USE OF THE SPECTRAL CHARACTERISTICS OF RYODIPINE TO STUDY THE DIHYDROPYRIDINE–RECEPTOR COMPLEX OF NEURON MEMBRANES

A. A. Timofeev, R. N. Glebov, M. V. Kalinina, and G. L. Grigoryan

UDC 615.217.24].015.4.07

KEY WORDS: synaptosomes; dihydropyridine receptor; fluorescence; ryodipine.

The molecular mechanisms of function of the dihydropyridine (DHP) receptor of neuron membranes, a component of electrically excitable Ca-channels of L-type, have still received little study. Investigations using radioactively labeled DHP-receptor agonists or antagonists basically give information on characteristics of binding of receptor with ligand [4, 7, 9, 11]. Isolation, purification, and reconstruction of subunits of this receptor have greatly extended our knowledge of the structure of the DHP complex, details of interaction between the subunits, and their functions [7, 8, 12], but this approach does not provide information on interaction of the DHP receptor itself and the native membrane, or on changes in these interactions with different functional states of the membrane. Information of this kind can be obtained by the use of various molecular probe methods [1].

In this investigation we suggested using a known DHP-receptor antagonist, ryodipine, as molecular probe on the DHP-receptor complex. By using the spectral properties of ryodipine, we attempted to study the DHP complex of native synaptic membranes.

EXPERIMENTAL METHOD

Experiments were carried out on synaptosomes isolated from the cerebral cortex of male Wistar rats, kept on the standard animal house diet. The synaptosomes were isolated by the method in [10]. A synaptosomal membrane preparation was suspended in medium containing 0.32 M sucrose, 10 mM HEPES-Tris, pH 7.4, at 5°C. The synaptosomes were used immediately after their isolation, and the protein concentration was determined by Lowry's method. Some experiments were carried out on proteoliposomes prepared as follows: the necessary quantity of dipalmitoyl-lecithin, cholesterol, and stearic acid, with N-ethylmaleimide attached in the $-COO^-$ position [2] in the proportion of 1:1:0.05 by concentration, was mixed in chloroform, which was then evaporated off in a rotor vaporizer. Buffer (0.1 M NaCl, 0.1 M Na_2HPO_4, 5 mM Na citrate, 2 mM EDTA, pH 5.5) was added at 20°C gradually to the thin film formed on the flask walls, the contents were shaken constantly until a turbid suspension of multilamellar liposomes had formed, and this was then filtered through a type HA filter (pore diameter 0.45 μ, Nillipore, USA) to obtain a uniform suspension. Next, albumin (bovine serum albumin) was attached to the liposomes thus prepared. To do this, 1 mg of albumin in the same buffer was added to the suspension of liposomes in an amount equivalent to 1 μg of lipid, and the sample was incubated for 12 h at 5°C. After incubation the mixture was centrifuged at 100,000g for 2 h and the residue was suspended in the same medium as the synaptosomes.

Research Institute of General Pathology and Pathological Physiology, Russian Academy of Medical Sciences. Institute of Higher Nervous Activity and Neurophysiology, Russian Academy of Sciences, Moscow. (Presented by Academician of the Russian Academy of Medical Sciences G. N. Kryzhanovskii.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 114, No. 7, pp. 29-32, July, 1992. Original article submitted January 13, 1992.

0007-4888/92/0007-0943$12.50

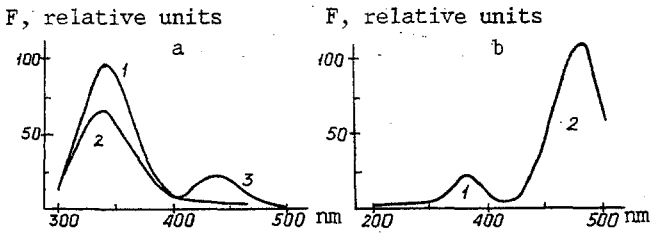

Fig. 1

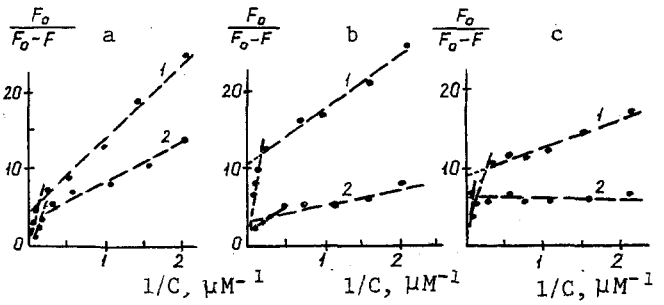

Fig. 2

Fig. 1. Spectrum of natural fluorescence of synaptosomal protein (1), quenched by 10 μM ryodipine (2), and fluorescence spectrum of ryodipine (3) due to IRTE from synaptosomal membrane proteins (a), and excitation spectrum of fluorescence of ryodipine in synaptosomal membranes (b) (1 – peak of excitation of ryodipine fluorescence due to tryptophan residues of membrane proteins, 2 – peak of excitation of ryodipine fluorescence due to natural excitation).

Fig. 2. Dependence of quenching of natural fluorescence of synaptosomal membrane proteins on ryodipine concentration in control (1) and during depolarization (2) (a); dependence of quenching of albumin fluorescence on ryodipine concentration in proteoliposomes (1) and in aqueous solution of protein (2) (b); dependence of quenching of synaptosomal membrane protein fluorescence on ryodipine concentration in presence of 5 μM nifedipine (1) and 5 μM nicardipine (2) (c). Abscissa, ryodipine concentration (in μM^{-1}), ordinate, ratio $F_0/F_0 - F$, where F_0 denotes fluorescence of membrane proteins without ryodipine, F the same with ryodipine.

In the course of the experiments synaptosomes and proteoliposomes in a concentration of 0.2 mg protein/ml were added to medium (in mM): NaCl 132, KCl 5, $MgSO_4$ 1, $CaCl_2$ 1, NaH_2PO_4 1, glucose 10, HEPES-Tris 20, pH 7.4, at 30°C. The synaptosomes were depolarized for 5 min by the addition of 60 mM KCl with a corresponding change in the NaCl concentration to preserve isotonicity of the solution. The membrane preparations were incubated in a thermostated cell, into which were added the necessary amounts of ryodipine and the other constituents, the specimen was incubated for 2-3 min, and fluorescence spectra were recorded on an MPF-4 ("Hitachi," Japan) spectrofluorometer in a 10-mm cuvette; the spectral widths of the monochromator slits were 5 nm for a wavelength of excitation of 285 nm and a fluorescence of 335 nm. A correction for scattering of light by the specimens and a correction for optical screening were made as in [1]. The results were subjected to statistical analysis by Student's test.

The reagents for this investigation were obtained from "Serva" (Germany), and "Sigma" (USA). All salts and organic solvents (double distilled) were of USSR origin and of the "very pure" and "chemically pure" grades. Ryodipine – 2,6-dimethyl-3,5-dicarbomethoxy-4-(*o*-difluoromethoxyphenyl)-1,4-dihydropyridine – was synthesized at the Institute of Organic Synthesis, Academy of Sciences of Latvia, and generously provided by G. Ya. Dubur. Ryodipine, nifedipine, and nicardipine were used in the form of alcoholic solutions, in a quantity such that the final ethanol concentration in the sample did not exceed 1%.

EXPERIMENTAL RESULTS

On the basis of the spectral properties of ryodipine (wavelength of excitation 360 nm, of fluorescence 440 nm) we postulated that inductive-resonance transfer of energy (IRTE) of fluorescence of the membrane proteins to ryodipine would take place in the synaptosomes, as has been demonstrated on other biological membranes [5]. If this is so, it would be possible to judge from the qualitative and quantitative parameters of IRTE not only a change in protein–lipid interactions in the DHP-complex in response to a change in status of the membranes, but also a change in the quantitative characteristics of ryodipine binding.

Since, according to the theory of IRTE [1], energy is transferred when the donor and acceptor of energy are quite close together (Forster's critical radius of transfer), which for ryodipine does not exceed 3 nm [1]. It will henceforward be considered that ryodipine, at a distance of under 3 nm from the donor (protein tryptophan groups) will be bound. In making this assumption, we accept that it is a rough approximation. Hence, it was first necessary to show that IRTE of fluoresce of the proteins to ryodipine was observed in the synaptosomal preparation.

The maximum in the excitation spectrum of fluorescence of the synaptosomal membrane proteins occurred at 285 nm, indicating excitation of the region of the fluorescence spectrum of tryptophan and tyrosine amino acid residues [3]. The maximum of fluorescence under these circumstances occurred at 335 nm, and this in turn corresponded to the parameters of tryptophan fluorescence of proteins located in a sufficiently hydrophobic environment, i.e., of membrane proteins [3] (Fig. 1). The DHP receptor bound with the Ca channel is an inseparable part of the membrane proteins [4, 5, 7, 8, 12]. Accordingly it can be postulated that the natural fluorescence of the receptor subunits takes place in the integral fluorescence spectrum of the membrane preparation. We hoped that with the aid of IRTE from proteins to ryodipine we would be able to distinguish the fraction of protein fluorescence due to fluorescence of the DHP complex.

In fact, IRTE from protein tryptophan residues to ryodipine was observed in membrane preparations from both synaptosomes and proteoliposomes, for the conditions of transfer were satisfied [1]: 1) quenching of the natural fluorescence of the membrane proteins took place; 2) the intensity of ryodipine fluorescence was increased; 3) a band of excitation of tryptophan fluorescence appeared in the fluorescence excitation spectrum of ryodipine (Fig. 1a, b).

Considering this situation and also the fact that ryodipine is a specific Ca-antagonist [6], we suggested that the fraction of natural protein fluorescence, quenched by ryodipine, may correspond to that fraction of DHP receptors that are bound with Ca channels of synaptosomal membranes.

The curve reflecting quenching of protein fluorescence by ryodipine is illustrated in Fig. 2a. The graph has a change of direction in the region of 1 μM. Extrapolation of the two straight lines to their point of intersection with the ordinate gives values of F_0-F corresponding to the maximal degree of quenching of fluorescence, expressed as a percentage [5] (see Table 1). As Fig. 2a shows, we were dealing with two populations of membrane proteins, unequally accessible to ryodipine. Extrapolation to the abscissa gives values of ryodipine concentrations that are saturating for these populations of synaptosomal proteins (K_{sat}). The fraction of each group of proteins was expressed as a percentage of the initial level of fluorescence, and is shown in Table 1 along with K_{sat}. It can be tentatively suggested that the I protein population (K_{Isat} = 1 μM) incorporates DHP-receptor-sensitive Ca channels, whereas population II combines nonspecific interaction of ryodipine with the membrane.

To study specificity of the changes in fluorescence characteristics of the membrane proteins during ryodipine binding, proteoliposomes (liposomes – albumin) were used. In this case ryodipine quenched albumin fluorescence (Fig. 2b). Moreover, it was found that the character of dependence of the changes in albumin fluorescence on the addition of ryodipine does not differ significantly from that in the synaptosomal preparation (Fig. 2a). Fractions of albumin populations I and II (binding sites) and K_{Isat} and K_{IIsat} were relatively close to the corresponding values for synaptosomes: fraction I of proteoliposomes was 8.6, fraction II 33%, K_{Isat} 1.2, and K_{IIsat} 16.6 μM. The data on synaptosomes are also given in Table 1 for comparison. In another series of experiments a simple solution of albumin in the same buffer was titrated with ryodipine under the same conditions (Fig. 2b). As the graph clearly shows, the character of the dependence in principle still remains here also, although the absolute values of the amplitude of fluorescence were significantly less, evidently due to hydration of the albumin and, correspondingly, to the poor accessibility of ryodipine for the hydrophobic regions of the protein.

TABLE 1. Dependence of Ryodipine Adsorption in Synaptosomal Membranes on Functional State of DHP Complex (M ± m)

	Type I sites		Type II sites	
	%	K_{Isat}, μM	%	K_{IIsat}, μM
Control	20,0±2,5	1,4±0,5	48,3±3,3	8,1±2,5
Depolarization	28,6±1,8*	1,9±0,4	50,1±3,4	9,4±3,0
100 μM Ni^{2+}	21,3±1,8	1,3±0,5	48,7±2,9	11,1±2,7
100 μM Cd^{2+}	22,5±2,0	1,9±0,3	52,6±3,7	9,1±2,0
Depolarization + 100 μM Ni^{2+}	30,1±2,4*	2,2±0,4	52,6±3,3	6,0±3,0
Depolarization + 100 μM Cd^{2+}	22,6±1,5**	0,9±0,4	52,2±2,7	11,0±2,3

Legend. *$p < 0.05$ Compared with control; **$p < 0.05$ compared with depolarization.

Taking into account the hydrophobicity of ryodipine [5], it can be tentatively suggested that interaction of ryodipine, both with albumin and proteoliposomes (when the quantum yield of protein fluorescence was sharply increased due to the lipid environment and, correspondingly, accessibility of ryodipine to tryptophan), and with synaptosomal membranes, when it was mainly due to hydrophobicity of the binding sites (sorption) for ryodipine. Just as on albumin, two types of binding sites more (K_{Isat} close to 1 μM) and less (K_{IIsat} over 10 μM) accessible to ryodipine exists in solution on proteoliposomes. With an increase in thermodipine concentration, the type I sites are mainly filled to begin with, followed by type II. Hence, the model experiments did not answer the question of specificity of ryodipine binding with synaptosomal membranes (ligand–receptor interaction), even for the type I sites. It remained to be explained whether the system of membrane protein–ryodipine is "sensitive" to functional changes in the DHP–receptor complex of synaptic membranes.

On depolarization of neuron membranes the affinity of the binding sites of dihydropyridines is reduced, but their number is increased [9]. We observed that during depolarization of synaptosomes the fraction of type I sites increased, whereas that of the II sites remained unchanged (Fig. 2a; Table 1) This indirectly confirms our suggestion that the DHP-receptor complex is one of a group of proteins whose fluorescence is quenched by ryodipine. Evidence in support of this view was given by the data given below on the effect of ionic inhibition of the Ca channel on interaction of ryodipine with the synaptosomal membranes. Nickel and cadmium ions caused voltage-dependent inhibition of Ca channels [12]. Nickel blocks the T type of Ca channels more than cadmium, whereas cadmium blocks the N- and L-types more than nickel, and the last of these is blocked by DHP [12]. The results of these experiments showed that neither cadmium nor nickel changes the values of the membrane parameters studied provided that the transmembrane potential is maintained. Meanwhile, during depolarization nickel does not affect the increase in the fraction of type I sites, whereas cadmium prevents any such effect. The fraction of the type II sites, less accessible to ryodipine, undergoes no changes whatever in response to different conditions of incubation of the membranes (Table 1).

The investigation thus showed that type I sites in synaptosomal membranes, those most accessible to ryodipine, "react" to changes in the conditions of functioning of the voltage-dependent Ca channels, unlike the type II sites. For that reason, the type I binding sites may belong to a class of DHP-sensitive Ca channels. The second group, evidently, combines nonspecific lipid and protein binding sites of DHP in the synaptosomal membranes.

To complete this group of experiments, we studied competitors of ryodipine for the DHP receptor: nifedipine and nicardipine. Addition of 5 μM nifedipine to medium with the synaptosomal preparation reduced by half the fraction of I sites (10%): K_{Isat} 0.3 μM, whereas the fraction of type II sites was unchanged (50.5%): K_{IIsat} 13 μM (Fig. 2c). After addition of 5 μM nicardipine, quenching of fluorescence of the type I ryodipine sites did not take place, but the fraction of type II sites rose by 20% and K_{IIsat} increased to 28 μM, evidently reflecting a redistribution of ryodipine into less specific regions.

We were thus dealing with competitive interaction between nifedipine, nicardipine, and ryodipine for the DHP receptor, to which the type I binding sites of ryodipine with synaptosomal membranes, recorded in the form of extinction of protein fluorescence by ryodipine, on account of IRTE, probably belong.

We thus attempted to use fluorescence characteristics of the DHP-receptor ligand ryodipine for quantitative evaluation of its binding with synaptosomal membranes. Clearly, besides obtaining binding parameters, this approach also possessed many other advantages and, in particular, its ability to record changes in the properties of a membrane adjacent to a DHP receptor, and changes in conformation of the DHP complex itself. However, judging from the experiments described above, it is impossible to study the parameters of ryodipine binding (using IRTE) with the DHP receptor of synaptosomal membranes strictly quantitatively, for several fundamental reasons: 1st, the concentration of the receptor in the synaptic membranes and, correspondingly, the fraction of its fluorescence in the integral spectrum is very small, judging from the value B_{max} = 1 pM/mg protein [7, 11]; 2nd, the DHP receptor was exposed to the aqueous phase [8, 12] and, consequently, was sufficiently hydrated, leading to a decrease in the quantum yield of its fluorescence. Finally, the sensitivity of standard fluorometers is clearly insufficient to record fluorescence of a substance with such parameters.

Nevertheless, these experiments provided evidence of the real possibility of studying the DHP complex of native membranes in accordance with the membrane protein–ryodipine system, at least at the qualitative level, for this system actually reflects the functional state of the DHP-receptor Ca-channel complex.

LITERATURE CITED

1. G. V. Belevich, G. Ya. Dubur, N. K. Kurek, et al., Biol. Membr., **5**, No. 7, 768 (1988).
2. L. D. Bergel'son, E. V. Dyatlovitskaya, Yu. T. Molotkovskii, et al., Preparative Biochemistry of Lipids [in Russian], Moscow (1981).
3. É. A. Burshtein, Progress in Science and Technology. Series: Biophysics [in Russian], Vol. 6, Moscow (1976).
4. P. G. Kostyuk, Calcium and Cellular Excitability [in Russian], Moscow (1986).
5. A. M. Rubtsov, N. Z. Barkalaya, A. A. Boldyrev, et al., Biol. Membr., **6**, No. 1, 18 (1989).
6. V. Z. Simkhovich, R. Ya. Mezhapuke, and G. Ya. Dubur, Farmakol. Toksikol., **51**, 30 (1988).
7. N. M. Soldatov, Biokhimiya, **53**, No. 9, 1418 (1988).
8. K. P. Campbell, A. T. Leung, and A. H. Sharp, Trends Neurosci., **10**, 425 (1988).
9. S. M. J. Dunn, Biochemistry, **27**, 5275 (1988).
10. F. Hajos, Brain Res., **93**, 485 (1975).
11. L. Massien and R. Tapia, J. Neurochem., **51**, No. 4, 1184 (1988).
12. P. W. Tsien, D. Lipscombe, D. V. Madison, et al., Trends Neurosci., **10**, 431 (1988).