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PURIFICATION OF A SOLUBLE PROTEIN CONTROLLING Ca^{++} CHANNEL ACTIVITY IN *PARAMECIUM*

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Genetic dissection of ion-channel function has been possible primarily in two model systems, the fruit fly *Drosophila* (for review see reference 1) and the single-celled eukaryotic protozoan *Paramecium* (for review see reference 2). *Paramecium* in particular is well suited to this approach to ion-channel function because biochemical and electrophysiological analysis can be combined with genetic analysis. In this organism, membrane depolarization causes voltage-sensitive Ca^{++} channels to open, transiently increasing the intracellular Ca^{++} concentration. The cilia, the motile apparatus of the cell, respond to the increase in Ca^{++} concentration by reversing their beat, thus causing the cell to swim backward. Mutants affecting Ca^{++}-channel function have been isolated in *Paramecium* as cells that do not swim backward when stimulated. These mutants, called "pawn" in *P. tetraurelia* and "*cnr*" in *P. caudatum*, map to three complementation groups in each species. Electrophysiological analysis of these mutants has revealed a loss in Ca^{++}-channel function in every case (for example, references 3 and 4).

Recently, we have shown that microinjection of cytoplasm from a donor wild-type cell into a recipient *cnr* or pawn cell can restore the ability of the mutant cell to swim backward when stimulated (5–7). The restoration is first observed 4 h after cytoplasmic transfer, reaches a maximum at 8 h, and lasts for two to three days. Electrophysiological analysis of such "cured" mutant cells has demonstrated that the curing represents a restoration of Ca^{++}-channel activity. We have begun then to fractionate wild-type *P. tetraurelia* cytoplasm in an attempt to isolate and characterize the factors which can cure the pawn or *cnr* mutational defects. The curing factors for the three pawn mutations appear to be microsomal membrane proteins, making their further purification by the assay system employed (see Methods) difficult.[1] However, one of the *cnr* mutants, *cnrC*, is cured by a soluble cytoplasmic factor (8). In this report, we describe briefly the purification of this curing factor and some of the electrophysiological properties of cured *cnrC* cells.

METHODS

The curing of Ca^{++}-channel mutants by the microinjection of wild-type cytoplasm is described in detail in reference 5. Crude cytoplasmic fractions from *P. tetraurelia* were prepared as described by Haga et al. (5).[1] Further purification of the *cnrC* factor was performed by standard ammonium sulfate precipitation, gel-filtration and ion-exchange chromatography. Approximately 50 pl of each fraction was injected into each *cnrC* recipient cell. The assay used to follow purification was the duration of backward swimming in response to depolarization by 20 mM KCl following microinjection of protein fractions. This duration is quantitatively related to Ca^{++}-channel activity (8). Thus, the specific activity is defined as the seconds of backward swimming induced per milligram protein injected. Electrophysiological experiments were performed as described by Saimi and Kung (9).

RESULTS AND DISCUSSION

We have attempted to purify the protein which "cures" the *cnrC* mutational defect from wild-type *Paramecium tetraurelia* cytoplasm. Purification was followed by the microinjection of biochemical fractions into *cnrC* mutant cells, and the restoration of Ca^{++}-channel activity was assayed using a rapid, behavioral assay (above). As described in Table I, by a combination of differential centrifugation, ammonium sulfate precipitation, gel filtration, and ion-exchange chromatography on DEAE-Sephadex, we have been able to purify the *cnrC*-curing activity ~1,000-fold. Fig. 1 shows that the curing activity is associated with a small protein (<30,000 mol wt). The restoration of Ca^{++}-channel activity following microinjection of partially purified fractions can be demonstrated electrophysiologically by voltage-clamp analysis, as shown

[1]Haga, N., M. Forte, Y. Saimi, and C. Kung. 1983. Characterization and partial purification of factors controlling the generation of action potentials in *Paramecium*. In preparation.

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TABLE I
PURIFICATION OF THE *cnrC* CURING FACTOR

Fraction	Specific activity	Total activity	Protein
	s/mg protein $\times 10^7$	$s \times 10^9$	*mg*
Cytoplasm (S_1)	1.2	7.4	616.7
Soluble Cytoplasmic Fraction (S_2)	4.3	7.7	179.5
>60% $AmSO_4$	17.9	3.5	19.6
Sephadex G-75	44.5	2.7	6.0
DEAE-Sephadex			
9	92.6	0.14	0.15
10	325.6	0.26	0.08
11	1000	0.30	0.03

The duration of backward swimming is measured in response to 20 mM KCl stimulation 6 to 8 hrs. following microinjection of the indicated fractions into *cnrC* cells. >60% $AmSO_4$ refers to those proteins not precipitated by the addition of $AmSO_4$ to the soluble cytoplasmic fraction to 60% saturation. The soluble proteins are then concentrated and chromatographed on Sephadex G-75. Active fractions are then combined, loaded onto a DEAE-Sephadex column, and eluted with a linear gradient of KCl from 0.1 to 0.4 M. The activities of the three most active fractions are shown.

in Fig. 2. Control injection of a similar fraction prepared from *cnrC* cells does not effect the restoration.

Further investigation of the *cnrC* curing activity in the near future will be aimed at two central questions. First, is the activity which is purified the product of the *cnrC* locus itself? Attempts now being made to compare purified curing protein from wild-type *P. caudatum* and various *cnrC* mutants should aid in answering this question. Second, is such a soluble protein an integral component of active Ca^{++}-channels in this organism? In this light, it is tempting to speculate that this activity represents the soluble factor or one of the soluble factors thought to be required for Ca^{++}-channel function in other organisms

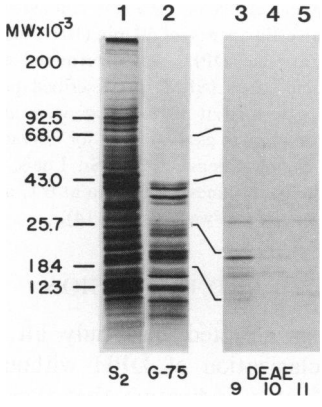

FIGURE 1 SDS-PAGE of purified *cnrC* curing factor. SDS-polyacrylamide gel electrophoresis was performed by the method of Adoutte et al. (3). *Lane* 1, soluble cytoplasmic fraction (S_2). *Lane* 2, combined active fractions following chromatography on Sephadex G-75. *Lanes* 3, 4, 5, are the most active fractions following elution from DEAE-Sephadex and are those described in Table I.

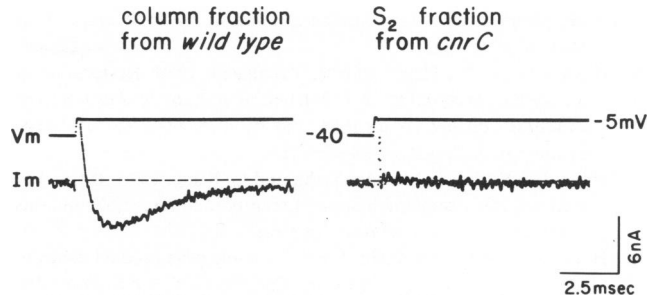

FIGURE 2 Restoration of the Ca current in the *cnrC* mutants. The Ca current was recorded at 6 h after an injection of a Sephadex G-75 column fraction from the wild type (left). A voltage step from the holding level at −40 mV to −5 mV induced the maximal peak Ca current of ~6 nA in this "cured" *cnrC*, after subtraction of linear leakage. The same voltage step in this series of experiments induced up to 9.5 nA current in the uninjected wild type (not shown). A similarly injected soluble cytoplasmic fraction, S_2, from *cnrC*, however, is not capable of curing their sister cells (right). Not more than 1.5-nA Ca current was ever detected in *cnrC* even after leakage correction. The bath contained 10 mM TEAC1, 4 mM CsCl, 1 mM $CaCl_2$, 1 mM HEPES at pH 7.3; electrodes filled with 4 M CsCl; all designed to better the resolution of the Ca current.

(for example, references 10 and 11). While at present no information is available on this question in *Paramecium*, recent success in the incorporation of Ca^{++}-channels from *Paramecium* into planar lipid bilayers may provide a system in which this question can be directly addressed (12).

Recently, we have been attempting to produce monoclonal antibodies against the *cnrC*-curing protein. These antibodies should be useful in the further purification of this factor, its functional analysis, and the identification of its intracellular location.

The purification of the *cnrC*-curing factor described briefly here demonstrates the advantages of *Paramecium* as a model system in which the disciplines of genetics, biochemistry, and electrophysiology can be combined to study ion channel function. Advances have been made recently in the purification of proteins that can cure other mutational defects in Ca^{++}-channel function (8). Ultimately, this approach may lead to the identification of a number of proteins controlling ion-channel activity in this organism.

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DIFFERENTIAL EFFECTS OF MEMBRANE PERTURBANTS ON VOLTAGE-ACTIVATED SODIUM AND CALCIUM CHANNELS AND CALCIUM-DEPENDENT POTASSIUM CHANNELS

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One theory of the action of intoxicant-anesthetic drugs is that they disorder membrane lipids and thereby alter the function of membrane proteins. Chemically diverse compounds such as alcohols, ethers, barbiturates, and halogenated hydrocarbons all disorder membrane lipids, produce a similar spectrum of intoxication and anesthesia, and possess potencies which are correlated with their solubility in membrane lipids (1, 2). These observations provide support for the "disordered lipid" hypothesis of anesthesia, but fall short of identifying the consequences of membrane perturbation; i.e., the specific membrane proteins affected by the anesthetics. Identification of membrane proteins that are sensitive to membrane order is an important issue because some investigators suggest that it is unlikely that the changes in membrane order produced by the anesthetics are of sufficient magnitude to alter membrane function (3). Ion channels represent transmembrane proteins that may be sensitive to the physical properties of their surrounding lipids and have been suggested as a site of action for anesthetic drugs (4). There is electrophysiological evidence that *n*-alkanols decrease neuronal sodium conductance and barbiturates decrease calcium conductance (5, 6), but these studies did not compare different anesthetics and do not provide a correlation of membrane perturbation with changes in channel function. My approach to this problem was to select chemically diverse compounds and to compare their effects on sodium, calcium, and potassium transport and on membrane order.

MATERIALS AND METHODS

Synaptosomes were isolated from mouse (male DBA/2) brain by Ficoll gradient centrifugation (7). Uptake of ^{24}Na was determined by the method of Krueger and Blaustein (8) using 0.09 mM veratridine (Sigma Chemical Co., St. Louis, MO) and a 2 s uptake. The use of a short time and low concentration of sodium (5 mM) essentially eliminates sodium efflux and changes in membrane potential and allows accurate measurement of influx through voltage-sensitive channels (8). Uptake of ^{45}Ca was measured by the procedure of Nachshen and Blaustein (9) using a 3 s uptake, 68 mM KCl as the depolarizing stimulus and 0.02 mM calcium, again allowing unidirectional influx through the voltage-dependent channel. Calcium-dependent efflux of ^{86}Rb was studied in resealed human erythrocytes using an efflux period of 60 min (10). Fluorescence polarization of diphenylhexatriene (DPH) was determined using mouse brain synaptic plasma membranes (SPM) as described previously (11, 12). Experiments were performed at 30°C. Drugs were added to the membrane solutions 5 min prior to assay, except for *cis*-vaccenic acid methyl ester (*cis*-VAME, Sigma Chemical Co., St. Louis, MO), which was incorporated into the membranes for 30 min at 0°C and the unincorporated fraction was removed by washing (13, 14).

RESULTS AND DISCUSSION

The seven drugs selected for study all decreased the fluorescence polarization of DPH without altering the fluorescence lifetime, indicating that they increased the mobility of the probe in the membrane lipids (11, 12, 15). Although all of the drugs decreased fluorescence polarization, their potency in producing this effect varied over a 2,000-fold range (Table I). These results confirm and extend earlier demonstrations of the perturbation of the

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