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EFFECT OF GLUCOSE AND PYRUVATE METABOLISM ON MEMBRANE POTENTIAL IN SYNAPTOSOMES

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Fluorescence changes of rhodamine 6G in synaptosomal suspension, which are correlated to changes in membrane potential in synaptosomes, were measured in the presence of various monosaccharides and organic acids. Addition of D-glucose, D-mannose, pyruvate and L-lactate hyperpolarized the membrane potential, whereas D-fructose, L-glucose, D-galactose, citrate, succinate and L-glutamate were without effect on the membrane potential. Hyperpolarization induced by D-glucose was inhibited by cytochalasin B, phloretin, iodoacetate, F^- and 2-deoxy-D-glucose, but not inhibited by oligomycin or phlorizin. On the other hand, hyperpolarization induced by pyruvate was inhibited by α-cyanocinnamate or phloretin, but not inhibited by cytochalasin B or F^-. Elimination of Na^+ in physiological saline depressed hyperpolarization of membrane potential induced by addition of D-glucose, L-lactate or pyruvate. These results suggest that the activity of ($Na^+ + K^+$)-ATPase in plasma membranes of synaptosomes is increased by ATP formed by glycolysis, and that the accumulated K^+ in synaptosomes hyperpolarizes the membrane potential.

Introduction

The nerve ending particles (synaptosomes) isolated from brains have been investigated as an in vitro system for the study of various functions of synapses [1]. The membrane potential, which is a function of K^+ concentration across the plasma membranes, is thought to be important for various synaptic functions. The K^+ concentration in synaptosomes (synapses) is kept constant by the function of plasma membrane ($Na^+ + K^+$)-dependent ATPase, whose activity is controlled with ATP in cytosol. It is known that ATP content and K^+ concentration in synaptosomes increased in the presence of glucose [1,2], probably because of the functions of mitochondria and the enzymes of glycolysis. We have previously developed the procedure to estimate the membrane potential in synaptosomes by measuring the fluorescence of rhodamine 6G in synaptosomal suspensions and proved using this procedure that glucose hyperpolarizes membrane potential in synaptosomes [3]. The present study was undertaken to elucidate the relation between membrane potential and energy metabolism in synaptosomes. For this purpose, the membrane potential in synaptosomes was measured by adding various substrates of carbohydrate metabolism and inhibitors of the transport of glucose and pyruvate.

Materials and Methods

Solutions

Physiological salines used were: (1) high-K^+ medium containing 137 mM KCl and 20 mM Tris-HCl (pH 7.4); (2) normal K^+ medium con-

Abbreviation: DiS-C_3-(5); 3,3'-dipropylthiodicarbocyanine.

taining 132 mM NaCl, 5 mM KCl, and 20 mM Tris-HCl (pH 7.4). All solutions for these experiments were prepared with deionized water.

Preparation of synaptosomes

Synaptosomes were prepared from rat brains according to the method described previously [3]. The whole brains were homogenized in 0.32 M sucrose. The homogenate was centrifuged for 10 min at $1500 \times g$, and the resultant supernatant was centrifuged for 20 min at $12000 \times g$. The pellets (P_2) were resuspended in 0.32 M sucrose and layered on discontinuous density gradients consisting of 0.8 M and 1.2 M sucrose. Synaptosomes were obtained from the 0.8 M/1.2 M sucrose interface after centrifugation for 60 min at $74700 \times g$. In many experiments, the P_2 fraction (crude synaptosomes) was used.

Measurements of fluorescence

Fluorescence was measured with a Hitachi MPF-4 spectrofluorimeter at 25°C with an excitation wavelength of 520 nm and an emission wavelength of 550 nm. A given volume of fluorochrome solution was added to 2 or 3 ml of a saline in a fluorometer cuvette (300–800 nM final concentration), and initial fluorescence, F_i (i.e. due to dye alone), was measured. Then, 0.1 or 0.07 ml of synaptosomes (0.3–0.7 mg protein/ml final concentration) was added to the cuvette, and fluorescence in the steady state, F_s, was measured. In the following discussion, fluorescence, F, is defined as

$$F = F_s/F_i \tag{1}$$

where F_i and F_s stand for the fluorescence intensities of the dye in the absence and in the presence of synaptosomes, respectively.

Calculation of membrane potential from fluorescence

The relationship between the membrane potential, E, in synaptosomes and the fluorescence of rhodamine 6G in synaptosomal suspensions was expressed by the following equation (Eqn. 2) [3],

$$\frac{(F^{-1}-1)}{(F_k^{-1}-1)} = (RT/FE)[1-\exp(-FE/RT)] \tag{2}$$

where F is the fluorescence with synaptosomes in a given medium, F_k is the fluorescence with synaptosomes in high-K^+ medium, and R, T and F are usual thermodynamic significances. Fig. 1 shows the relation between the membrane potential in synaptosomes and the calculated value of right term of Eqn. 2. Membrane potential in the presence of various agents was determined by measuring the fluorescence of rhodamine 6G in a given (F) and in high-K^+ medium (F_k) under the same conditions as in Fig. 1.

Determination of lactate concentration

The crude synaptosomes (4–6 mg proteins in 0.25 ml) were incubated under various controlled conditions for 7 min at 27°C. The reaction was terminated by addition of cold perchloric acid (0.3 M final concentration). After centrifugation at $15000 \times g$ for 3 min, the concentration of lactate in the perchloric acid extracts was measured by the method of Gutmann and Wahlefeld [4].

Reagents

Rhodamine 6G(CI.45160) and α-cyanocinnamate were obtained from Tokyo Kasei Kogyo. Cytochalasin B, phloretin, phlorizin, 2-deoxy-D-glucose, 3-*O*-methyl-D-glucose, and oligomycin were from Sigma Chemical Co. DiS-C_3-(5) (3,3′-dipropylthiodicarbocyanine) was from Nippon Kankoh-Shikiso Kenkyusho Co., Ltd. (Shimoishii, Okayama-shi, 700 Japan). Solcoseryl was gift from Taiho Pharmaceutical Co., Ltd. (2-9 Kanda, Tsukasa-cho, Chiyoda-ku, Tokyo, 101 Japan).

Results

Some monosaccharides and organic acids, which are thought to be metabolyzed in synaptosomes, were added to synaptosomal suspension and the membrane potential in synaptosome was determined using the graph show in Fig. 1 (Table I). As seen from Table I, D-glucose, D-mannose, pyruvate and L-lactate made the membrane potential more negative (hyperpolarization). Solcoseryl, which is a drug to activate respiration of some tissue [5–7], also hyperpolarized the membrane potential. On the other hand, D-galactose, D-fructose, L-glutamate, ATP, succinate and citrate did not decrease the membrane potential. Also, no significant change was observed by addition of

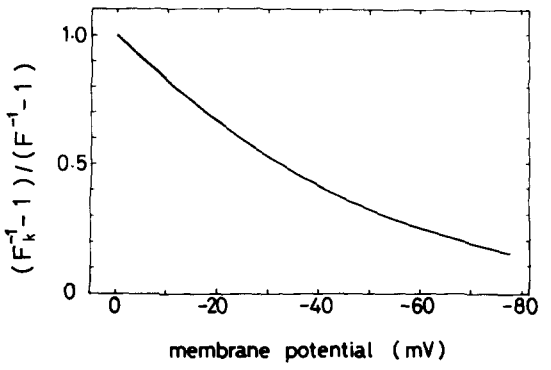

Fig. 1. Values of $(F_k^{-1}-1)/(F^{-1}-1)$ vs. membrane potential (E) were plotted according to the Eqn. 2. Membrane potential was determined by measuring the fluorescence of rhodamine 6G in a given medium (F) and in high-K^+ medium (F_k).

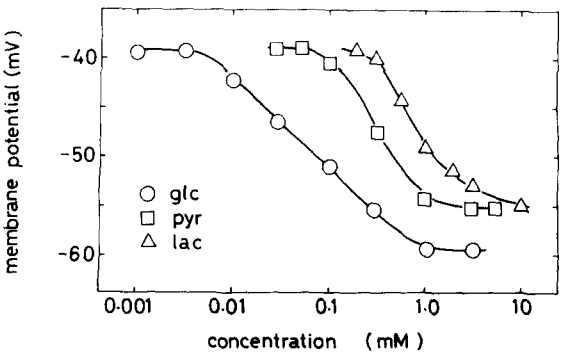

Fig. 2. The relation between membrane potential (E) and the concentration of D-glucose (○), pyruvate (□) and L-lactate (△).

D-ribose, D-maltose, L-glucose, 2-deoxy-D-glucose, 3-*O*-methyl-D-glucose, D-fucose, malonate, maleate, D,L-malate, glucose 6-phosphate or NADH (results were not shown). The same results as described above were obtained when crude synaptosomes (P_2 fraction) were used instead of purified synaptosomes.

Fig. 2 shows the relation between membrane potential changes and the concentrations of D-glucose, pyruvate, and L-lactate. As seen from the figure,glucose is the best substrate for the generation of membrane potential. The curve of mem-

TABLE I

EFFECT OF VARIOUS SUBSTRATES OF SUGAR METABOLISM, ATP, AND SOLCOSERYL ON RHODAMINE 6G FLUORESCENCE IN NORMAL-K^+ MEDIUM (F) AND IN HIGH-K^+ MEDIUM (F_k) WITH SYNAPTOSOMES

Membrane potential (E) was determined using the graph shown in Fig. 1 as described in Material and Methods. The fluorescence values were averages from five measurements and have standard deviations of ± 0.01. Each membrane potential has a standard deviation of ± 3 mV. The concentrations of synaptosomal proteins and rhodamine 6G were 0.5 ± 0.8 mg protein/ml and 400–600 nM, respectively.

Chemical	F	F_k	E (mV)
Buffer only	0.39	0.60	−39
+ D-glucose (1 mM)	0.28	0.59	−58
+ D-mannose (1 mM)	0.28	0.59	−58
+ D-fructose (1 mM)	0.39	0.60	−39
+ D-galactose (1 mM)	0.39	0.60	−39
+ pyruvate (3 mM)	0.27	0.56	−54
+ L-lactate (10 mM)	0.30	0.59	−53
+ citrate (1 mM)	0.38	0.59	−40
+ L-glutamate (1 mM)	0.38	0.59	−40
+ succinate (1 mM)	0.39	0.60	−39
+ ATP (1 mM)	0.39	0.60	−39
+ solcoseryl (0.05 ml)	0.30	0.58	−52

TABLE II

EFFECT OF VARIOUS CHEMICALS ON RHODAMINE 6G FLUORESCENCE IN NORMAL-K^+ MEDIUM (F) AND IN HIGH-K^+ MEDIUM (F_k) CONTAINING SYNAPTOSOMES AND 1 mM D-GLUCOSE

Membrane potential (E) was determined using the graph shown in Fig. 1. The fluorescence values were averages from five measurements and ave standard deviations of ± 0.01. Each membrane potential has a standard deviation of ± 3 mV. The concentrations of synaptosomal proteins and rhodamine 6G were 0.5–0.8 mg protein/ml and 400–600 nM, respectively.

Chemical	F	F_k	E (mV)
buffer with 1 mM D-glucose	0.28	0.59	−58
(without 1 mM D-glucose	0.39	0.60	−39)
+ cytochalasin B (0.001 mM)	0.38	0.60	−41
+ phloretin (0.025 mM)	0.41	0.63	−41
+ phlorizin (0.1 mM)	0.29	0.59	−57
+ ICH_2COOH (0.5 mM)	0.38	0.62	−42
+ 2-deoxy-D-glucose (10 mM)	0.37	0.60	−42
+ 3-*O*-methyl-D-glucose (10 mM)	0.28	0.59	−58
+ NaF (20 mM)	0.39	0.60	−39
+ α-cyanocinnamate (0.01 mM)	0.41	0.63	−41
+ DiS-C_3-(5) (75 ng/ml)	0.38	0.60	−41
+ oligomycin (0.004 mg/ml)	0.33	0.63	−55
low O_2 buffer (15% saturation)	0.28	0.59	−58

brane potential changes against the concentration of mannose was the same as that of glucose. The concentrations of substrate giving half maximum changes in membrane potential were 0.1 mM for glucose, 0.3 mM for pyruvate and 1.0 mM for lactate.

The membrane potential in synaptosomes was next measured by addition of glucose in the presence of some chemicals, which affect transport and metabolism of glucose (Table II). Phloretin, phlorizin and cytochalasin B are inhibitors of sugar transport [8–12]. Phloretin and cytochalasin B inhibited the hyperpolarization by glucose, whereas phlorizin was without effect. Both NaF and iodoacetate, inhibitors for glycolysis, inhibited hyperpolarization by glucose. DiS-C_3-(5), which is known as a fluorescent dye capable of measuring membrane potential of many cells and organella [13–16], inhibited the decrease of membrane potential in synaptosomes. D-Glucose hyperpolarized membrane potential in the medium with low O_2 or with oligomycin, where oxidative phosphorilation of mitochondria in synaptosomes were moderately inhibited. α-Cyanocinnamate, a potent inhibitor of pyruvate transport in mitochondria [17,18], inhibited the hyperpolarization.

3-*O*-Methylglucose and 2-deoxyglucose are penetrated into synaptosomes by the glucose transporters [19]. Therefore, the amount of glucose transported into cytosol was expected to decrease in the presence of these derivatives of glucose. To examine this possibility, membrane potential was measured with varying glucose concentration in the presence of 10 mM these derivatives (Fig. 3).

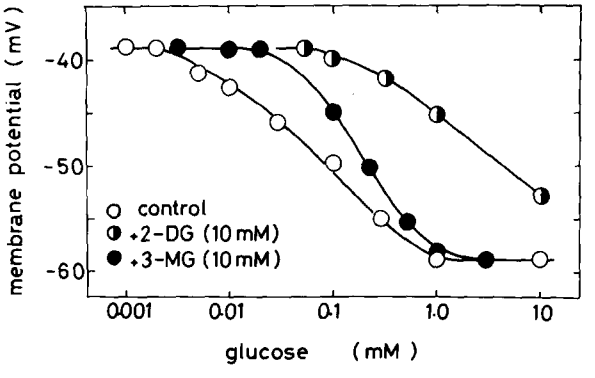

Fig. 3. Effect of 3-*O*-methyl-D-glucose (10 mM) and 2-deoxy-L-glucose (10 mM) on hyperpolarization of the membrane potential induced by glucose.

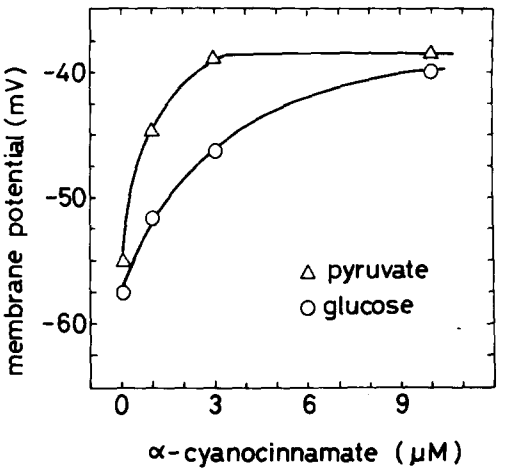

Fig. 4. Membrane potential (E) changes induced by glucose (○) or pyruvate (△) were plotted as a function of α-cyanocinnamate concentration.

As seen from the figure, 3-*O*-methyl-D-glucose inhibited hyperpolarization at low concentration of glucose but 2-deoxyglucose inhibited it at high concentration of glucose. This indicates that 3-*O*-methylglucose is a weak inhibitor of glucose transport but 2-deoxyglucose is a potent one.

α-Cyanocinnamate, an inhibitor of pyruvate transport in mitochondria, inhibited, hyperpolarization induced by pyruvate and by glucose as well (Fig. 4). Next, the effect of inhibitors of glucose transport on hyperpolarization induced by pyruvate was examined. NaF and cytochalasin B inhibited the hyperpolarization induced by glucose, but they did not inhibit the hyperpolarization induced by pyruvate (Fig. 5). Phloretin inhibited hyperpolarization induced by pyruvate and also inhibited that induced by glucose (Fig. 6). Therefore, the effect of phloretin seem to be similar to that of α-cyanocinnamate. Phlorizin did not inhibited hyperpolarization induced by glucose and pyruvate.

NaCl in normal-K^+ medium was replaced by several monovalent cations and the fluorescence was measured by addition of glucose, pyruvate and lactate, following estimation of membrane potential. As seen from Table III, hyperpolarization induced by glucose, pyruvate or lactate is largest in normal-K^+ medium, and replacement of NaCl in the medium by sucrose inhibited hyper-

TABLE III

EFFECT OF REPLACEMENT OF Na^+ BY SOME CATIONS AND SUCROSE IN THE MEDIUM ON HYPERPOLARIZATION INDUCED BY SUBSTRATES.

NaCl (132 mM) in normal-K^+ medium as replaced by each salt (chloride) or sucrose (300 mM). The concentration of D-glucose, pyruvate, and L-lactate was 1 mM, 3 mM, and 10 mM, respectively. Membrane potentials without substrate represent in the first column. Each value is a mean ± S.D. and the numbers of determinations represents in parentheses. The concentration of synaptosomal proteins and rhodamine 6G were 0.4–0.6 mg protein/ml and 500 nM, respectively.

Cation	Membrane potential (mV)	Hyperpolarization (mV)		
	Without substrate	With glucose	With pyruvate	With lactate
Na^+	−40 ± 2 (12)	−18 ± 3 (4)	−14 ± 2 (4)	−13 ± 2 (4)
$Choline^+$	−49 ± 3 (12)	−3 ± 3 (4)	−5 ± 2 (4)	−2 ± 2 (4)
Li^+	−45 ± 3 (12)	−6 ± 3 (4)	−6 ± 2 (4)	−2 ± 2 (4)
$Tris^+$	−46 ± 2 (12)	−1 ± 3 (4)	−3 ± 3 (4)	−2 ± 2 (4)
Sucrose	−50 ± 3 (12)	−1 ± 1 (4)	−1 ± 2 (4)	−1 ± 1 (4)

polarization. Li^+, $choline^+$ or $Tris^+$ is scarcely substituted for Na^+.

Velocity of the formation of lactate is known as an indicator of activity of glycolysis. Table IV shows the activity of lactate formation by crude synaptosomes (P_2) under various conditions. As seen from the table, addition of D-glucose and D-mannose increased lactate formation, but neither D-galactose or D-fructose had any effect (Table IV). Lactate formation from glucose was inhibited by 2-deoxy-D-glucose and cytochalasin B, but did not inhibited by 3-*O*-methyl-D-glucose. Taking in account the results in Table I, II and IV, we suggest that efficiency of lactate formation is correlated to that of changes in membrane potential in synaptosomes.

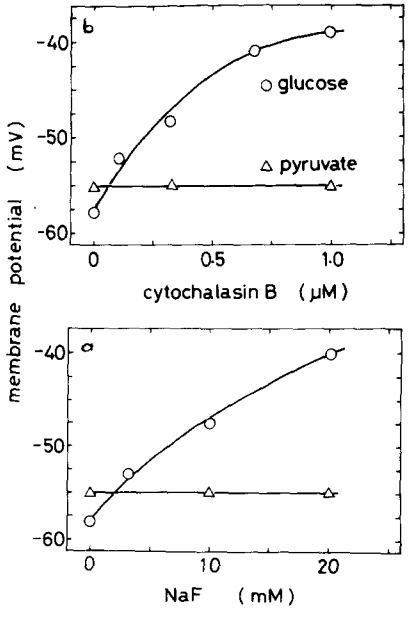

Fig. 5. Membrane potential (E) changes induced by glucose (○) or pyruvate (△) were plotted as a function of NaF (a) or cytochalasin B (b) concentration.

TABLE IV

FORMATION OF LACTATE UNDER VARIOUS CONDITIONS

Each value is a mean ± S.D. and the numbers in parentheses represents the number of determinations. Synaptosomes were incubated with various substrated and concentration of lactate was determined as described in Materials and Methods.

	Lactate production (mol/mg protein) ($\times 10^7$)
Without substrate	1.0 ± 0.1 (4)
+ 10 mM D-glucose	2.4 ± 0.2 (4)
+ 10 mM D-mannose	2.3 ± 0.2 (2)
+ 10 mM D-galactose	0.9 ± 0.1 (2)
+ 10 mM D-fructose	1.1 ± 0.2 (2)
+ 10 mM pyruvate	1.6 ± 0.2 (2)
+ 10 mM D-glucose, 50 mM 3-*O*-methyl-D-glucose	2.3 ± 0.3 (2)
+ 10 mM D-glucose, 50 mM 2-deoxy-D-glucose	1.5 ± 0.3 (2)
+ 10 mM D-glucose, 0.003 mM cytochalasin B	1.1 ± 0.2 (2)

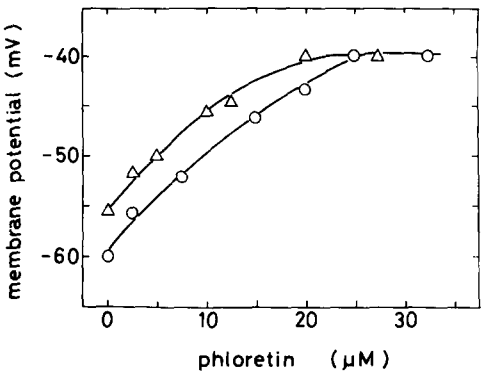

Fig. 6. Membrane potential (E) changes induced by glucose (○) or pyruvate (△) were plotted as a function of the phloretin concentration.

Discussion

The membrane potential of synaptosomes is a function of K^+ concentration in both cytosol and suspending medium [3,20]. Since synaptosomes are ordinarily prepared in medium with a low ionic strength at low temperature, the membrane potential may be somewhat depolarized from the normal value because of the lower K^+ content in synaptosome thus prepared. The results shown in Fig. 2 and Tables II and III suggest that membrane potential in synaptosomes reverted to the normal level by ATP which was formed from glucose, mannose, pyruvate and lactate. The effectiveness of substrates for generation of membrane potential was in the following order; D-glucose-D = mannose > pyruvate > lactate > glutamate = succinate. The order is different from that of O_2 uptake rate of synaptosomes: succinate > glutamate > pyruvate > D-glucose [21]. The result obtained above is consistent with the fact that D-glucose is a sole substrate of carbohydrate metabolism in brain in vivo under normal conditions and D-mannose is exchangeable for D-glucose.

Cytochalasin B inhibits sugar transport in blood red cells and in some organella whose transport system is a facilitated diffusion responding the concentration gradient across the membranes [9,10,12]. Phlorizin is a inhibitor for active transport, which require the Na^+ concentration difference across the membrane [11,12]. Hyperpolarization in the membrane potential in synaptosomes induced by glucose was inhibited by cytochalasin B but did not phlorizin (Table II). This result indicates that the glucose transport in synaptosomes was a facilitated diffusion depending on the glucose concentration across the membrane. Considering the fact that glucose enters into synaptosomes in the medium without Na^+[19] and phlorizin did not inhibit the hyperpolarization, Na^+ seems to be unnecessary for the glucose transport system. Table III shows that hyperpolarization requires Na^+ in external medium. Na^+ may penetrate cytosol independent of transport of substrate and activate ($Na^+ + K^+$)-ATPase in cytosol.

Fig. 3 shows that 2-deoxy-D-glucose inhibited the hyperpolarization induced by glucose more strongly than 3-*O*-methylglucose. It is, therefore, suggested that the affinity of glucose transporter for 2-deoxyglucose is stronger than that for 3-*O*-methylglucose. This view is compatible with the result that glucose inhibits the transport of 2-deoxyglucose more strongly than 3-*O*-methylglucose [19].

Hyperpolarization induced by glucose and pyruvate was inhibited by phloretin (Fig. 6). This suggests the possibility that phloretin is not only a inhibitor of the transport of glucose [19], but also a inhibitor of the transport or metabolism of pyruvate. Halestrap and Denton [18] reported that α-cyanocinnamate has no effect on glycolysis below 0.1 mM in adipose and heart tissue. The present study shows that α-cyanocinnamate inhibited hyperpolarization induced by glucose as well as by pyruvate above 0.01 mM (Fig. 4). It is not clear whether inhibition of glucose-mediated hyperpolarization by α-cyanocinnamate is attributed by the inhibition of pyruvate transport into mitochondria or by the direct inhibition of glucose metabolism in synaptosomes.

Since solcoseryl contained D-glucose (12 mM) and L-lactate (75 mM), about 80% of the membrane potential changes induced by solcoseryl may result from these two substrates. Solcoseryl may contain unknown activators and/or another substrate(s) of glucose metabolism other than those tested in this experiment, since the drug also increase the velocity of O_2 uptake of the synaptosomes (results not shown).

Hyperpolarization by glucose and pyruvate was also observed when the membrane potential in synaptosomes was measured with the tetraphenylphosphonium ion, which is used to measure the membrane potential in some cells [22,23]. However, the phenomenon of hyperpolarization of synaptosomes induced by glucose and pyruvate could not be observed when diS-C_3-(5) was used as a fluorescence dye (unpublished data). The fluorescence if diS-C_3-(5) was dependent on the K^+ concentration of media. As shown in Table II this dye inhibited the rhodamine 6G fluorescence at a lower concentration than that ordinarily used to measure the membrane potential. DiS-C_3-(5) may inhibit energy metabolism in synaptosomes. Further study of the effect of diS-C_3-(5) on the membrane potential measured with the tetraphenylphosphonium ion and on the energy metabolism in synaptosomes was required.

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