USE OF THE FLUORESCENT PROBE 1-ANILINONAPHTHALENE-8-SULFONATE TO STUDY THE SYNAPTOSOMAL TRANSMEMBRANE POTENTIAL

E. V. Nikushkin, I. M. Antonikov, and R. N. Glebov

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The transmembrane potential (TMP) of synaptosomes isolated from the rat cerebral cortex was studied with the aid of 1-anilinonaphthalene-8-sulfonate (ANS). Addition of valinomycin to the synaptosomes was accompanied by an increase in the intensity of fluorescence of the probe at 464 nm (λ_{exc} = 365 nm), which is interpreted as reflecting hyperpolarization of the synaptosomal membranes. A decrease in the K^+ gradient on the synaptosomal membrane (a decrease in TMP), achieved by preincubation of the synaptosomes with ouabain (1 mM) or an increase in the concentration of extrasynaptosomal K^+ from 5 to 20 mM appreciably reduced the effect of valinomycin. Valinomycin had no effect on synaptosomes exposed to osmotic shock. It is suggested that valinomycin-induced changes in the intensity of fluorescence of ANS be used as a test of preservation of the K^+ gradient (the presence of a TMP) by synaptosomal preparations, i.e., of the active state of the synaptosomes.

KEY WORDS: synaptosomes; valinomycin; transmembrane potential; fluorescent probe.

Synaptosomes preserve the metabolic, osmotic, secretory, and other properties of intact nerve endings [2], and also a transmembrane potential (TMP) which is close to the K^+-diffusion potential [4, 7, 9]. This last property, one of the most important features of tissue viability, can be used to assess the native state of synaptosomal preparations.

The small size of synaptosomes (under 1 μ in diameter [2]) does not allow the TMP of these particles to be recorded by a microelectrode method. The fluorescent probe method [4] has been used in recent years for this purpose and its adequacy has been demonstrated on the squid giant axon [5, 6]. The intensity of fluorescence (I_{fl}) of probes of the carbocyanine group has been shown to depend on the K^+ gradient on the membrane and on the velocity constant of permeability of the membrane to K^+ (P_{K+}) [4, 10]. With a potential-sensitive probe available, preservation of the K^+ gradient (the presence of a TMP) on the synaptosomal membrane can be tested most easily and adequately, without preliminary calibration of I_{fl} of the probe, which is somewhat laborious, by two methods: 1) adding a definite quantity of KCL to the synaptosomal preparation, which changes the $[K^+]_o/[K^+]_i$ ratio, lowers TMP, and causes a corresponding change in I_{fl} of the probe; 2) addition of a K^+-ionophore, such as valinomycin, to synaptosomes incubated in a medium with low K^+ concentration, which leads to an increase in P_{K+}, hyperpolarizes the membrane, and causes a change in I_{fl} of the probe.

However, the addition of KCl is accompanied by a change in the ionic and osmotic strength of the solution, and also by a change in the surface charge of the membrane, which may affect I_{fl} of the probe. The addition of KCl to synaptosomes which have not preserved the K^+ gradient and, consequently, the TMP on their membrane, but which have a considerable P_{K+} value, may lead to the appearance of a K^+-diffusion potential. In that case the change in I_{fl} of the probe will not be evidence of the native state of the resulting preparation. For that reason, the second alternative seems preferable as a test for the native state of synaptosomes.

The compound 1-anilinonaphthalene-8-sulfonate (ANS), previously used for this purpose on mitochondria and submitochondrial particles (SMP) [3], was used as the potential-sensitive probe in this investigation.

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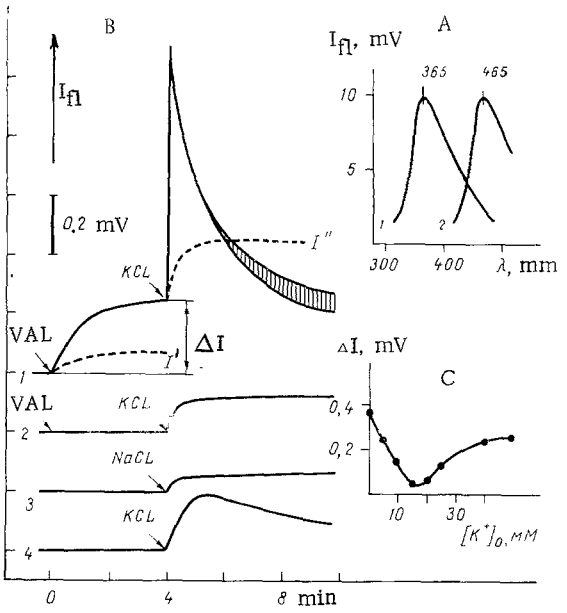

Fig. 1. Excitation (1) and fluorescence (2) spectra of ANS bound with synaptosomes (A); effect of valinomycin (val), K^+, and Na^+ on I_{fl} of ANS bound with synaptosomes suspended in Krebs–Ringer medium (B); and effect of extrasynaptosomal K^+ concentration on valinomycin-induced increase in I_{fl} of ANS (Δ_I) (C). Final concentrations of KCl and NaCl 30 mM. Ouabain added before ANS to the system (1'). Remainder of explanation in text.

EXPERIMENTAL METHOD

Measurements were made on a Hitachi-204 (Japan) spectrofluorometer, equipped with magnetic mixer. To increase the sensitivity of the instrument, an electrical compensator similar to that described in [1] was used. Fluorescence of ANS was excited at 365 nm and recorded at 464 nm (Fig. 1A).

Synaptosomes isolated from rat cerebral cortex by Hajos' method [8] were kept for not more than 3-4 h at 0-4°C in Krebs–Ringer solution (in mM): Tris-HCl 20, NaCl 132, KCl 5, NaH_2PO_4 1.2, $MgCl_2$ 1.3, $CaCl_2$ 1.2, glucose 10; pH 7.4. The protein concentration in the cuvette, determined by Lowry's method, was 0.2 mg/ml. ANS and valinomycin, from Sigma and Calbiochem (USA) respectively, were added in the form of an ethanol solution after preincubation of the synaptosomes for 5 min (for ANS) or 20 min (for valinomycin) at 30°C to a final concentration of ANS of 10^{-5} M, of valinomycin of $(2\text{-}10)\times10^{-8}$ M, and ethanol of 0.2%. The original signal measured 10 mV. Curves were drawn with allowance for the dilution effect.

EXPERIMENTAL RESULTS

The addition of valinomycin to the synaptosomes was accompanied by an increase in I_{fl} of ANS (Fig. 1B). It was observed previously on mitochondria and SMP that I_{fl} of ANS decreases with the appearance of K^+-diffusion potentials, if an excess of negative charges appears within these formations, and rises if an excess of positive charges appears [3]. The membrane of mitochondria and SMP is permeable for ANS, and a sharp decrease in the ANS concentration within the formations has a greater effect on I_{fl} of the suspension than the comparatively small increase externally [3]. Conversely, the membrane of the squid giant axon is impermeable to ANS [5]. Its depolarization was accompanied by a decrease in I_{fl} of ANS added externally, whereas hyperpolarization was accompanied by an increase [5]. Addition of valinomycin to the synaptosomes is bound to lead to an increase in P_{K^+} and, if the K^+ gradient is preserved on the synaptosomal membrane (K^+ is greater inside), TMP must approximate to the K^+-equilibrium potential, i.e., it must lead to hyperpolarization of the membrane.

As a result, for an excitable membrane (impermeable to ANS [5]) I_{fl} of ANS ought to increase, and this has been observed experimentally. The mechanism of potential-induced changes in I_{fl} of ANS has already been discussed previously [3].

Preincubation of the synaptosomes with ouabain (1 mM), leading to a marked decrease in the intrasynaptosomal K^+ concentration and to a decrease in TMP [4]), appreciably reduced the effect of valinomycin (Fig. 1B, curve 1). Addition of valinomycin to synaptosomes exposed to osmotic shock (in 10 volumes of distilled water followed by freezing and thawing), however, caused no change whatsoever in I_{fl} of ANS (Fig. 1B, curve 2). These observations confirm that the valinomycin-induced increase in I_{fl} of ANS can be used as a test of preservation of the K^+ gradient and TMP of synaptosomes, i.e., a test of the native state of these formations.

An increase in the K^+ concentration in the incubation medium from 5 to 20 mM also lowered TMP and was accompanied by diminution of the valinomycin-induced effect (Fig. 1C). However, an increase in $[K^+]_o$ to 40-50 mM led to an increase in the response of ANS, possibly due to an increase in the quantity of bound probe when the negative charges on the outer surface of the membrane were screened by potassium. The increase in I_{fl} of ANS in response to addition of KCl against the background of valinomycin can evidently be explained similarly, as is shown by the increase in I_{fl} of the probe on the addition of KCl to synaptosomes exposed to osmotic shock (Fig. 1B, curve 2), and on the addition of NaCl to native synaptosomes (Fig. 1B, curves 1 and 3). Addition of valinomycin, leading to hyperpolarization of the membrane and to the appearance of an excess of positive charges on its outer surface, may perhaps increase the concentration of bound probe or enable it to penetrate into more hydrophobic regions of the membrane. As a result, I_{fl} of the suspension increases, as also does the screening effect against the background of valinomycin. For instance, addition of NaCl after valinomycin caused a greater increase in I_{fl} of ANS than in the absence of the ionophore. The phase of the decrease in I_{fl} of ANS on the addition of KCl against the background of valinomycin to the original level (or slightly below it) was probably connected with depolarization of the membrane. It appeared also in the absence of valinomycin (Fig. 1B, curve 4). It should be noted that the influence of valinomycin on I_{fl} of ANS was unchanged for 3-4 h after isolation of the synaptosomes and their preservation at 0-4°C, after which this effect diminished.

The results are thus evidence that valinomycin-induced changes in the intensity of fluorescence of ANS can be used as a test for the native state of synaptosomes.

LITERATURE CITED

1. A. N. Bessonov, N. B. Gusev, et al., Vopr. Med. Khim., 20, 218 (1974).
2. R. N. Glebov and G. N. Kryzhanovskii, Functional Biochemistry of Synapses [in Russian], Moscow (1978).
3. V. P. Skulachev, Transformation of Energy in Biomembranes [in Russian], Moscow (1972).
4. M. P. Blaustein and J. M. Goldring, J. Physiol. (London), 247, 589 (1975).
5. L. B. Cohen, Physiol. Rev., 53, 373 (1973).
6. H. V. Davila, B. M. Salzberg, et al., Nature New Biol., 241, 152 (1973).
7. A. V. Escueta and S. H. Appel, Biochemistry (Washington), 8, 725 (1969).
8. F. Hajos, Brain Res., 93, 485 (1975).
9. P. Keen and T. D. White, J. Neurochem., 18, 1097 (1971).
10. P. J. Sims, A. S. Waggoner, et al., Biochemistry (Washington), 13, 3215 (1974).