and −0.492) was found between the ability of the animals to respond correctly to the conditioned stimulus and the shift of the L:R ratio toward an increase. It was also found that animals with left-sided asymmetry of most of the free amino acids determined predominated in the group of untrained rats (Fig. 2).

Asymmetry of distribution of the free amino acid pool was thus demonstrated in the brain of these animals, and it probably makes a definite contribution to their learning ability.

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EFFECT OF THE CALCIUM IONOPHORE A23187 ON PLASMA AND MITOCHONDRIAL POTENTIALS OF RAT BRAIN SYNAPTOSOMES: FLUORESCENCE STUDY

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The use of isotopic methods has shown that A23187, in the presence of Ca^{++}, lowers the potential of intrasynaptosomal mitochondria [5] and induces slow depolarization of the synaptosomal plasma membrane [5, 10]. Meanwhile Bartschat and Blaustein [6], using the potential-sensitive fluorescent probe diS-C_3-(5), obtained hyperpolarization of the synaptosomal plasma membrane by the addition of Ca^{++} in the presence of A23187. These workers linked hyperpolarization with activation of Ca-dependent K channels. Several investigations [1, 3, 4, 9] on different objects, including synaptosomes [4, 9], have now shown that positively charged voltage-sensitive probes respond to changes in both plasma potential and intracellular mitochondrial potential. In this connection the problem of whether hyperpolarization of the plasma membrane during the action of pharmacologic agents (A23187, valinomycin), simultaneously inducing mitochondrial depolarization also, can be recorded with the aid of diS-C_3-(5), is not yet clear. The aim of this investigation was to study, with the aid of diS-C_3-(5), the effect of A23187 and of Ca^{++} on the synaptosomal plasma and mitochondrial potentials and to evaluate the possibility of using this method to study the Ca-activated K channel in synaptosomes.

EXPERIMENTAL METHOD

Synaptosomes were isolated from the rat cerebral cortex [8] and suspended in modified Krebs–Ringer medium of the following composition (in mM): NaCl 132, KCl 5, NaH_2PO_4 5, glucose 10, $MgSO_4$ 1.3, HEPES 20, pH 7.4 (at the temperature of the measurements). The fluorometric measurements were made on a "Hitachi MPF-4" spectrofluorometer. Fluorescence of the probe was excited by light with wavelength 650 nm and was recorded at 670 nm. Except where

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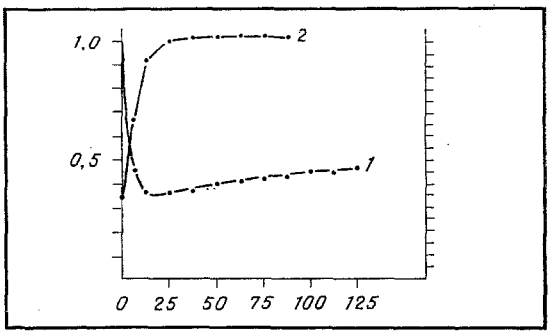

Fig. 1. Fluorometric titration of diS-C_3-(5) probe with synaptosomes. Abscissa, concentration of synaptosomes, mg protein/ml; ordinate: on left I_{670} at 670 nm (1), relative units; on right, I_{688}/I_{670} at 688 and 670 nm (2). Concentration of diS-C_3-(5) 1.25 μM. Temperature 37°C. Averaged results of 4-5 experiments shown.

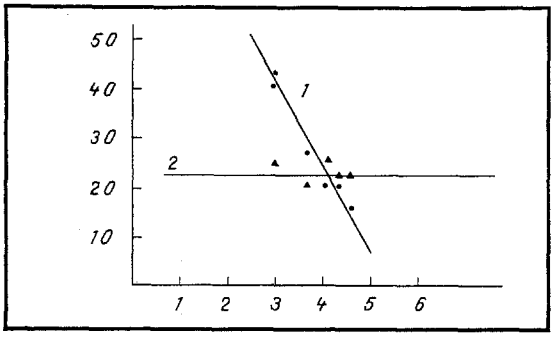

Fig. 2. Dependence of quenching of fluorescence of probe on K^+ concentration in solution. Abscissa: $\ln([K^+]_0 + 0.05[Na^+])_0$ indicates concentration of ions (in mM); ordinate, quenching of fluorescence on addition of synaptosomes, conventional units. 1) Native synaptosomes; 2) synaptosomes subjected to osmotic shock. Concentration of diS-C_3-(5) 1 μM. Temperature 30°C.

stated to the contrary, the experiments were in accordance with the following scheme: a suspension of synaptosomes was added in a volume of 20-30 μl to a constant-temperature cuvette containing 2 ml of Krebs–Ringer solution. The protein concentration was determined by Lowry's method. After incubation for 15-30 min at 37, 30, or 25°C, 3-5 μl of an ethanol solution of the probe was added to the cuvette up to a final concentration of 0.5 or 1-1.25 μM. An ethanol solution of A23187 was then added to a final concentration of 1.25 μM and of 1 mM $CaCl_2$. Qualitatively identical effects were observed at different temperatures and with different concentrations of probe and synaptosomes. Osmotically disintegrated synaptosomes were used in some experiments. For this purpose, the synaptosomes were suspended in distilled water with 5 mM Tris-HCl, pH 7.4 (25°C) and frozen (1 h, −20°C). After thawing and centrifugation (20,000g) the residue was suspended in Krebs–Ringer solution. The A23187 was obtained from "Calbiochem" (USA) and the oligomycin, valinomycin, and carbonylcyanide-m-chlorophenylhydrazone (CCCP) were obtained from "Serva" (West Germany).

EXPERIMENTAL RESULTS

The character of distribution of the probe among the membranes and aqueous medium and its redistribution in response to changes in transmembrane potentials were determined both by the properties of the probe itself and by the structure and features of the membranes. Voltage-dependent changes in fluorescence of the probe depend on its concentration in the fluorescent monomer form in the membranes. To optimize the conditions of the measurements, ti-

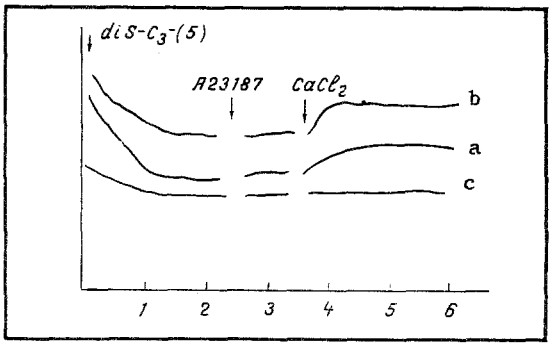

Fig. 3. Effect of A23187 and Ca^{++} on intensity of fluorescence of diS-C_3-(5). Abscissa, time, min; ordinate, I_{fl} of diS-C_3-(5). a) Incubation of synaptosomes in Krebs–Ringer solution, b) incubation in Krebs–Ringer solution with 75 mM KCl, c) preliminary incubation (15 min) with oligomycin. Arrows indicated time of addition of diS-C_3-(5) (final concentration 0.5 μM), of A23187 (1.25 μM), and of $CaCl_2$ (1 mM). Temperature 25°C.

tration curves were obtained of a solution of the probe (Fig. 1), reflecting, on the one hand, the distribution of the monomer form between the incubation medium and membranes, and on the other hand, the distribution of the probe between the monomer form and nonfluorescent aggregates [2]. The intensity of fluorescence (I_{fl}) decreased even after addition of small quantities of synaptosomes, and subsequent additions of the membranes led to ever-diminishing quenching of fluorescence, and later, the value of I_{fl} began to rise gradually. To reflect changes in the fluorescence spectrum of the probe, a convenient parameter is the ratio of intensities on the slope of the spectrum I_{688}/I_{670} [2] (Fig. 1, curve 2). The optimal concentration of monomers in the membranes for a concentration of probe of 1.25 μM is evidently achieved by synaptosomes in a concentration of 55-65 μg protein/ml. With this level of synaptosomes the contribution of aqueous monomers becomes negligibly small, since the shift of the spectrum does not take place (curve 2 in Fig. 1 flattens out on a plateau) and I_{fl} is proportional to the concentration of monomers in the membrane (the linear region of curve 1 in Fig. 1).

As a test of the native state of the synaptosomes thus obtained we used their ability to undergo potassium depolarization. To record K^+-depolarization, I_{fl} was measured in medium with different K^+ concentrations. Changes of ionic strength on account of an increase in KCl concentration were compensated by a corresponding decrease in the NaCl concentration. Dependence of I_{fl} on $\ln([K^+]_0 + (P_{Na}/P_K)[Na^+]_0)$, where P_{Na} and P_K denote membrane permeability for Na^+ and K^+, respectively, and the ratio between them is taken to be 0.05 [7], is illustrated in Fig. 2. The linear character of this dependence (see Fig. 2, curve 1) is evidence that the isolated synaptosomes preserve a plasma potential (PP) that is close to the K^+-diffusion potential. The value of PP is proportional to the coefficient of linearity (k). Thus, the synaptosomes obtained as described above preserved their PP, so that they could be used in the experiments.

On the addition of Ca^{++} to the suspension of synaptosomes in the presence of A23187 an increase in I_{fl} of the probe was observed (Fig. 3a). Replacement of NaCl in the solution by choline chloride or addition of the sodium channel blocker tetrodotoxin (10 μM) did not change the effect of A23187 and Ca^{++}. Consequently, the increase in I_{fl} was not caused by an increase in sodium conductance of the plasma membrane. In medium with a raised KCl concentration (75 mM), but with unchanged ionic strength, the effect of A23187 and of Ca^{++} was preserved (Fig. 3b). In the presence of oligomycin, a blocker of the mitochondrial H pump (1.25 μg/ml) of of the protonophore CCCP (1 μM) A23187 and Ca^{++} caused no change in I_{fl} (Fig. 3c). Valinomycin (0.1-1 μM, data not shown) had a similar action on I_{fl}, in agreement with results obtained by Heinonen and coworkers [9] in experiments on synaptosomes of the guinea pig brain [9]. The authors cited, and also Akerman [4], showed that valinomycin depolarizes intrasynaptosomal mitochondria.

It can be concluded from the foregoing facts that the increase in I_{fl} induced by A23187 and Ca^{++} reflects lowering of the mitochondrial potential, in agreement with results obtained by other methods [5].

The fact that our results do not agree with those obtained by Bartschat and Blaustein [6] can be explained on the grounds that they recorded hyperpolarization of the plasma membrane under conditions when the probe reacts mainly to changes in PP. To reproduce their results, we attempted experimentally to choose conditions (temperature, relations between probe and synaptosomes, time of addition of the agent) under which the sensitivity of the method to a change in mitochondrial potential would be low, but to a change of plasma potential, it would be high. However, when the magnitude of the response to valinomycin or to A23187 and Ca^{++} was lowered, the response to addition of KCl also was depressed. Thus, we were unable to record hyperpolarization of the plasma membrane due to activity of the Ca-activated K channel. We consider that without the development of a quantitative model of the distribution of diS-C_3-(5), depending on the plasma and mitochondrial potentials, this probe can be used to advantage only in order to study the action of factors which modify only one of the potentials (for example, K^+-depolarization of the plasma membrane). So far as protonophores (CCCP, FCCP) are concerned, although they do not act on PP [3], their effect on fluorescence of the probe must be taken into account. Thus, CCCP quenches I_{fl} of the probe in Krebs–Ringer solution substantially and to a degree that depends on concentration (data not given).

It can thus be concluded from these results that A23187 and Ca^{++} induce depolarization of intrasynaptosomal mitochondria and that diS-C_3-(5) cannot be used to study the Ca-activated K channel until a quantitative model of dependence of I_{fl} of the probe on the plasma and mitochondrial potentials is available.

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