TRANSMEMBRANE ELECTRICAL POTENTIAL MEASUREMENTS IN RAT BRAIN SYNAPTOSOMES

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1. Introduction

During dispersion of mammalian brain cortex in isotonic sucrose with mild shear forces the endings of the nerves tear away from their axons and post-synaptic cell surface and seal off, forming vesicular structures termed 'synaptosomes'. The synaptosomal preparation is complex because it contains nerve endings of diverse origin. Nevertheless, a substantial fraction of the preparation is derived from neurons of the central nervous system and thus the preparation offers a unique opportunity to study the structure and metabolic behaviour of the synaptic membranes. The necessary requirement for this approach is that properties of the synaptosomes were similar to those of synapses in vivo. In recent years, several studies have been undertaken to characterize this synaptosomal preparation with respect to its electrical properties [1], biochemistry [2–4] and the characteristics of neurotransmitter uptake [5–12], release [1,4,13–15] and synthesis [13,16–18].

Most of this work has been done, however, on a preparation which is isolated by a hypertonic sucrose procedure [19]. This technique often yields preparations which are contaminated with non-synaptosomal vesicles and fragments and are metabolically relatively inactive. Recently, a new method has become available which uses an iso-osmotic Ficoll separation [20] in place of hypertonic sucrose. Preparations purified on a Ficoll gradient contain $\geqslant$ 75% vesicle-containing synaptosomes as estimated by electron microscopy (in preparation) and have been shown to exhibit high metabolic activity and levels of ATP, ADP and other energetic parameters similar to those in the intact brain [21].

Here we report the determination of transmembrane electrical potentials in synaptosomal preparations which have been purified on a Ficoll gradient and then maintained under physiological conditions or treated with drugs which are known to affect the behaviour of intact nervous tissue.

2. Experimental

2.1. *Preparation of rat brain synaptosomes*

Rat brain synaptosomes were prepared essentially by the method in [20] as detailed in [21].

2.2. *Determination of the transmembrane electrical potential*

Synaptosomes were suspended at ~4 mg protein/ml in Krebs-Henseleit medium (144 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 5 mM $NaHCO_3$, 1 mM P_i) containing 10 mM Tris–Hepes at pH 7.4. Glucose (10 mM) and $CaCl_2$ (2.5 mM) were added and the suspension was incubated at room temperature with gentle shaking for 10 min. The suspension was transferred to stirred vials and sodium tetraphenylboron (TPB) and tritiated triphenylmethyl phosphonium ([^{3}H]TPMP) were added. Samples (100 μl) were pipetted into 0.4 ml polypropylene micro-centrifugation tubes containing 10 μl silicone oil (density 1.03, General Electric) and centrifuged for 2 min in a Beck-

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man Microfuge (12 000 X *g*). An aliquot of the supernatant was added directly to a scintillation vial, the residuum removed by vacuum, and the tube swabbed to remove any of the supernatant aqueous layer. The pellet was resuspended with the aid of a glass microrod in the same microtube with 0.1 ml half-saturated ammonium chloride. The entire tube was placed in a vial, the contents thoroughly mixed with scintillation fluid (Searle aqueous counting solution) then counted (Delta 300 Searle two channel scintillation counter).

2.3. *Synaptosomal water volume*

Intrasynaptosomal water volume was measured as above, using ^3H_2O and the extracellular marker, C-14 polyethylene glycol (PEG, mol. wt 4000). Protein was measured by the Lowry method [22] with bovine serum albumin as a standard.

^3H_2O and C-14 PEG were obtained from California Bionuclear Corp. [^{3}H]Triphenylmethyl phosphonium bromide was generously supplied by Dr H. R. Kaback, Roche Inst. Mol. Biol., Nutley, NJ. TPB and veratrine were obtained from Sigma Chemical Co.

3. Results

3.1. *Synaptosomal water volume*

The measurements of the intravesicular water volume gave a value of 4.02 ± 1.3 (n=19) μl/mg protein for the PEG impermeable space for synaptosomes suspended in Krebs-Henseleit Hepes medium at pH 7.4. This value is in agreement with the estimates in [1]. Extracellular fluid carried down with the synaptosomes during centrifugation through silicone oil (trapped space) averaged ~50% of the water volume of the pellet (cell suspensions of ~4 mg protein/ml).

3.2. *Transmembrane electrical potential*

TPMP equilibrates across synaptosomal membranes within 30 min; and the rate of equilibration is enhanced by addition of TPB. When TPB increased from 0–10 μM, the rate of TPMP (25 μM) accumulation progressively increased with no effect on the final equilibrium value (fig.1). TPMP uptake was measured using 7–25 μM extracellular TPMP. These results are shown in fig.2 and demonstrate that the calculated transmembrane electrical potential is independent of TPMP concentration in this range. Equilibration of

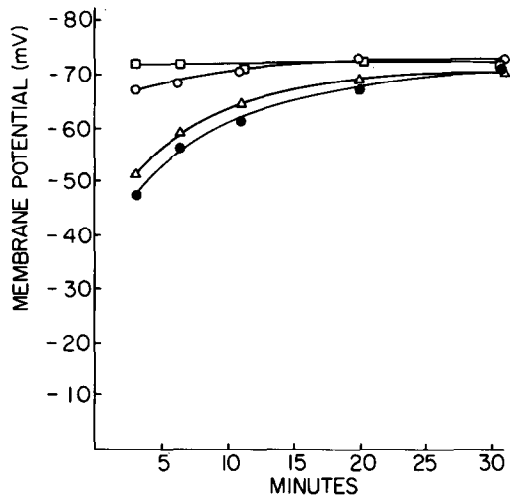

Fig.1. Concentration dependence of tetraphenylboron on the TPMP equilibration across the synaptosomal membrane. Synaptosomes were suspended at 4.2 mg/ml in Krebs-Henseleit–Hepes incubation medium at room temperature, the TPB added and then, 25 μM TPMP added. The TPB concentrations were 1 X 10^{-6} M (△), 5 X 10^{-6} M (○), 10 X 10^{-6} M (□). The filled circles represent the TPMP uptake in the absence of TPB. Each point represents duplicate samples.

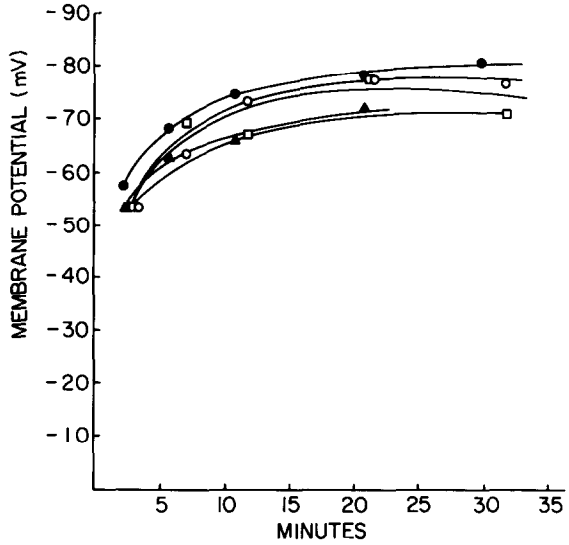

Fig.2. Dependence of the calculated membrane potential across synaptosomal membranes on TPMP concentration. Conditions are those described in fig.1. TPMP was 7 μM (●), 16 μM (▲), 18 μM (○) and 25 μM (□) and the TPB is 1 μM. Each point represents duplicate samples.

TPMP (10 μM) in the presence of 2 μM TPB, was complete within 10 min and the value of the transmembrane electrical potential calculated from the distribution of TPMP is −86.8 + 7.8 mV (n=11). Although this value includes bound TPMP (which must be assessed in order to determine the actual value of the membrane potential), it is in reasonably good agreement with the potassium diffusion potential of −72 mV calculated from potassium gradients measured by atomic absorption spectroscopy (C.D., unpublished).

Both the rate and the final steady-state level of TPMP accumulation were independent of extracellular pH over extracellular pH 6.8–7.8 (unpublished data). Addition of 50 mM K^+ to the synaptosomes suspended in Krebs-Henseleit–Hepes buffer caused a decrease in the TPMP accumulation of ~30% and the calculated membrane potential decreased by ~20 mV. Further addition of K^+ decreased the TPMP accumulation only slightly. It should be mentioned here that the synaptosomes accumulate potassium when the extracellular potassium concentration is raised, which decreases the amount of expected depolarization. Therefore, the expected decreases in TPMP accumulation are smaller, and the value of the calculated membrane potential is larger than that predicted on the basis of an increased extracellular [K^+] and a constant intracellular [K^+].

Both oxygen consumption and [ATP]/[ADP] ratios were essentially unchanged in synaptosomal suspensions upon addition of 2 μM TPB and 10 μM TPMP.

The distribution of a lipophilic anion, C-14 thiocyanate was also measured in this preparation. It was found that SCN was only slightly excluded from the synaptosomes (which suggests substantial binding of this anion) and that its accumulation increased somewhat upon the addition of K^+. The binding of SCN in conjunction with the relatively high proportion of trapped space in the synaptosomal pellet, make the distribution of this ion unsuitable for calculation of the membrane potential. We are currently searching for lipophilic anions which are bound less by the vesicles.

Figure 3 shows the effects of various pharmacologic agents on TPMP uptake. Incubation of the synaptosomes for ~10 min with ouabain (10^{-4} M) decreased the value of the calculated membrane potential by

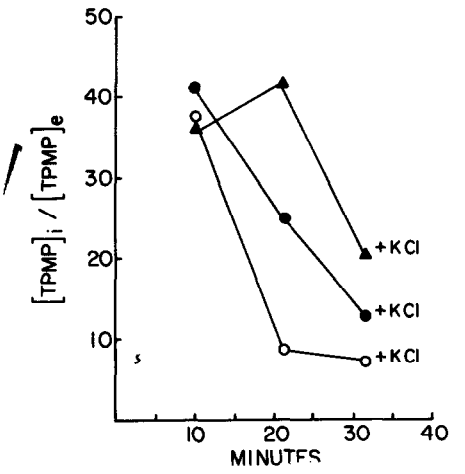

Fig.3. Effect of ouabain (10^{-4} M), veratrine (10^{-4} M) and TTX (10^{-6} M) on TPMP uptake by synaptosomes. Synaptosomes were suspended at 4 mg/ml in Krebs-Henseleit with 2 μM TPB and 25 μm TPMP. At 10 min, control samples were taken and ouabain (●), veratrine (○) and TTX plus veratrine (▲) added to 3 separate suspensions. After 10 min incubation, samples were taken and 50 mM KCl added to each of the residual suspensions. After an additional 10 min, samples were again taken. Each point represents duplicate samples.

~15 mV. This value was further decreased to ~−65 mV by 50 mM KCl. Veratrine, one of the veratrum alkaloids that increase sodium permeability of the plasma membrane in various cell systems [23–25] including synaptosomes [1,26], was added to synaptosome suspensions at 10^{-4} M final conc., resulting in a large depolarization of ~40 mV. Subsequent addition of 50 mM KCl gave even greater depolarization by ~5–10 mV. The effect of veratrine on the accumulation of TPMP was totally blocked by 10 μM tetrodotoxin (TTX); yet, this TTX-plus veratrine-treated synaptosome suspension could be induced to exclude TPMP upon subsequent addition of 50 mM KCl.

4. Discussion

A study of the membrane potentials in synaptosomes using the voltage-sensitive fluorescent probe, 3,3′-dipentyl 2,2′-oxacarbocyanine has been conducted [1]. Our observations are in general agreement with these qualitative results; however, we have attempted

in our study to quantitate the membrane potential by using the technique of lipophilic ion distributions and the Nernst equation.

These results show that the distribution of the lipophilic cation, TPMP, faithfully reflects transmembrane electrical potential in suspensions of rat brain synaptosomes: TPMP is accumulated or excluded as would be predicted upon treatment of the synaptosomes with drugs or modified media which are known to hyperpolarize and depolarize the plasma membrane, respectively.

The permeability of the synaptosome membrane to TPMP is similar to that observed in other nervous tissue [27], equilibration of the probe requiring ~25–30 min. The rate of TPMP accumulation is substantially increased upon the addition of TPB. TPB (at μM levels) has been shown to increase the rate of uptake of lipophilic cations into erythrocytes [28], lymphocytes [28] and neuroblastoma [27]. Furthermore, a decrease of the internal dipole potential of lipid bilayers has been shown with 10^{-7} M TPB, enhancing cation permeation by one order of magnitude [29]. Our results are consistent with this mechanism of catalytic action since as fig.1 shows, upon increasing TPB from 0–10 μM the rate of TPMP uptake increased but the final accumulation value for the distributions was not altered. The final accumulation corresponds to a transmembrane potential of -86.8 ± 7.8 mV. This value is calculated from total (free intracellular and bound) cell TPMP and therefore represents a maximum potential difference and is consistent with the values obtained with microelectrodes in mammalian central nervous system neurons [30,31]. Moreover it is only slightly (~16 mV) greater than the Nernst potential assuming free diffusion of K^+ (unpublished). The distribution of TPMP gives a calculated electrical gradient which is independent of external pH. This implies that the membrane potential itself is unaltered by pH since TPMP accumulation appears to respond to changes in synaptosome potential and that the changes in cell associated TPMP are not a function of surface charge–pH-dependent binding, but reflect changes in electrical field.

The accuracy of the calculated value of –86.8 mV for the transmembrane potential in isolated synaptosomes is contingent upon assessing the heterogeneity of the synaptosomal vesicles, the extent of binding of TPMP and the size and properties of intrasynaptosomal compartments. Binding of TPMP results in an increased apparent intracellular TPMP concentration and therefore a larger calculated negative potential. Although there are as yet no suitable methods for directly measuring binding, an indirect assessment was made in cultured neuroblastoma [27] by assuming that voltage-independent binding of TPMP was responsible for its presence in cells suspended in high K^+ medium (depolarized). Introducing this binding correction, gave a calculated value of membrane potential which was in excellent agreement with that obtained from microelectrode measurements. Similar assumptions for the synaptosomes, i.e., that veratrine + K^+ cause complete depolarization of the synaptosomal membranes, would lower the calculated transmembrane electrical potential by 5 mV. With respect to heterogeneity, if as much as 50% of the vesicle population is composed of a non-synaptosomal or damaged vesicle-fraction of equal water volume and possessing no membrane potential, then the calculated membrane potential of the intact synaptosomes would be –104 mV. This indicates that vesicle heterogeneity does not appear to introduce a very large discrepancy in our calculated value even for the afore-mentioned theoretical extreme case and is certainly not a problem in our studies since electron microscopic examination of our preparation shows that $\geqslant 75\%$ of the synaptosomes contain the characteristic vesicles associated with neurotransmitter storage and release. (I. A. Silver, unpublished). (The presence of a non-synaptosomal vesicle fraction with a membrane potential > -86 mV is highly unlikely and this case is not discussed here.)

The consequences of subcellular compartments with significant membrane potentials have been discussed in [27] and are under further evaluation for synaptosomes.

Ouabain (10^{-4} M) decreases the TPMP accumulation to an extent that is equivalent to a 15 mV depolarization. This observation implies that the ion gradients are maintained by energy-dependent processes and is in agreement with the studies [1] which show the responses of synaptosomes to ouabain with fluorescent dyes.

In nerve cells, the plasma membrane can be depolarized via two mechanisms: an increase in sodium permeability and a decrease in the potassium

concentration gradient. Veratrum alkaloids have been shown to increase sodium permeability in synaptosomes [1] as well as to induce Ca^{2+} uptake [32], release of neurotransmitter [32] and release of ATP [33,34] in synaptosomes. Our results are consistent with veratrum alkaloid-induced increase in sodium permeability in that they show a depolarization of the plasma membrane upon exposure to veratrine which is completely blocked in the presence of TTX. (TTX blocks sodium channels in nervous tissue and appears thereby to prevent any veratrine-induced alteration of membrane permeability.) However, TTX does not block depolarization induced by the addition of potassium since decrease in the electrochemical gradient for potassium in the presence of TTX would still be expected to depolarize the membrane. Our observations are consistent with the latter prediction and the results in [1].

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