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FLUORESCENCE CHANGES OF RHODAMINE 6G ASSOCIATED WITH CHANGES IN MEMBRANE POTENTIAL IN SYNAPTOSOMES

TOSHIHIRO AIUCHI [a], TAKASHI DAIMATSU [b], KAZUYASU NAKAYA [a] and YASUHARU NAKAMURA [a]

[a] *Laboratory of Biological Chemistry, School of Pharmaceutical Sciences, Showa University, Hatanodai, Shinagawaku, Tokyo 142 and* [b] *Department of Nutrition, Tokyo University of Agriculture, Setagayaku, Tokyo 156 (Japan)*

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The intensity of rhodamine 6G fluorescence was found to be a useful scale for measuring the membrane potential in synaptosomes. The fluorescene of rhodamine 6G in synaptosomal suspensions increases with depolarization in the synaptosomes induced by the replacement of cations in the medium or by the addition of agents known to depolarize the membrane potential. Considering the character of the dye, we have derived an equation which gives the relation between the fluorescence intensity of the dye and the membrane potential. The change in membrane potential (diffusion potential) of synaptosomes was calculated using the equation. The calculated membrane potential was proportional to the logarithm of the K^+ concentration above 20 mM, and the slope of membrane potential against log[K^+] was about 52 mV per decade of concentration. The permeability ratio (P_X/P_K; the ratio of the permeability constants of a given cation, X^+, and K^+) was estimated from the calculated membrane potential.

Introduction

In squid axons, more than 300 dyes have been shown to alter their fluorescence and/or absorption in response to changes in membrane potential [1,2]. The membrane potential in red blood cells [3], synaptosomes [4] and Ehrlich ascites cells [5] has also been investigated using some of these dyes. The results of these investigations suggest that fluorescence may become a useful tool for monitoring the membrane potential in small cells, organelles, and vesicle preparations that cannot be penetrated or are damaged by penetrations by microelectrodes. In the previous paper [6], we reported the use of rhodamine 6G fluorescence to monitor a change in the membrane potential in *Tetrahymena* and have found that the changes in fluorescence intensity are associated closely with the chemotactic responses, which are considered to be induced by changes in membrane potential. We inferred that the change in membrane potential caused the change in the dye binding to the membrane phase and, thus, the change in the fluorescence.

The present study was undertaken to confirm whether changes in membrane potential in synaptosomes (presynaptic nerve ending preparations), which have a K^+ diffusion potential [4,7], lead to parallel changes in the fluorescent intensity of rhodamine 6G. For this purpose the fluorescence for rhodamine 6G in synaptosomal suspensions was measured with varying K^+ concentrations in the suspending medium and with the addition of agents which affect the permeability of synaptosomal membranes to ions. The present results indicate that various agents known to induce depolarization in membrane potential lead to an increase in fluorescence. Based on these results, we calculated the change in membrane potential of synaptosomes by using measurements of fluores-

cence under the assumption that the electric potential gradient within the membrane is constant.

Materials and Methods

Solutions

Physiological salines used were: (1) high K^+ medium containing 137 mM KCl, 20 mM Tris-HCl (pH 7.4) and 10 mM glucose; (2) low K^+ medium containing 132 mM NaCl, 5 mM KCl, 20 mM Tris-HCl (pH 7.4) and 10 mM glucose. In order to alter the K^+ concentration, these salines were mixed in different proportions. In some experiments salines without glucose were used. For the experiment to study the selectivity of the potassium channel of synaptosomes for monovalent cation, 132 mM of several chlorides replaced sodium chloride in low K^+ medium. All solutions for these experiments were prepared with deionised water.

Preparation of synaptosomes

Synaptosomes were prepared from rat brains according to the procedure of Gray and Whittaker [8]. For each experiment four or five adult rats were used. The whole brains were homogenized in a Potter-Elvejhem homogenizer in 10 vol. of 0.32 M sucrose adjusted to pH 7.4 with Tris-HCl. 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 were resuspended in 0.32 M sucrose (pH 7.4) and layered carefully on discontinuous density gradients consisting of 0.8 and 1.2 M sucrose. The gradients were centrifuged for 60 min at $74700 \times g$, and the synaptosomes were gently sucked off the 0.8–1.2 M sucrose interface. The synaptosomes were diluted with 4 vol. of a saline containing 70 mM NaCl, 2.5 mM KCl and 5 mM Tris-HCl (pH 7.4), and centrifuged for 5 min at $6000 \times g$. The resulting pellets were resuspended in low K^+ medium (6–16 mg synaptosomal protein/ml) to form the 'synaptosome suspension' which was used within 4 h of preparation.

Measurements of fluorescence

Fluorescence was measured with a Hitachi MPF-4 spectrofluorimeter at 27°C (an excitation wavelength of 520 nm and an emission wavelength of 550 nm). A given volume of fluorochrome solution was added to 3 ml of a saline in a fluorometer cuvette, and the initial fluorescence, f_i (i.e. due to dye alone), was measured. Then, 100 μl of synaptosome suspension (6–16 mg protein/ml) was added to the cuvette, and the fluorescence in the steady state, f_s, was measured. In the following discussion, the 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.

Reagents

Rhodamine 6G (CI-45160) was obtained from Tokyo Kasei Kogyo. Ouabain was from E. Merck. Veratrine was purchased from Sigma Chemical Co. All other materials were reagent grade obtained from commercial sources.

Results and Discussion

Fluorescence changes associated with change in K^+ concentration in medium

When synaptosome suspensions were added to salines containing 1.0 μM rhodamine 6G and various concentration of K^+, the fluorescence of the dye decreased markedly and reached respective minimum levels after 3–4 min (Fig. 1a). In Fig. 1b the fluorescence intensity at the steady level was plotted against the K^+ concentration in the medium. As seen in the figure, the fluorescence of synaptosomal suspension is increased from 0.23 to 0.58 as the concentration of K^+ is increased from 5 to 137 mM. In the absence of synaptosomes, on the contrary, no change in the fluorescence of the dye was observed with varying K^+ concentration. When the two fractions other than the synaptosomal fraction of the sucrose density gradient (see Preparation of synaptosomes) were added similarly to rhodamine 6G solutions, the fluorescence of the dye did not change appreciably with varying K^+ concentration in suspending medium. As inferred from the previous study on the interaction between rhodamine 6G and liposomes [6], the increase in the fluorescence of the dye is consid-

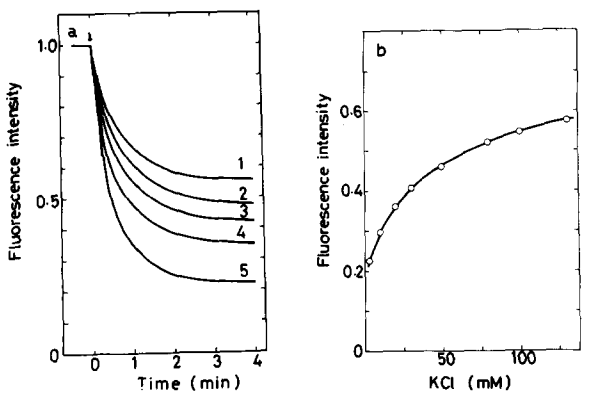

Fig. 1a. Effects of varying K^+ concentrations in the media on the fluorescence intensity of rhodamine 6G in synaptosomal suspensions. At the time indicated by the arrow, synaptosome suspensions (final concentration, 0.6 mg protein /ml) were added to various media containing 1 μM of the dye. Fluorescence was recorded at 550 nm with excitation at 520 nm. The concentration of K^+ in the media was altered by mixing low K^+ medium with high K^+ medium in different proportions (see Materials and Methods). The fluorescence intensity is given by Eqn. 1. 1, 137 mM KCl (high K^+ medium); 2, 80 mM KCl; 3, 50 mM KCl; 4, 20 mM KCl; 5, 5 mM KCl (low K^+ medium). 1b. The fluorescence intensity of the dye in synaptosomal suspension at the plateau level as shown in 1a plotted as a function of K^+ concentration.

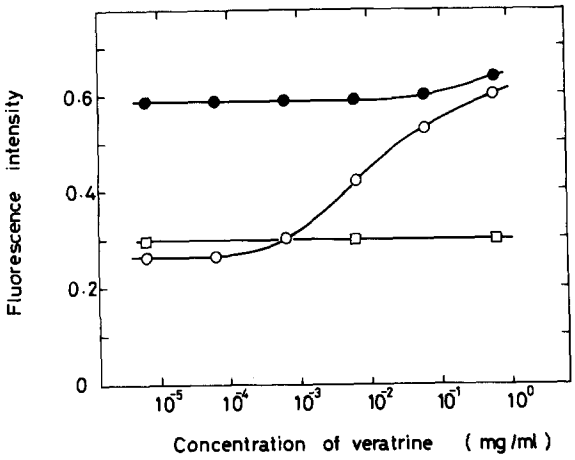

Fig. 2. Fluorescence intensity of rhodamine 6G in synaptosomal suspensions in various media was plotted as a function of veratrine concentration. Addition of veratrine (about 10^{-3} mg/ml) depressed the quenching of fluorescence in low K^+ medium (○). Veratrine did not influence the fluorescence in the high K^+ medium (●) or the choline medium (□), which was prepared by replacing Na^+ with choline$^+$ in the low K^+ medium. The concentrations of the dye and synaptosomal protein were 0.33 μM and 0.21 mg protein/ml, respectively.

ered to be associated with the depolarization of the membrane potential, which is induced by increasing of K^+ concentration. This interpretation is consistent with the view that synaptosomes have a membrane potential which approximates the K^+ diffusion potential, as is the case for most intact neurones [4,7].

Effect of various agents

The two physiological salines, low K^+ and high K^+ media, without glucose were used in the following experiments. Veratrine is known to activate the membrane's permeability by Na^+ and to depolarize the membrane potential [9,10]. In Fig. 2, fluorescence in the synaptosomal suspension is plotted against the concentration of veratrine. As seen in the figure, the fluorescence increased with increasing concentrations of veratrine in the low K^+ medium. In the high K^+ medium, on the contrary, the addition of veratrine did not affect the fluorescence. When Na^+ was replaced by choline$^+$ in the low K^+ medium, the fluorescence

TABLE I

EFFECT OF VARIOUS CHEMICALS ON FLUORESCENCE OF RHODAMINE 6G IN SYNAPTOSOMAL SUSPENSIONS

The values in the table are the averaged values of the data obtained with three or four measurements. Each value has a mean error of 0.02 in low K^+ medium and of 0.01 in high K^+ medium. The concentration of rhodamine 6G was 0.7 μM and that of synaptosomal protein was 0.4−0.5 mg/ml. The composition of low K^+ and high K^+ medium is given in Materials and Methods. CPC, cetylpyridinuim chloride.

Chemicals	Fluorescence	
	Low K^+ medium	High K^+ medium
Buffer only	0.30±0.02	0.58±0.01
+Glucose (1 mM)	0.25	0.58
+Succinate (1 mM)	0.30	0.58
+ATP (1 mM)	0.30	0.58
+Ouabain (1 mM)	0.46	0.59
+Veratrine (0.06 mg/ml)	0.53	0.59
+Triton X-100 (0.01%)	0.65	0.72
+CPC (0.1 mM)	0.75	0.80
Toluene-saturated buffer	0.64	0.68

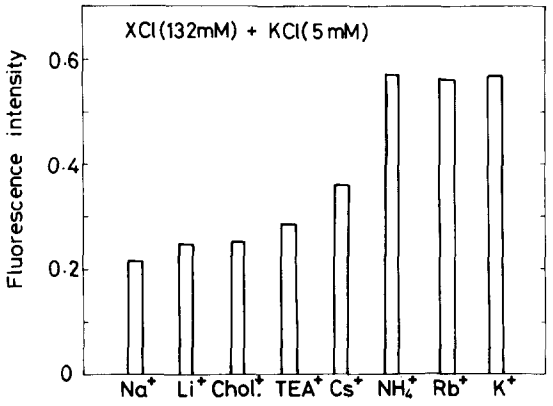

Fig. 3. Effects of different cations in media on the fluorescence of rhodamine 6G. Na^+ in the low K^+ medium was replaced by each monovalent cation. The concentrations of the dye and synaptosomal protein were 0.66 μM and 0.5 mg protein/ml, respectively. TEA^+, tetraethylammonium$^+$.

was also unaffected by the addition of veratrine. The fluorescence changes induced by veratrine in the low K^+ medium (Fig. 2) are quite similar to the membrane potential changes observed for crustacean axons in response to veratrine [10].

The effects of various agents on the fluorescence of rhodamine 6G in synaptosomal suspension are summarized in Table I. As described in our previous paper [6], an increase or decrease in fluorescence in low K^+ media indicates, respectively, depolarization or hyperpolarization of the membrane potential in the synaptosomes.

The membrane potential in synaptosomes is a function of the Na^+ and K^+ concentration gradients across the plasma membrane which is brought about by the action of $(Na^+ + K^+)$-ATPase. Ouabain, an inhibitor of $(Na^+ + K^+)$-ATPase, increases fluorescence in a low K^+ medium, indicating depolarization. The addition of 1 mM ATP had no appreciable effect on the fluorescence. This is probably due to the fact that ATP cannot permeate the plasma membranes of synaptosomes. While the addition of 1 mM succinate caused no change in the fluorescence, glucose, which is more effective for ATP synthesis than succinate, caused a slight decrease in the fluorescence.

Triton X-100, cetylpyridinium chloride and toluene are expected to damage the permeability of synaptosomal membranes. In the presence of these chemicals, the fluorescence of the dye in synaptosomes (in low K^+ medium) was much greater than that in intact synaptosomes and was practically the same as that observed in the high K^+ medium. These results indicate that the fluorescence of the dye in a synaptosomal suspension with Triton X-100, cetylpyridinium chloride or toluene was independent of the K^+ concentration in the medium. It should be noted that all of the agents tested have no appreciable effect on the fluorescence of the dye in the absence of synaptosomes under the same experimental conditions.

If synaptosomes were first lysed by exposure to a hypotonic buffer (a 1:50 dilution of low K^+ medium with water) and suspended in a low K^+ or high K^+ medium containing rhodamine 6G, the degree of quenching fluorescence in the low K^+ medium was practically the same as that in the high K^+ medium (data not shown). When heated (60°C for 5 min) or frozen synaptosomes were added to a rhodamine 6G solution, the fluorescence of the dye did not change with varying K^+ concentration in the medium (data not shown). These results are different from those obtained with intact synaptosomes shown in Fig. 1a.

Effect of replacement of cation in medium

Na^+ in low K^+ medium was replaced by several monovalent cations, and the resulting fluorescence of rhodamine 6G in synaptosomal suspension was measured (Fig. 3). As seen in the figure, the fluorescence decreased in the following order of ions; $K^+ \simeq Rb^+ \simeq NH_4^+ > Cs^+ >$ tetraethylammonium$^+$ > choline$^+ \simeq Li^+ > Na^+$. When synaptosomes were absent, the fluorescence in the various media were practically the same. The above order of ions is similar to that reported by Blaustein and Goldring [4] for synaptosomes using a cyanine dye. Moreover, the above order of ions resembles the order of the effectiveness of cations in inducing depolarization in excitable cells [11,12].

Interaction between synaptosomal membranes and rhodamine 6G

We examined the relation between the concentration of free dye in the media and the amount of dye bound to the synaptosomal membranes. As mentioned in our previous paper [6], the fluorescence intensity and the amount of fluorescence

quenching after the addition of synaptosomes were considered to be proportional to the concentration of free dye in the medium and the amount of bound dye, respectively (see Eqns. 8 and 9). Therefore, we measured fluorescence at varying dye concentrations before and after the addition of synaptosomes, and calculated the concentration of free dye in the medium and the amount of dye bound to the synaptosomal membranes (Fig. 4). The amount of bound dye increased with increasing concentration of free dye, and approached plateau levels in both low K^+ and high K^+ media. The initial slopes of the curves in Fig. 4 correlate with the binding affinity of the dye for synaptosomal membranes. The results show that the binding affinity of the dye for synaptosomal membranes in the low K^+ medium is much higher than that in the high K^+ medium. It is reasonable to consider that the change in binding affinity resulted from a change in the electrical interaction between the dye and the synaptosomal membranes, because depolarization in membrane potential decreases the binding affinity of the dye for membranes (compare the initial slope of each curve in Fig. 4).

Derivation of an equation relating membrane potential to fluorescence intensity

The results, as shown in Figs. 1–3 and Table I, provide convincing evidence that the membrane potential across the plasma membranes of synaptosomes in suspension can be monitored by measuring the fluorescence of rhodamine 6G. The result shown in Fig. 4 suggests that the distribution of the cationic dyes between the membrane phase and the extracellular medium depends on the membrane potential.

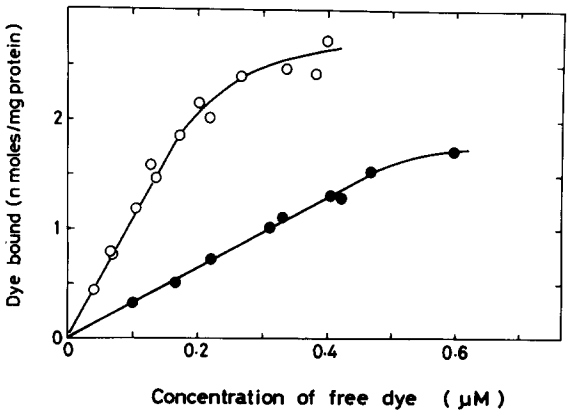

Fig. 4. Relation between the amount of dye bound to synaptosomes and the concentration of free dye in the medium. ○, low K^+ medium; ●, high K^+ medium. The amount of dye bound is calculated using Eqn. 9, and the concentration of free dye in medium is calculated using Eqn. 8.

Tanabe (unpublished data) obtained the equation which gives the relation between the amount of bound dye and membrane potential. The amount of bound dye in a membrane, Q, is given by the equation.

$$Q = V_m C_m \tag{2}$$

where V_m and C_m are the effective volume of membrane with bound dye and the mean concentration of the dye in the membrane, respectively. The value of C_m is calculated using the equation,

$$C_m = C_{mo}\frac{1}{L}\int_0^L \exp\left(-\frac{F\psi(x)}{RT}\right) dx, \tag{3}$$

where C_{mo} is the dye concentration at the outer boundary of the membranes, L is the thickness of the membrane and $\psi(x)$ is the electric potential within membranes. F, R, and T have their usual thermodynamic meanings. If we assume that the gradient of the electrical field within the membrane is constant, the definite integral in Eqn. 3 is calculated easily to give the following equation,

$$C_m = \frac{C_{mo}RT}{F\Delta\psi_d}\left(1-\exp\left(-\frac{F\Delta\psi_d}{RT}\right)\right), \tag{4}$$

where $\Delta\psi_d$ is the diffusion potential within the membrane. The value of C_{mo} is given by the equation,

$$C_{mo} = KC_0 \exp(-F\Delta\psi_0/RT), \tag{5}$$

where K is the non-electrostatic binding constant, C_0 is the free dye concentration in medium, and $\Delta\psi_0$ is the surface potential of the membrane. Eqn. 2 can be rewritten as follows using Eqns. 4 and 5:

$$Q = \frac{RTV_m C_0 K}{F\Delta\psi_d} \exp\left(-\frac{F\Delta\psi_0}{RT}\right) \times \left(1 - \exp\left(-\frac{F\Delta\psi_d}{RT}\right)\right) \quad (6)$$

The amount of bound dye in the high K^+ medium, Q_k, is approximately expressed as Eqn. 7, because $\Delta\psi_d$ is estimated to be much smaller than RT/F (26 mV at 25°C) in high K^+ medium and all but the first and second terms in the expansion of $\exp(-F\,\Delta\psi_d/RT)$ in Eqn. 6 can be neglected.

$$Q_k = V_m K C_0 \exp(-F\Delta\psi_0/RT) \quad (7)$$

As previously mentioned, the fluorescence and the amount of quenching fluorescence after the addition of synaptosome suspension are considered to be proportional to the concentration of free dye in the medium and the amount of bound dye, respectively. From this, we obtained Eqns. 8 and 9:

$$f = k_1 C_0 \quad (8)$$

$$Q = k_2(1-f) \quad (9)$$

In Eqns. 8 and 9, k_1 and k_2 are proportional constants. Combining Eqns. 6, 7, 8, and 9, we get the following equation:

$$\frac{f_k/(1-f_k)}{f/(1-f)} = (RT/F\Delta\psi_d) \times (1 - \exp(-F\Delta\psi_d/RT)) \quad (10)$$

Eqn. 10 shows that diffusion potential is synaptosomes can be calculated from the measurement of the two fluorescence intensities, f (fluorescence with synaptosomes in a given medium) and f_k (fluorescence with synaptosomes in high K^+ medium).

Calculation of diffusion potential and permeability for ions in synaptosomes

The diffusion potentials (membrane potential) of synaptosomes in media with varying K^+ concentrations were calculated from the results shown in Fig. 1 using Eqn. 10, and was plotted against the external K^+ concentration (Fig. 5). As seen in the figure, there is a linear relation between the diffusion potential and $\log[K^+]$ above 20 mM KCl. The slope of the straight line was about 52 mV per decade concentration, which is nearly equal to the ideal value (2.3 $RT/F = 59$ mV). This result is consistent with the view that the membrane potential in synaptosomes follows the constant field equation [13] as shown later (see Eqn. 11).

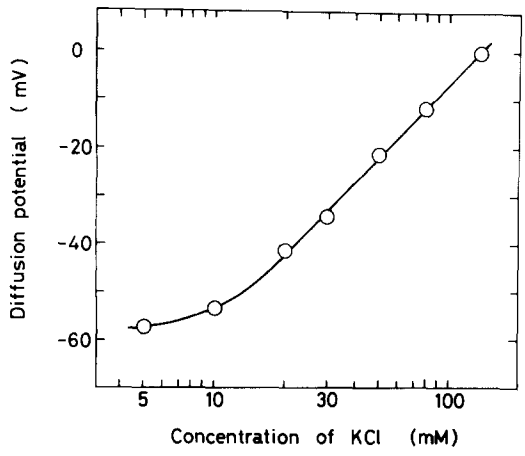

Fig. 5. Relation between the diffusion potential across synaptosomal membranes and logarithm of the KCl concentration in the medium. The diffusion potential was calculated from the results shown in Fig. 1 using Eqn. 10.

TABLE II

CALCULATED MEMBRANE POTENTIAL (DIFFUSION POTENTIAL) IN MEDIA CONTAINING VARIOUS CATIONS, AND THE RATIOS OF THE PERMEABILITY CONSTANTS

The diffusion potentials were calculated using Eqn. 10. The ratio of permeability constants of X^+ and K^+ (P_X/P_K) was calculated using Eqn. 11. TEA^+, tetraethylammonium$^+$.

Cation	Diffusion potential (mV)	(P_X/P_K)
K^+	0	1.0
Rb^+	0	1.0
NH_4^+	0	1.0
Cs^+	−37±2	0.21
TEA^+	−44±3	0.15
Choline$^+$	−53±4	0.094
Li^+	−53±4	0.094
Na^+	−55±5	0.084

The diffusion potential (membrane potential) for each medium containing a different monovalent cation was calculated from the results shown in Fig. 3 using Eqn. 10. These are listed in Table II.

The membrane potential (diffusion potential) in synaptosomes can be expressed by the equation [13]:

$$\Delta\psi_d = (RT/F)\ln([K^+] + (P_X/P_K)[X^+]) + \text{constant} \quad (11)$$

where $[K^+]$ and $[X^+]$ are the concentrations, of K^+ and X^+ (a selected ion) in the medium, and (P_X/P_K) is the ratio of the permeability constants of X^+ and K^+. The values of (P_X/P_K) were calculated from the diffusion potential shown in Table II using Eqn. 11, and are also listed in Table II. The order of (P_X/P_K) is similar to that estimated by measuring the fluorescence of a cyanine dye in synaptosomes [4] or that obtained by using merocyanine 540 in synaptic membrane ghosts [14]. The order of permeability of ions is in good agreement with that obtained in synaptosomes by Keen and White [15] with light scattering methods, and also with that in other excitable membranes with microelectrodes [12]. The values obtained from the above mathematical analysis, as shown in Fig. 5 and Table II, are reasonable for synaptosomes, and indicate that the above mathematical analysis is valid.

Fluorescence quenching of rhodamine 6G

Rhodamine 6G fluoresces in an aqueous solution but it is less fluorescent in nonpolar solvents such as benzene, toluene and ethyl ether. The fluorescence of the dye also decreases when negatively charged chemicals such as sodium dodecyl sulfate, tetraphenylboron and dextran sulfate are added to aqueous dye solutions [6]. The addition of proteins such as serum albumin, trypsin and lysozyme dose not change the fluorescence. These results suggest that an increase in the amount of the dye bound to hydrophobic regions or negative charges in synaptosomal membranes cause quenching of rhodamine 6G fluorescence.

At present, it is not clear on molecular grounds why rhodamine 6G becomes less fluorescent in membranes. We did not take account of existence of aggregated dyes in the calculation of membrane potentials, though the possibility is not excluded completely. When rhodamine 6G is bound to synaptosomes, the absorption maximum of the dye shifts toward longer wavelengths as shown in the previous paper [6]. The shift of the absorption maximum is different from that of some cyanine dyes, which are known to form nonfluorescent dimers [2]. If the aggregated dyes have charges corresponding to their aggregated numbers, the above mathematical analysis can be applicable with a few minor corrections in which the meaning of K in Eqns. 5, 6, and 7 is changed. We do not think that the dye loses its charge even if it is aggregated.

Distribution of rhodamine 6G

Rhodamine 6G is thought to permeate synaptosomal membranes easily. The dye accumulates as a lipophilic cation in intracellular space according to the membrane potential [7]. We think that the dye in cytosol fluoresces for two reasons. (1) Addition of protein such as bovine serum albumin to the aqueous dye solution did not affect the fluorescence. (2) No shift in the dye spectrum was observed with changes in the concentration of the dye. Also the Lambert-Beer low is applicable below 50 μM. The concentration of the dye in cytosol is about 10-fold that in the suspending medium, provided that the membrane potential is 60 mV and the dye accumulates in cytosol according to the Nernst equation. In this experiment, the concentration of the dye in cytosol had to be smaller than 5 μM, because the concentration of free dye was below 0.5 μM.

In the above calculation of membrane potential in synaptosomes, the dye in cytosol was disregarded in Eqn. 8, because an intrasynaptosomal space is 3.5–4.5 μl/mg protein [4,15], and is much smaller than the extracellular volume in this experiment.

The addition of synaptosomes to high K^+ medium appreciably decreased fluorescence (Fig. 1), whereas no membrane potential can exist in synaptosomes in high K^+ medium. This decrease in fluorescence probably results from the dye binding to synaptosomal membranes as well as to membranes of intracellular particles. In the

above mathematical analysis, the amount of the dye bound to these membranes was estimated by Eqn. 7, and assumed to be independent of membrane potential. Therefore, the value of V_m in Eqn. 2, 6 or 7 is an apparent value and may be different from the value determined by a geometrical calculation.

Fluorescence was increased by additions of detergents and toluene in high K^+ medium as well as in low K^+ medium, as shown in Table I. This increase in fluorescence probably results from release of the dye from the membranes of synaptosomes and of intracellular vesicles, since these reagents alter the membrane structure and affect dye binding to these membranes. It is difficult to calculate the exact membrane potential in synaptosomes with fluorescence measurements, because part of the dye may bind to intracellular particles regardless of the membrane potential in synaptosomes. Therefore, further studies using simple systems, such as synaptic membrane ghosts [14] and liposomes, will be useful to find the exact relation between dye binding to membranes and membrane potential.

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