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EFFECT OF NEUROTROPHIC COMPOUNDS ON TRANSMEMBRANE POTENTIAL OF SYNAPTOSOMES

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Besides the secretory, metabolic, osmotic, and other properties of intact nerve endings [2], synaptosomes also preserve a transmembrane potential (TMP) that is closely similar to the K^+-diffusion potential [5, 6]. The very existence of the TMP, its magnitude, sign, and its other characteristics are determined by several properties of nerve endings and processes taking place in them, and they are a reflection of these properties. In particular, the TMP is determined by membrane permeability for ions (its integrity, the chemicophysical state of the membrane components, functioning of the pores and channels, and so on), by the distribution of ions relative to the membrane (the existence of gradients), the work of ion pumps (in particular, Na,K-ATPase), and metabolic activity (ATP synthesis, and so on). At the same time, it is very probable that the TMP can control activity of membrane-dependent processes and can influence the structural and functional state of the membrane, which implies also the function of nerve endings. It is also possible that the action of various neurotropic agents and drugs is determined by their ability to influence the TMP of nerve endings.

In the investigation described below the properties of TMP of the synaptosomes and the action of the various neurotropic agents and drugs on TMP were investigated.

EXPERIMENTAL METHOD

Synaptosomes were isolated from the cerebral cortex of rat weighing 180-200 g by Hajos' method [7]. The synaptosomes thus obtained were suspended in Krebs–Ringer medium of the following composition (in mM): NaCl 132, KCl 5, NaH_2PO_4 1.2, $MgCl_2$ 1.3, $CaCl_2$ 1.2, glucose 10, Tris-HCl buffer 20, pH 7.6 (20°C). The synaptosomes were kept at 0°C for not more than 3-4 h. TMP of the synaptosomes was measured by the technique of fluorescent potential-sensitive probes. That this method was possible and suitable for recording and studying the properties of TMP of different cells and subcellular particles was demonstrated previously [8, 13]. Fluorometric measurements were made at 37°C on a "Hitachi-204" spectrofluorometer (Japan). Fluorescence of the 3,3-dipropylthiodicarbocyanine probe [diS-C_3-(5)] was excited

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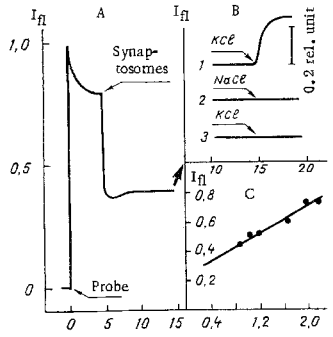

Fig. 1

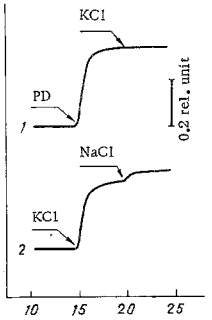

Fig. 2

Fig. 1. Investigation of properties of synaptosomal TMP with the aid of fluorescent probe diS-C_3-(5). A) Change in intensity of fluorescence (I_{fl}) of diS-C_3-(5) on addition of synaptosomes to Krebs–Ringer medium; B) effect of K^+ and Na^+ concentration in incubation medium on I_{fl} of diS-C_3-(5) in suspension of synaptosomes: 1, 2) native synaptosomes; 3) synaptosomes subjected to osmotic shock. $[K^+]_o$ and $[K+]_o$ increased to 100 mM; C) dependence of I_{fl} of diS-C_3-(5) in synaptosomal suspension on $[K^+]_o$ and $[Na^+]_o$. Abscissa: A, B) time (in min), C) log $\{[K]_o + 0.05\,[Na^+]_o\}$; ordinate, intensity of fluorescence of diS-C_3-(5) (in relative units).

Fig. 2. Effect of psychotropic drugs (PD) on intensity of fluorescence diS-C_3-(5) in suspension of synaptosomes. 1) native synaptosomes; 2) synaptosomes depolarized by KCl. Final concentrations of substances added: PD 10^{-4}–10^{-3} M, KCl 100 nM. Abscissa, time (in min); ordinate, I_{fl} (in relative units).

by light with a wavelength of 624 nm, and recorded at 674 nm at an angle of 90° to the excitation light. The sequence of the operations during the experiments was as follows: 10 µl of an ethanol solution (100%) of the probe was added to a cuvette containing 1 m of Krebs–Ringer medium to a final concentration of 5×10^{-7} M, and 5 min later, with constant mixing of the solution, when the fluroescence signal flattened out to a plateau (Fig. 1A), 50 µl of the suspension of synaptosomes was added to the cuvette up to a final concentration of 0.1-0.6 mg protein/ml. The protein concentration was determined by Lowry's method. After incubation for 10-15 min the suspension of synaptosomes was treated with 50-100 µl of 1 M KCl or NaCl solution made up in Krebs–Ringer medium, and 1-10 µl of ethanol solutions of the neurotrophic drugs chosen for study. The peptides and colchicine were added in the form of aqueous solutions.

EXPERIMENTAL RESULTS

Addition of the diS-C_3-(5) probe to the Krebs–Ringer medium caused the appearance of fluorescence in the 630-700 nm region. The intensity of fluorescence of the probe (I_{fl}) fell exponentially with time (adsorption of the probe on the cuvette walls [8]), to reach a practically stable level after 5-7 min (Fig. 1A). The addition of synaptosomes to the cuvette was accompanied by abrupt quenching of fluorescence (Fig. 1A), which can evidently be explained both by TMP-independent and by TMP-dependent adsorption of the probe on synaptosomes or inside these structures. Evidence in support of the existence of TMP-independent adsorption is given by the fact that quenching of I_{fl} also was observed on the addition of synaptosomes to incubation medium containing 100-130 mM K^+ (TMP = 0), and also on the addition of synaptosomes previously subjected to osmotic shock (TMP = 0). Evidence of the presence of TMP-dependent adsorption is given by the effect on the degree of quenching of I_{fl} observed on the addition of synaptosomes to the solution of the probe and of the K^+ concentration in the incubation medium ($[K^+]_o$), i.e., the value of TMP. The degree of quenching of I_{fl} was reduced with an increase in $[K^+]_o$ to 80-100 mM and remained unchanged with a further increase in $[K^+]_o$. Immediately after the abrupt quenching, I_{fl} of the probe remained virtually unchanged for quite a long time (up to 1 h). Addition of KCl to 50-100 mM (it was initially 5 mM) after incubation of the synaptosomes for 10-15 min led to an increase in I_{fl} of diS-C_3-(5) due to membrane depolarization and the outflow of probe, initially bound by synaptosomes (TMP-dependent desorption), into the incubation medium (Fig. 1B) [8, 13]. This was confirmed by the absence of any such effect on the addition of the corresponding quantity of NaCl to the cuvette and the absence of an increase in I_{fl} of the probe on the addition of KCl to synaptosomes previously subjected to osmotic shock (Fig. 1B). Characteristically I_{fl} of the probe

TABLE 1. Effect of Cytostatics, Peptides, and Psychotropic Drugs on TMP

Substance	C_a, M	Effect on I_{fl} of system: probe $C > C_a$	probe $C \leq C_a$ synaptosomes	probe $C \leq C_a$ synaptosomes	C_{ef}, M	C_M, M	Result of action on TMP
Cytocholasin B	10^{-3}	—	$+\Delta$	—	—	—	—
Colchicine	10^{-3}	—	—	—	—	—	—
Leu-enkephalin	10^{-4}	—	—	—	—	—	—
Met-enkephalin	10^{-4}	—	—	—	—	—	—
Tuftsin	10^{-4}	—	—	—	—	—	—
Chlorpromazine	10^{-5}	$+\Delta$	0	$+\Delta$	10^{-4}	$5 \cdot 10^{-4}$	Depolarization
Trifluoperazine	10^{-5}	$+\Delta$	$+\Delta$	$+\Delta$	$3 \cdot 10^{-5}$	10^{-4}	»
Haloperidol	10^{-6}	$+\Delta$	0	$+\Delta$	10^{-4}	$5 \cdot 10^{-4}$	»
Imipramine	10^{-5}	$+\Delta$	$+\Delta$	$+\Delta$	$5 \cdot 10^{-5}$	$2 \cdot 10^{-4}$	»
Diazepam	10^{-4}	$-\Delta$	$+\Delta$	$+\Delta$	$5 \cdot 10^{-4}$	10^{-3}	»
Phenazepam	10^{-4}	$-\Delta$	$+\Delta$	$+\Delta$	$5 \cdot 10^{-4}$	10^{-3}	»

Legend. C) concentration of substance tested, C_a) concentration of substance at which no interaction took place with the probe, C_{ef} and C_M; concentrations of the substance at which it reduced the effect of addition of 100 mM KCl to the synaptosomes roughly by half, and prevented it. $+\Delta$) and $-\Delta$) increase and decrease in I_{fl} respectively.

rose with an increase in $[K^+]_o$ to 80-100 mM, after which it remained unchanged. Consequently, in these concentrations the K^+ gradient disappeared on the membrane, so that the intrasynaptosomal K^+ level ($[K^+]_i$) was 80-100 mM. This conclusion is in good agreement with data obtained by other workers [6].

Investigation of the effect of $[K^+]_o$ on I_{fl} of diS-C_3-(5) showed that it is described by a linear function within coordinates I_{fl} vs. log $\{[K^+]_o + 0.05[Na^+]_o\}$ (Fig. 1C). The coefficient $\alpha = P_{Na^+}/P_{K^+}$ is assumed equal to 0.05 for the basis of operation [6, 10]. The change in $[K^+]_o$ in these experiments was compensated by a corresponding change in $[Na^+]_o$, so that the ionic strength of the incubation medium of the synaptosomes remained unchanged. The result is evidence that synaptosomes isolated by Hajos' method [7] preserve TMP at a value close to the K^+-diffusion potential, and, given known values of $[K^+]_i$ and $[K^+]_o$, and considering that $P_{K^+} \gg P_{Na^+}$, TMP can be estimated by means of the Nernst equation:

$$-E_M = 2.3 \frac{RT}{F} \lg \frac{[K^+]_i}{[K^+]_o}.$$

On the basis of our results it follows that $[K^+]_i$ = 80-100 mM, and consequently the value of TMP of the synaptosomes at 37°C in medium with $[K^+]_o$ = 5 mM must be 70-80 mV, in agreement with data in the literature [6].

The results of the study of the effect of colchicine, cytochalasin B, met- and leu-enkephalins, tuftsin, chlorpromazine, trifluoperazine, haloperidol, imipramine, diazepam, and phenazepam on synaptosomal TMP are given in Table 1. To begin with the possibility of interaction of the probe with the given compound was studied in the absence of synaptosomes. It was found that diS-C_3-(5) virtually did not interact with colchicine, cytochalasin B, and the peptides and it appreciably modified I_{fl} when psychotropic drugs were added in concentrations of more than 10^{-5}-10^{-4} M: I_{fl} was increased by chlorpromazine, trifluoperazine, haloperidol, and imipramine and reduced by diazepam and phenazepam (Table 1).

Addition of colchicine and cytochalasin B, and also of the peptides in concentrations of up to 10^{-4}-10^{-3}M did not change I_{fl} of the probe in the presence of synaptosomes also (Table 1), i.e., these compounds did not affect TMP of synaptosomes during incubation (40-50 min); under these circumstances $[K^+]_o$ was 5-100 mM. It might be supposed that cytostatics (colchicine, which destroys the structure of the microtubules, and cytochalasin B, which destroys the structure of actin microfilaments) would damage the cytoskeletal reticulum which is associated with the inner surface of the axolemma and nerve ending membranes, and would modify the functioning of the ion-selective channels in the "denuded" part of the membrane. However, the results actually obtained are evidence that the functioning of at least the K^+- and Na^+-channels is unchanged by colchicine and cytochalasin B. Consequently, without discussing here the validity of the previous hypothesis that during excitation the cytoskeletal reticulum becomes "disassembled" [9, 11, 12], it can be tentatively suggested that this process evidently could not be "triggered" in the opposite direction. Addition of dibutyryl-cAMP (10^{-4} M) to a suspension of these formations, which ought to facilitate phosphorylation of the membrane proteins that participate in the structure of the ion-selective channels, and opening of the channels [1], likewise did not affect the synaptosomal TMP.

Cytochalasin B, in a concentration of over 4×10^{-4} M, caused an increase in the scatter of light by the synaptosomal suspension in the absence of the probe, and this can be taken as evidence of aggregation of these formations.

The peptides likewise did not affect synaptosomal TMP (Table 1). It can be tentatively suggested that receptors for leu- and met-enkephalins and tuftsin either were absent on the synaptosomal membranes or were blocked and thus could not exhibit their action on the synaptic membrane through TMP, or the mechanism of action of these compounds is not connected with their effect on structures and processes responsible for the existence of TMP on the nerve ending membrane.

Addition of psychotropic compounds in concentrations of up to 10^{-5}-10^{-4} M to the suspension of synaptosomes in the presence of the probe was accompanied by an increase in I_{f1} for trifluoperazine, imipramine, diazepam, and phenazepam (Table 1). These compounds evidently caused a decrease in TMP of the synaptosomes, i.e., their depolarization. The most marked increase in I_{f1} of the probe was observed on addition of the drugs in concentration of 10^{-4}-10^{-3} M. However, in these concentrations interaction of the probe with the tested drug ought to take place (Table 1), and for that reason accurate measurements of changes in TMP induced by the psychotropic drugs were impossible. Only the qualitative effect of the pharmacological agent on synaptosomal TMP could be estimated. For this purpose the concentrations of agents in which their addition to the synaptosomes either sharply depressed the reaction of the probe (increase in I_{f1}) to a rise of $[K^+]_o$ to 50-100 mM (i.e., depolarization), or prevented this effect altogether (Fig. 2). Of course this was possible only if the compound tested had a depolarizing action. Additionally, the following experiment was undertaken: Synaptosomes were depolarized by increasing $[K^+]_o$ to 100 mM, after which the compound for testing was added to it in a concentration completely preventing the effect of KCl (Fig. 2). If the increase in I_{f1} of the probe observed in response to addition of the drug was due to its depolarizing action on synaptosomes, against the background of 100 mM $[K^+]_o$, it (the increase in I_{f1}) ought to be appreciable reduced (Fig. 2). As a result of analysis of this kind it was shown that the neuroleptics chlorpromazine, trifluoperazine, and haloperidol, and the antidepressant imipramine, in a concentration of about 10^{-4} M or more, and also the tranquilizers diazepam and phenazepam in a concentration of 10^{-3} M or more, completely depolarized the synaptosomes (Table 1).

The depolarizing action of chlorpromazine was demonstrated previously on the squid axon and frog heart muscle [3]. It has been suggested that many drugs with inhibitory properties act exclusively or mainly on the inward Na^+ current [3], and that this is possibly connected with their inhibitory effect on Na,K-ATPase [4]. The above-mentioned effect for chlorpromazine (5×10^{-5} M), trifluoperazine (2×10^{-5} M), diazepam (5×10^{-3} M) and the other substances is evidently effected through the action of the drug on "flowability" of the membrane lipids [4]. Very probably the decrease in TMP found in the present experiments is also connected with an increase in viscosity of the synaptic membrane lipids under the influence of the drug, with the consequent inhibition of Na,K-ATPase.

The use of the potential-sensitive probe diS-C_3-(5) thus showed that synaptosomes, isolated by Hajos' method, retain a TMP close to the K^+-diffusion potential, and equal to 70-80 mV. Chlorpromazine, trifluoperazine, haloperidol, imipramine, diazepam, and phenazepam, in high concentrations (10^{-4}-10^{-3} M) depolarize isolated nerve endings.

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MICROCALORIMETRIC STUDY OF ERYTHROCYTE MEMBRANE SUSPENSION FROM NORMAL SUBJECTS AND PATIENTS WITH ESSENTIAL HYPERTENSION

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It has recently been shown both in patients with essential hypertension [7] and in the experimental model of this disease, namely rats with spontaneous genetic hypertension [1, 6], disturbances of the functions of the erythrocyte membrane (its permeability for monovalent cations, binding and ATP-dependent transport of calcium) are based on a substantial disturbance of its structure. One line of further research into this problem is the discovery of the components of the membrane that are responsible for the membrane defect described above.

It was accordingly decided to study erythrocyte membranes from patients with essential hypertension by means of differential scanning microcalorimetry. Blood from patients with a clinically established diagnosis of essential hypertension, stage III-II according to the WHO classification (six men and five women, aged 30-56 years, BP 160-190/100-120 mm Hg) and blood from normotensive patients (six men and seven women aged 30-50 years, BP 110-130/70-90 mm Hg) were used.

The method of obtaining erythrocyte ghosts was described previously [2]. The total protein concentration was determined by Lowry's method [5]. In every case 5 mM Na-phosphate buffer, pH 7.4, was used as the solvent. The protein concentration in the suspension of erythrocyte ghosts was 3-5 mg/ml. Microcalorimetric investigations were carried out on a DASM-1M high-sensitivity differential scanning instrument (Special Design Office, Biochemical Center, Academy of Sciences of the USSR), the design and principle of operation of which have been fully described previously [8]. The working volume of the measuring cell was 1 ml. The rate of heating was 1°C/min. The sensitivity of the calorimeter for heat capacity at this rate of heating was 5×10^{-5} J/°C. To obtain a base line, both calorimetric cells were filled with solvent. High reproducibility of the base line enabled the specific heat of the membranes to be recorded with an error of not more than 5%. The accuracy of temperature recording was ±0.1°C.

A microcalorimetric recording of the change in excess heat capacity of the heated human erythrocyte membrane suspension (normal) in a protein concentration of 4.55 mg/ml is given in Fig. 1. The heat uptake contour of the suspension of erythrocyte ghosts is complex (multistage) in appearance. Some workers have made a detailed study of the five most clearly defined thermoinduced transitions in erythrocyte membranes [3, 4, 9, 10]. The elementary components of the contour which we distinguished in a pure form are named in accordance with the terminology suggested by the authors cited above, allowing for the thermal irreversibility of each transition.

The procedure of obtaining elementary contours was as follows. The suspension was first heated to 45°C, which is close to the temperature maximum of the first presumptive elementary transition (A-transition). The suspension was then cooled to 10°C (the temperature at the beginning of heating), after which the membranes were heated at the same rate to 51°C — a

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