sodium ion in the presence of gramicidin established sodium ion membrane diffusion potentials. Vesicles resuspended in choline or acetylcholine chloride became hyperpolarized upon addition of gramicidin. Hyperpolarization was subsequently partially reversed spontaneously by choline or acetylcholine influx, which was confirmed by gel filtration, to yield a new, less negative, stable membrane potential. Thus, acetylcholine and choline are taken up electogenically by synaptic vesicles.

Acetylcholine is stored in synaptic vesicles and is recognized as the mediator of nerve impulses at many types of nerve terminals (1), including the electric organ of *Torpedo californica* (2). The organ is embryologically derived from muscle and is therefore a useful model system for characterization of their structure and dynamic function (3).

Vesicles from the *Torpedo* electric organ are membrane-bounded, 880 Å in diameter, and contain approximately 0.08 m ATP and 0.30 m acetylcholine, and an acidic curve protein called vesiculin (2). Binding of exogenous acetylcholine to intact *Torpedo* vesicles (4) and to vesicle ghosts (5) has been demonstrated. Acetylcholine but not ATP spontaneously leaks slowly from isolated vesicles (3). Isolated vesicles have been found to contain significant quantities of Na+*, K+*, Mg**+, Ca**+, and Zn**+ (6). We present here preliminary results on transport properties exhibited by purified *Torpedo* vesicles for some physiologically important ions.

MATERIALS AND METHODS

Gramicidin (Dubos) and valinomycin were from Sigma. [3H]Choline chloride (2 Ci/mmol) was from Amersham/Searle. The dye 3,3'-dipropylthiadicarbocyanine was synthesized as described (7). Synaptic vesicles were isolated similarly as described (3) from *Torpedo californica* electric organ by isopycnic zonal density gradient centrifugation. A well resolved vesicle peak was obtained. 

Uptake of 13Hlcholine was studied as follows. Aliquots (10 ml) of vesicles were pelleted similarly to that above. One pellet was resuspended at 26° in a 0.9-ml aliquot of 0.8 M sucrose, 5 mM Hepes, pH 7.2 with NaOH, 5 mM HEPES, pH 7.2 with NaOH, and centrifuged 1 h at 45,000 rpm in an SW50.1 rotor at 4°. After centrifugation, the supernatant was poured off, the inside of the centrifuge tube was wiped dry, and the tube was stored on ice until used. Two milliliters of isosmotic (800 mosm) solution containing various ions and sucrose and buffered to pH 7.2 with sodium HEPES were added to 2 ml of 0.20 M choline chloride, 1.7 mM sodium chloride, 1.7 mM sucrose, 5 mM HEPES, 400 mM sucrose, and dye at 25° in the spectrofluorimeter.

Uptake of [3H]choline was studied as follows. Aliquots (10 ml) of vesicles were pelleted similarly to that above. One pellet was resuspended at 26° in a 0.9-ml aliquot of 0.8 M sucrose, 5 mM HEPES, pH 7.2 with NaOH, (1.5 mM final), and 2 µl of the gramicidin solution was added. [3H]Choline (0.1 ml, 30 Ci/ml, 1.5 x 10^-5 M) in the same sucrose medium was added and allowed to incubate 2 h at 26° and then was chilled to 4°. The solution was chromatographed on a column (0.9 x 25 cm) of Sephadex G-50 equilibrated in the same sucrose medium at 4°, 0.75-ml fractions were collected, and fluorescence was determined. Similar experiments were conducted on another two vesicle pellets, except that for one all incubations were conducted at 4° for and for the other vesicles were resuspended in 0.4 M NaCl, 5 mM HEPES, pH 7.2 with NaOH. Hyperpolarized vesicles loaded with [3H]choline at 26° above were subjected to hypotonic shock before fluorescence measurements by resuspending the pellet in 0.5 ml of 5 mM HEPES, pH 7.2 with NaOH (1.5 mM final) and incubating at 25° for 10 min. The suspension then was added to 2 ml of 0.20 M choline chloride, 1.7 mM sodium chloride, 1.7 mM HEPES, 400 mM sucrose, and dye at 25° in the spectrofluorimeter.

RESULTS

A thermodynamic driving force for ion transport across the synaptic vesicle membrane was established by resuspending pelleted vesicles in media of different ionic composition. Electrogenic ion fluxes were monitored with the cationic fluorescent dye 3,3'-dipropylthiadicarbocyanine iodide shown in Fig. 1. Very rapid electrogenic efflux of sodium ions from vesicles could be imposed when desired by the addition of gramicidin, which cannot pass typical organic cations (11). Vesicles resuspended in isosmotic mixed NaCl/lysine chloride solutions at 0.21 M constant ion strength produced approximately the same level of fluorescence at all sodium ion concentrations tested, as shown in Fig. 2, left. Lysine was chosen to maintain ion strength because it is taken up only

1 J. E. Rothlein and S. M. Parsons, unpublished observation.

2 The abbreviation used is: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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Addition of gramicidin after 1 min resulted in rapid fluorescence quenching to a new value which depended on the external sodium ion concentration (Fig. 2, left). Addition of valinomycin had no effect on fluorescence (not shown). This behavior is consistent with vesicle hyperpolarization (internal negative) due to sodium ion efflux with consequent increasing binding of the dye to the vesicles (7, 12). The data are plotted in Fig. 2, right, where it is evident that the amount of fluorescence quenching was approximately a linear function of the logarithm of the external sodium ion concentration in the presence of gramicidin. The null point where no change in fluorescence occurred upon addition of gramicidin was at 0.20 mM sodium ion. At this point, the actual membrane potential could be determined if the internal sodium ion activity were known (13), but this information is not available.

When vesicles were resuspended in isoosmotic 0.21 M choline chloride at 25° the fluorescence behavior seen in Fig. 3, left, was obtained. An initial nearly flat fluorescence baseline was observed. Addition of gramicidin to the vesicle suspension after 0.3 min resulted in an immediate large decrease in fluorescence followed by a delayed rebound of the fluorescence. The gramicidin-induced fluorescence quenching again is consistent with vesicle hyperpolarization due to sodium ion efflux. The fluorescence rebound is consistent with later spontaneous vesicle depolarization. This could occur either by endogenous anion efflux or choline influx through the vesicle membrane. Since little or no rebound was seen in the previous experiment, it is unlikely that endogenous anion efflux accounts for the spontaneous depolarization. Rather, choline uptake driven by the large negative membrane potential could explain the fluorescence rebound. When a similar experiment was repeated at approximately 7°, gramicidin addition again caused an immediate large decrease in fluorescence, but there was no fluorescence rebound (not shown). Also, vesicles subjected to hypoosmotic shock for 10 min before dilution into 0.15 M choline at 25° still exhibited 52% of the gramicidin-induced fluorescence quenching of unshocked vesicles and exhibited fluorescence rebound behavior.

3 R. S. Carpenter and S. M. Parsons, unpublished observation.
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Uptake of choline by the vesicles was confirmed directly in Fig. 4. Vesicles which were hyperpolarized in the presence of \(^{3}H\)choline at 26°C concentrated radioactivity approximately 6.5-fold which was retained after isoosmotic gel filtration to remove unbound \(^{3}H\)choline. Similarly, hyperpolarized vesicles lost 54% of the bound radioactivity after gel filtration into a hypoosmotic medium (not shown). Vesicles which were hyperpolarized at 4°C or were not hyperpolarized at 26°C incorporated much less \(^{3}H\)choline. Thus, choline and acetylcholine uptake at 25 to 26°C but not at 4 to 7°C can be demonstrated by their causing depolarization of hyperpolarized vesicles and by incorporation of radioactivity into hyperpolarized vesicles.

DISCUSSION

The fluorescent dye technique utilized in this study is gaining wide acceptance as a reliable method for quantitatively estimating membrane potentials in systems inaccessible to direct measurement with microelectrodes (12-15). The dye acts as a lipophilic cation which partitions between the free and bound state where it is fluorescence-quenched in response to the membrane electrical potential (7, 12). The change in dye fluorescence has been found in several systems to be a linear function of membrane potential (12, 13, 16). Since relatively low concentrations of dye are used, it will not significantly perturb the membrane potential.

An approximately linear relationship has been confirmed for synaptic vesicles by establishing a sodium ion Nernst potential with gramicidin. If the membrane potential of gramicidin-treated vesicles suspended in sodium ion is described by Equation 1, then the change in fluorescence in the presence of different external sodium ion concentrations can be described by Equation 2.

\[
E_M = \frac{RT}{F} \ln \left( \frac{[Na_{ext}]}{[Na_{int}]} \right)
\]

(1)

\[
\Delta F = a \ln([Na_{ext}]) - b
\]

(2)

where \(E_M\) is membrane electrical potential, \(\Delta F\) is the change in fluorescence from a constant reference value \(b\), \(a\) is a proportionality constant between \(\Delta F\) and the logarithm of the external sodium ion activity \([Na_{ext}]\), \([Na_{int}]\) is the internal sodium ion activity, \(R\) is the universal gas constant, \(T\) is the absolute temperature, and \(F\) is the Faraday constant. The above equations assume that fluxes of any other ions across the vesicle membrane are much less than that of sodium ion in the presence of gramicidin, and that the internal sodium ion concentration does not change significantly over the course of the measurement. Vesicles appeared to obey Equation 2 in the presence of gramicidin over a 50-fold decrease in external sodium ion concentration, which would correspond to a 90-mV decrease in the membrane potential if sodium ion actually contributes the primary flux across the gramicidin-treated membrane.

The fluorescence rebound after gramicidin-induced hyperpolarization in choline and acetylcholine solutions at 25°C means that choline or acetylcholine uptake partially reversed the sodium ion diffusion potential. A new less negative potential was established apparently after the internal choline or acetylcholine concentration achieved electrochemical equilibrium. Thus, the vesicle membrane potential in the presence of gramicidin appears to be a function of both sodium ion and choline or acetylcholine ion diffusion potentials (17).

Although the internal concentration of acetylcholine has been estimated at the very high value of 0.36 M, Whittaker et al. have suggested that most of this acetylcholine is tightly bound to a core complex of vesiculin and ATP2. Thus, most of the endogenous acetylcholine might not contribute to the transmembrane electrical potential because it is not free to diffuse across the membrane. Therefore, hyperpolarization of the vesicles due to endogenous acetylcholine efflux during the relatively short time period of the fluorescence experiments is not necessarily expected and was not observed here. The previously observed slow leakage of acetylcholine from synaptic vesicles which might suggest that such hyperpolarization should occur was measured in the presence of high concentrations of external sodium ion which might have stimulated electroneutral exchange with internal acetylcholine. Additional information about all ionic species which determine the membrane potential of native vesicles and whether vesicles swell in different ionic media is needed before further electrogenic quantitation is warranted.

Since both the characteristic gramicidin-induced fluorescence behavior and \(^{3}H\)choline uptake partially survived hypoosmotic conditions, Torpedo electric organ synaptic vesicles appear to be partially resistant to hypoosmotic disruption. Resistance also was suggested by Suszkiw (6) on the basis of electronmicrographs. This is similar to the behavior of brain synaptic vesicles which routinely are isolated by hypoosmotic disruption of synaptosomes (18).

The results presented here allow several new conclusions about the cholinergic synaptic vesicles from Torpedo californica electric organ. The vesicles as isolated contain a mobile cation, probably sodium ion, which is releasable by gramicidin. There is no evidence for valinomycin-releasable potassium ion. The vesicles can become hyperpolarized. The hyperpolarized vesicle membrane is very permeant to choline and acetylcholine at 25-26°C, but not to choline (and probably also acetylcholine) at 4-7°C, and choline and acetylcholine uptake can be driven by an internal negative potential. These properties suggest that acetylcholine uptake into, storage in, and release from synaptic vesicles in vivo might possibly be influenced by vesicle-associated electrogenic processes.

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