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IDENTIFICATION OF AN ANION CHANNEL PROTEIN FROM ELECTRIC ORGAN OF NARKE JAPONICA

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SUMMARY: The anion permeability of membrane vesicles prepared from the electric organ of Narke japonica was inhibited by the addition of 4,4'-diisothiocyano-stilbene-2,2'-disulfonic acid (DIDS). The permeability was measured by measuring changes in the scattered-light intensity caused by the osmotic volume change of vesicles; and also by the efflux measurement of ions from the vesicles using radioisotopes. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of membrane vesicles treated with dihydro analog of DIDS ($[^3H]H_2DIDS$) showed that the H_2DIDS binding protein has a molecular weight of 180,000, and exists in membrane vesicles as a dimer formed by a disulfide bond between monomers of molecular weight 90,000.

INTRODUCTION

White and Miller (1) recently reported that membrane vesicles, "microsacs", prepared from the electric organ of the electric ray, Torpedo californica, contained a voltage-dependent, anion-selective channel. The actual substance of the anion channel, however, remained unknown, because their results were obtained electrophysiologically by using the planner artificial bilayer membrane.

In this report, we confirm the existence of anion channels in membrane vesicles prepared from the electric organ of the electric ray, Narke japonica; a species similar to Torpedo. These results were obtained by measuring the permeability of membrane vesicles in two different ways; osmotic volume change of vesicles and efflux of radioisotopes from vesicles. The permeability of vesicles to anions was decreased by a potent anion channel inhibitor, 4,4'-diisothiocyano-stilbene-2,2'-disulfonic acid (DIDS).

Abbreviations: DIDS, 4,4'-diisothiocyano-stilbene-2,2'-disulfonic acid; H_2DIDS, 4,4'-diisothiocyano-1,2-diphenyl-ethane-2,2'-disulfonic acid; SDS, sodium dodecyl sulfate; PAS, periodic acid-Schiff.

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Also, polyacrylamide gel electrophoresis using sodium dodecyl sulfate (SDS), identified the anion channel protein as $[^3H]H_2$DIDS binding protein. The anion channel protein in the electric organ of N. japonica was a 180,000-dalton protein and this protein is a dimer which consists of two monomers of molecular weight 90,000 crosslinked by disulfide bond.

MATERIALS AND METHODS

Materials: The Japanese electric rays, N. japonica, were the kind gifts of Mr. T. Tasaka. The excised electric organs were kept frozen at -80°C. $[^3H]H_2$DIDS was the kind gift of Prof. A. Rothstein. DIDS was purchased from Pierce (U.S.A.). $^{22}NaCl$ and $Na^{35}SO_4$ were purchased from New England Nuclear (U.S.A.).

Preparation of membrane vesicles: Excitable membrane vesicles were prepared according to the Kasai and Changeux method (2), as follows. Electric organ (about 100 g) was thawed and cut in small pieces. The organ suspended in 80 ml of distilled water with 0.1 mM phenylmethanesulfonyl-fluoride was homogenized by a Waring blender at maximum speed for 1.5 min in ice bath. The homogenate was then centrifuged (80,000 x g for 1 hr) in a Hitachi RP-30 rotor. The pellet was rehomogenized in 80 ml of 5 mM Na-HEPES buffer, pH 7.4 at low speed for 1.5 min. After centrifuging the homogenate (6,000 x g for 20 min), the supernatant was carefully layered on sucrose of a discontinuous density gradient (10 ml of 1.4 M sucrose and 20 ml of 1.0 M sucrose) and then centrifuged (70,000 x g for 6 hr) in a RPS-25 II rotor. The fraction between 1.4 M and 1.0 M sucrose was collected and washed with 5 mM Na-HEPES, pH 7.4 under the same conditions as the first centrifugation. All centrifugations were performed at 4°C.

Assay of permeability: The radioisotope permeability of membrane vesicles was measured according to the Kasai and Changeux method (2). The osmotic volume change of the membrane vesicles was measured according to the Kasai and Kometani method (3,4).

Electrophoresis: SDS polyacrylamide gel electrophoresis was performed according to the method of Fairbanks et al (5). The molecular weight was determined using crosslinked hemoglobin and crosslinked bovine serum albumin (Sigma, U.S.A.). Periodic acid-Schiff (PAS) staining was performed according to the method of Zacharius et al (6).

Assay of protein: Protein was assayed by the method of Lowry et al (7).

RESULTS

The effect of DIDS on the permeability of membrane vesicles was first examined by measuring changes in the scattered-light intensity, i.e., the osmotic volume change of membrane vesicles. By this method, net movement of ions can be followed (4). As shown in Fig. 1, DIDS decreased the ion permeability of both the normal membrane vesicles and the vesicles treated with valinomycin. The result suggested that DIDS decreased the anion permeability of membrane vesicles, although the effect on cation permeability was not clear.

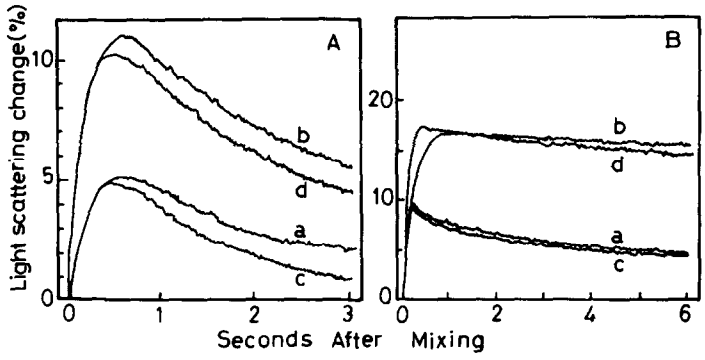

Fig. 1. Change in the scattered light intensity of membrane vesicles caused by the volume change. Using a stopped flow apparatus, the suspension of membrane vesicles incubated in 5 mM Na-HEPES, pH 7.4 and 0.5 mg protein/ml was mixed with equal amounts of either (A) 100 mM KCl, or (B) 67 mM K_2SO_4 and 5 mM Na-HEPES. The ordinate shows the percent change of the scattered light intensity, where the intensity before the mixing was taken as 100 %. Valinomycin was added to the suspension of membrane vesicles about 30 min before the mixing. The suspension of membrane vesicles containing DIDS was incubated for 10 min at 37°C. (a) No valinomycin and no DIDS treatment, (b) no valinomycin and treated with 100 μM DIDS, (c) 1 μM valinomycin and no DIDS treatment, (d) 1 μM valinomycin and treated with 100 μM DIDS.

As shown in Fig. 2, the effect of DIDS on anion permeability was confirmed by measuring the permeability of membrane vesicles using radioisotopes. DIDS did not change the cation ($^{22}Na^+$) permeability of vesicles,

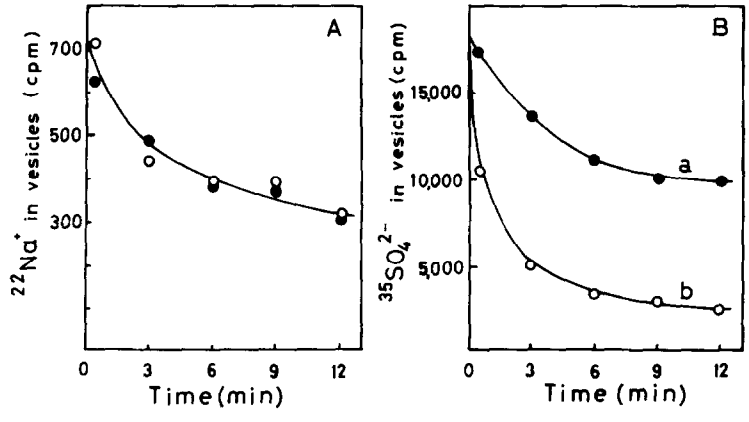

Fig. 2. The effect of DIDS on permeability of membrane vesicles. Suspended solution (50 μl of 10 mg protein/ml) containing either (A) 0.5 μCi $^{22}NaCl$, or (B) 2 μCi $Na_2{}^{35}SO_4$, was treated with (a)(●) 200 μM DIDS in 5 mM Na-HEPES, pH 7.4, or (b)(○) no DIDS for 10 min at 37°C. Before measurement, the mixture was diluted 100 times with 5 mM Na-HEPES, pH 7.4. At each measurement time, 1.0 ml of the diluted mixture was rapidly filtered through a Millipore filter (HAWP 02500, Millipore Co. U.S.A.). The radioactivity remaining on the filter was measured in a liquid scintillation counter LS-500 (Horiba, Japan).

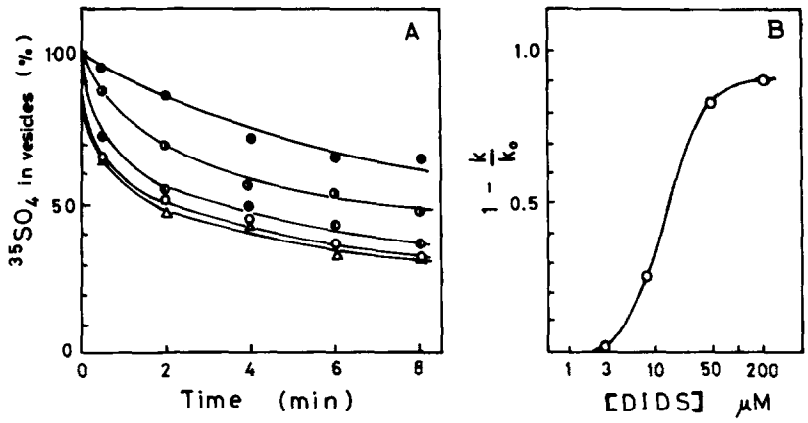

Fig. 3. Inhibition of the $^{35}SO_4^{2-}$ permeability by DIDS. Experiments were performed as described in Fig. 2. (A) $^{35}SO_4^{2-}$ efflux was measured over a period of 8 minutes. DIDS concentration during the treatment was as follows, (Δ) 0, (o) 3 μM, (◐) 10 μM, (◑) 50 μM, (●) 200 μM. (B) In order to calculate the inhibition of permeability, $(1 - k/k_o)$ was used, where k and k_o are the first order date constants of $^{35}SO_4^{2-}$ efflux from the vesicles treated or not treated with DIDS, respectively.

although it decreased anion ($^{35}SO_4^{2-}$) permeability. Fig. 3 (A) showed the concentration dependence of DIDS on $^{35}SO_4^{2-}$ permeability. When the concentration of DIDS during the treatment was increased, the $^{35}SO_4^{2-}$ permeability decreased. As a measure of the inhibitory effect of DIDS, the value $(1 - k/k_o)$ calculated from the data in Fig. 3 (A) were taken. These values were plotted against DIDS concentrations in Fig. 3 (B), where k and k_o are the first order rate constant in the presence and absence of DIDS, respectively. The results show the inhibition constant of DIDS to be about 10-20 μM.

The above results unequivocally suggested that the DIDS binding substance (protein) was an anion channel. The results of SDS polyacrylamide gel electrophoresis demonstrate as in Fig. 4 (A) that $[^3H]H_2$DIDS was bound to 180,000-dalton proteins in the absence of 2-mercaptoethanol and to 90,000-dalton proteins in its presence. We discovered the existence of two kinds of vesicles with the alkaline treatment (pH 11.4) of excitable membrane vesicles prepared from electric organ of N. japonica (8); the heavy vesicles contain acetylcholine receptor protein, and the light

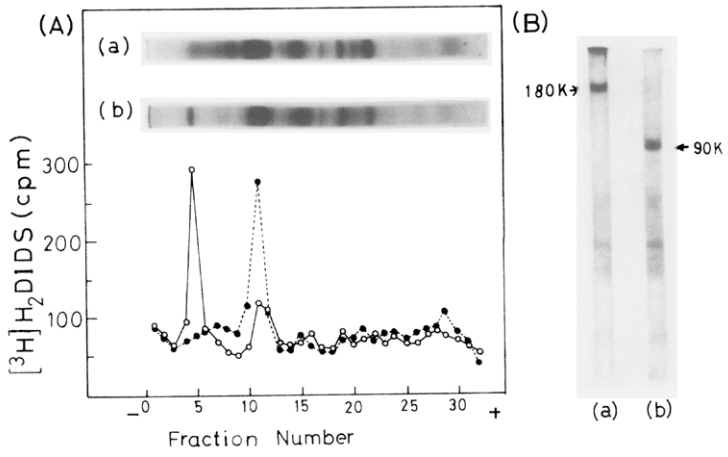

Fig. 4. (A) Identification of DIDS binding protein. SDS polyacrylamide gel electrophoresis was carried out using membrane vesicles (100 µg protein) treated with [^{3}H]H_2DIDS for 1 hr at 37°C, with (a), or without (b) 5 % 2-mercaptoethanol. After electrophoresis, the gel was stained with coomasie brilliant blue and destained in a solution of 5 % methanol and 7.5 % acetic acid. The destained gel was then sliced in 3 mm sections and each section was incubated in 0.5 ml of H_2O_2 for 1 hr at 90°C. After addition of 7 ml of Scintisol 500 (Dojin Kagaku, Japan), radioactivity was measured in the liquid scintillation counter. (B) Electrophoresis of partially purified 180,000-dalton protein. A suspension of excitable membrane vesicles was diluted with 10 times of distilled water, and the pH adjusted to 11.4. After centrifugation (100,000 x g for 30 min) of the suspension, two kinds of vesicles were precipitated. One was acetylcholine receptor-rich, and the other the 180,000-dalton protein-rich. A sample of the latter vesicles (30 µg protein) was processed through gel with 5 % 2-mercaptoethanol (b) or without (a).

vesicles contain the 180,000-dalton protein. The 180,000-dalton protein is a dimer of the 90,000-dalton proteins as shown in Fig. 4 (B). PAS staining showed the 180,000-dalton protein to be a glycoprotein (data not shown). We conclude from the results mentioned above that the 180,000-dalton protein (dimer of 90,000-dalton protein) is an anion channel.

DISCUSSION

The identification of the anion channel protein in the electric organ of N. japonica is the subject of this report. The protein of molecular weight 90,000 was named "anionin". The characteristics of band 3 protein, an anion channel in the red blood cell membrane, have been well documented (9). "Anionin" is similar to band 3 protein on some points, namely: i) The molecular weight (approximately 90,000), ii) DIDS binding protein, iii)

formation of dimer in membrane, iv) glycoprotein. Recently, it was reported that DIDS-sensitive anion channels also exist in sarcoplasmic reticulum vesicles (2), and that their molecular weight was about 100,000 (our preliminary data). Anion channels might exist generally in cells and be similar to the band 3 protein (9).

As mentioned above, "anionins" formed a dimer crosslinked by disulfide bond. Although band 3 protein has a sulfhydryl-containing site, through which two monomers of band 3 can be crosslinked to form a dimer in red cell membrane (10), band 3 protein is not crosslinked by a disulfide bond. Accordingly, the disulfide bond between two "anionins" might have been formed by mild oxidation during the preparation of membrane vesicles, and these two "anionins" might be united by a non-covalent bond in membrane.

The value of the inhibition constant of DIDS in these results were about 10-20 μM, which is comparable with the value 10 μM, obtained by White and Miller (1). The affinity of DIDS with anion channels might be underestimated because DIDS can be bound to membrane lipid (probably to phosphatidylethanolamine). As described under RESULTS, the alkaline treatment of excitable membrane vesicles is a good method for the rapid purification of "anionin". Further studies of the molecular properties of "anionin" are now in progress.

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REFERENCES

1. White, M. H., and Miller, C. (1979) J. Biol. Chem. 254, 10161-10166.
2. Kasai, M., and Changeux, J.-P. (1971) J. Membrane Biol. 6, 1-23.
3. Kasai, M., and Kometani, T. (1979) Biochim. Biophys. Acta 557, 243-247.
4. Kometani, T., and Kasai, M. (1978) J. Membrane Biol. 41, 295-308.
5. Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971) Biochemistry 10, 2606-2617.
6. Zacharius, P. G., Zell, T. E., Morrisory, J. H., and Woodlock, J. J. (1969) Anal. Biochem. 30, 148-152.

7. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
8. Taguchi, T., and Kasai, M. unpublished data.
9. Cabantchik, Z. I., Knauf, P. A., Rothstein, A. (1978) Biochim. Biophys. Acta 515, 239-302.
10. Steck, T. L. (1972) J. Mol. Biol. 66, 295-305.