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Dimeric structure of single chloride channels from *Torpedo* electroplax

(membrane excitability/planar bilayers/stilbenedisulfonates)

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ABSTRACT **The inhibition by 4,4′-diisothiocyano-2,2′-stilbenedisulfonate (DIDS) of Cl^- channels from *Torpedo* electroplax incorporated in planar phospholipid bilayer membranes is studied. DIDS irreversibly and rapidly inhibits the macroscopic conductance of membranes containing many channels. At the single-channel level, the effect of DIDS is more complicated. The uninhibited single channel displays three "substates" of conductances 20, 10, and 0 pS. Short exposure (5–30 s) to 10 μM DIDS converts this three-level active channel into a "conventional" channel of 10-pS conductance. Longer exposure eliminates all channel fluctuations. The results are taken as strong evidence that the Cl^- channel is constructed as a functional dimer of identical protein subunits.**

In the plasma membrane of the electric organ of *Torpedo californica* resides a Cl^--specific channel whose function is to establish the electroplax cell as a low-internal-resistance battery that the fish may use as a source of electric current in stunning its prey (1–5). This channel has been characterized in detail, after insertion into planar lipid bilayers (1–5) or giant lipid vesicles (6, 7), by observation of current fluctuations under voltage-clamp conditions. At the single-channel level, an unusual gating behavior is observed: a "bursting" process in which the open channel displays three distinguishable "substates" (4, 5). This open-channel substructure is uncommonly well-behaved and has led to a simple physical picture of the Cl^- channel as a functional dimer. We have proposed that the three active substates result from the independent opening and closing of two identical Cl^- diffusion pathways, or "protochannels" (4, 5). In this scheme, the dimeric channel complex may exist with both protochannels simultaneously open, with one open and one closed, or with both closed. In this way, the three substates, equally spaced in conductance, are generated. According to this view, the protochannels must be intimately associated in a complex because of the channel's bursting behavior: the simultaneous appearance and disappearance of both protochannels.

This "double-barreled shotgun" model for the channel is quite eccentric, but it is well supported by several lines of evidence (4, 5): the equal spacing in conductance of the three substates, the binomially distributed probabilities of the appearance of the substates, and the "dimeric" transition probabilities among the substates. But this stochastic evidence is, ultimately, indirect. In this report, we present much stronger evidence for the dimeric structure of this channel by examining the action of 4,4′-diisothiocyano-2,2′-stilbenedisulfonate (DIDS) on the channel's substate behavior. We argue that this irreversible inhibitor can be used to trap a "half-inhibited" channel complex in which only one of the protochannels is able to function and, thus, may be observed directly.

MATERIALS AND METHODS

Biochemical. Noninnervated face vesicles of *T. californica* electroplax were prepared immediately after dissection of the tissue as described (4). DIDS was obtained from Pierce, and phospholipids were from Avanti.

Planar Bilayers. The planar bilayer system into which Cl^- channels are inserted has been described in detail (2), as has the method of insertion (5). Briefly, a planar bilayer was formed from a decane solution of 30 mM phosphatidylethanolamine/10 mM phosphatidylserine on a small hole in a polystyrene partition separating two aqueous chambers filled with 150 mM NaCl/10 mM Hepes·NaOH, pH 7.3. *Torpedo* vesicles (10 mg/ml) sonicated for 2 min (5) were added to the "*cis*" chamber, in the presence of 1 mM $CaCl_2$ also added to this chamber. After the first incorporation event (detected as an abrupt increase in membrane conductance), further fusion was stopped by addition of 1.5 mM EDTA. Recordings were collected on FM tape and analyzed by hand. The "*trans*" chamber, opposite to the addition of vesicles, is defined as electrical ground.

RESULTS

In the experiments to be reported here, Cl^- channels were assayed by fusing plasma membrane vesicles from *Torpedo* electroplax into planar phospholipid bilayer membranes (1–5). With this method, channels are inserted with a high degree of orientation as seen from voltage-dependent gating (1, 2), activation by protons (5), and block by SCN^-, stilbenedisulfonates, and other compounds (2).

The bursting pattern of a single Cl^- channel is illustrated in Fig. 1. Four distinctly identifiable states of the single channel (labeled on Fig. 1) are apparent in such a record. A long-lived zero-conductance state, the "inactivated" state, designated "I," separates bursts of "active" channels. Each active burst displays three substates, called the "U," "M," and "D" states, with approximate conductances 20, 10, and 0 pS, respectively, in 150 mM Cl^-. A transition diagram among these states, previously derived to represent this channel's gating kinetics (5), is also shown in Fig. 1. Note that all three active states may be reached from the inactivated state, but that within a burst, transitions between the U and D states must proceed through the M state. Both the D and I states are nonconducting, but they are readily distinguished from each other by the great differences in their average dwell-times (typically 10 and 500 ms, respectively).

This study is concerned with the inhibition of this channel by DIDS, a stilbenedisulfonate that has been widely used as a specific inhibitor of a variety of Cl^--transport proteins (8–10). This compound irreversibly inhibits the *Torpedo* Cl^-

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Abbreviation: DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulfonate.

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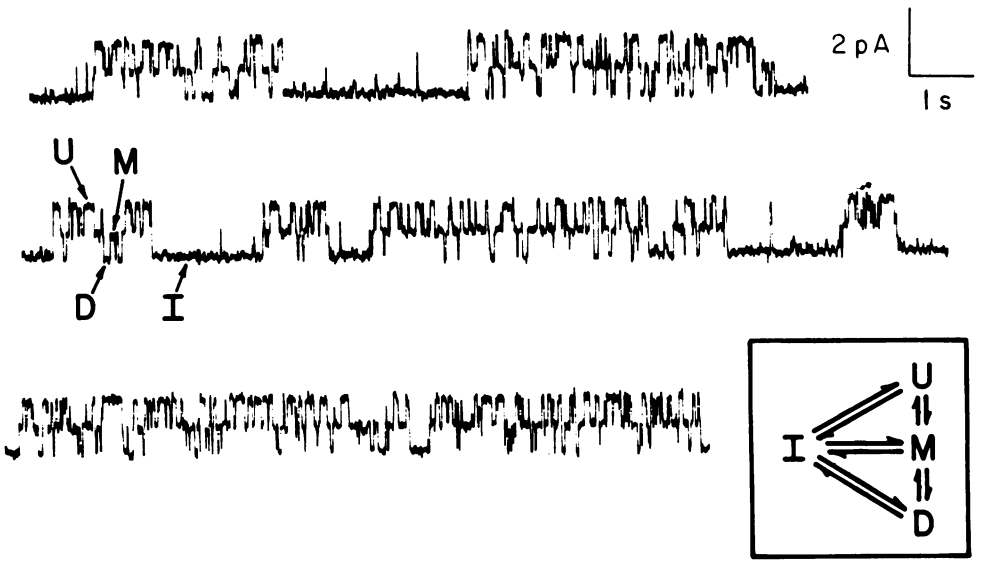

FIG. 1. Single Cl^- channel bursts. A single Cl^- channel was inserted into the bilayer, and recordings were collected at a holding potential of −90 mV. The four states of the single channel discussed here are labeled on the channel record: Inactivated state (I), and three substates of the active channel (U, M, and D). (*Inset*) Diagram previously proposed (4) to describe the allowed transitions among the four states. All rate constants in this diagram depend on both voltage and the pH of the *cis* solution (4, 5).

channel from the *cis* side of the bilayer but is ineffective from the opposite *trans* side (1, 2). Two kinds of DIDS inhibition experiments are reported here: macroscopic inhibition, in which many channels are inserted into the bilayer, and single-channel inhibition, with only a single channel complex in the bilayer.

Macroscopic Inhibition by DIDS. Fig. 2 presents a time course of DIDS action on the macroscopic conductance of a lipid bilayer containing many Cl^- channels (about 500). At a holding potential of −50 mV (Fig. 2, upper trace), a voltage at which most channels are in the active state, DIDS inhibition is complete and rapid, with a half-time of about 4 s at 20 μM DIDS; the lower trace demonstrates that DIDS also can inhibit when the channel is in its inactivated state. Here, channels are first activated at −50 mV to assay the "control" conductance. The voltage is then shifted to +50 mV and held at this value long enough (35 s) to achieve complete inactivation of all channels, as has been previously documented (1, 2). DIDS is then added to a final concentration of 20 μM, and 20 s later voltage is shifted back to −50 mV to assay the degree of inhibition that had occurred during the time of exposure to DIDS. The experiment shows that the channels are completely inhibited by DIDS during the 20 s in their inactivated state. Previous work (2) has shown that this inhibition cannot be reversed by extensive perfusion to remove the DIDS.

DIDS Inhibition of Single Channels. We are now in a position to examine the effect of DIDS on the single Cl^- channel. Fig. 3 shows an experiment in which only a single Cl^- channel was incorporated into the planar bilayer. The top trace shows the three-level bursting pattern just before addition of DIDS. The next two traces are continuous recordings begun 4 s after vigorously stirring 10 μM DIDS into the *cis* chamber. (Noise from settling of membrane vibrations due to stirring can be seen at the beginning of this part of the record.) Immediately after DIDS addition, the bursting channels appear normal, with U, M, and D state conductances of 22, 11, and 0 pS. At about 5 s after DIDS addition, during an inactivated interval (marked by the arrow), the first "hit" by DIDS occurred as indicated by a profound change in the appearance of the single-channel currents. After this time, the three-state active channel disappeared, being replaced by a "conventional" bursting channel with only a single conducting state of 11 pS. After about 25 s in the presence of DIDS, all channel fluctuations disappeared entirely as illustrated in the lowest trace.

This result shows that the three-substate structure of the single Cl^- channel must be due to the operation of two Cl^- diffusion pathways, each with a single open state, rather than to a single Cl^- diffusion pathway with multiple conduc-

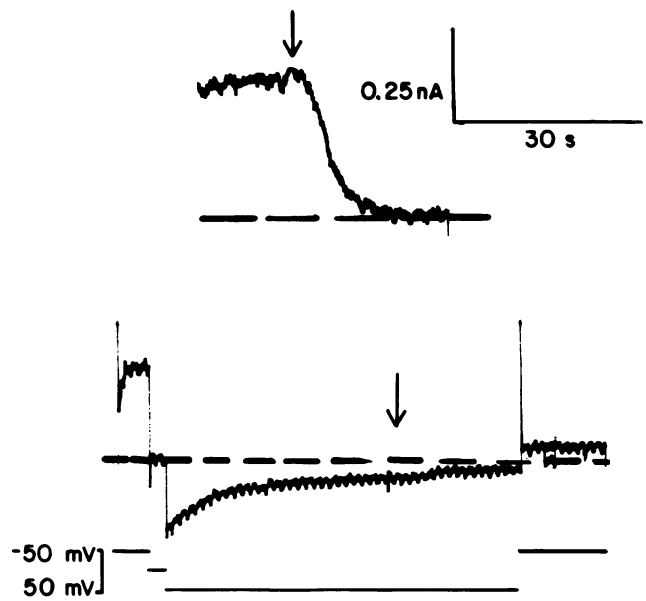

FIG. 2. Macroscopic inhibition by DIDS. Insertion of Cl^- channels into planar bilayers was carried out as in Fig. 1, except that about 100 fusion events were allowed to occur before EDTA was added to stop fusion. DIDS was added (arrow) to a final concentration of 20 mM (from an aqueous stock solution of 2 mM) to the *cis* chamber with a 2-s burst of vigorous stirring sufficient for complete mixing. The upper trace shows current monitored at a holding potential of −50 mV, at which most channels are active in steady-state conditions (1, 2). The lower trace shows inhibition in the inactivated state. Here current was monitored with the voltage command sequence shown below the current trace. DIDS was added only after complete inactivation had been achieved, approximately 30 s after applying a 50-mV holding potential.

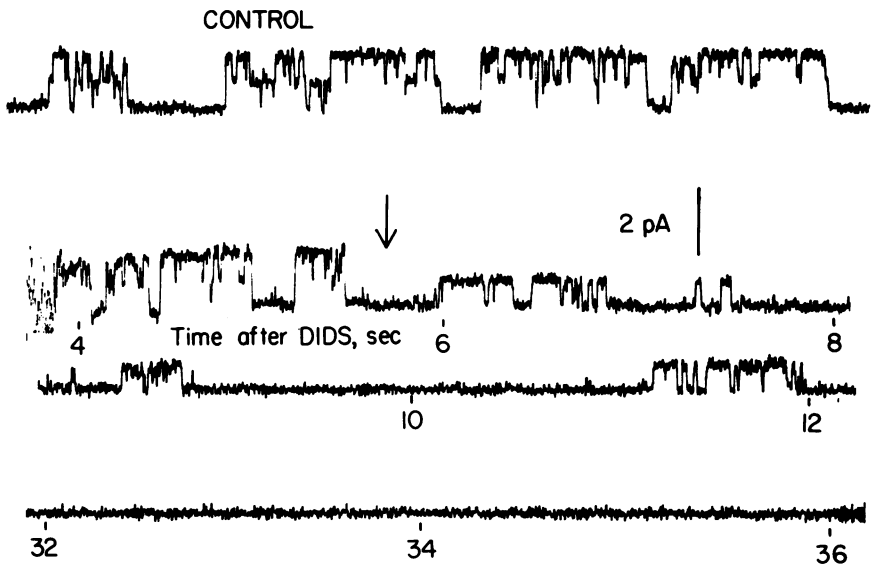

FIG. 3. Inhibition by DIDS of a single Cl^- channel. Conditions were as in Fig. 1, except that the aqueous phase contained 200 mM NaCl. The holding potential was −80 mV, and the pH was 7.2. This bilayer contained a single Cl^- channel. The upper trace shows a section of a control record before DIDS addition. The middle two traces show a continuous record taken at the times indicated after DIDS addition (final concentration, 10 mM). The stirrer was turned off just before the middle trace began. The first hit by DIDS occurred during the inactivated interval at about 5.5 s (arrow). The bottom trace shows a sample record taken after channel fluctuations had disappeared (approximately 30 s after DIDS addition). Baseline current was not different from that recorded on the bare bilayer before channel insertion.

tance states. Therefore, it provides strong support for the dimeric model under consideration here. We interpret the conventional channel fluctuations observed between 5 and 30 s after DIDS to be due to a dimeric channel complex in which one of the protochannels has been rendered nonconducting by covalent reaction with DIDS. This "half-inhibited" channel complex still enters and leaves the long-lived inactivated state, but now only one of the protochannels is observable in the active state. Furthermore, this isolated protochannel is still capable of opening and closing on a rapid time scale, much as it did in the channel complex before DIDS struck down its twin protochannel. The alternative possibility, that the half-inhibited complex represents two active but uncoupled protochannels, is ruled out by the fact that we never observe a "double-level" of 22 pS after the first hit by the inhibitor.

We have repeated this experiment in 20–30 planar bilayers between −90 mV and +25 mV and between pH 6.7 and 7.4. In most attempts, however, more than a single Cl^- channel complex was present in the bilayer (usually 3–5). This complication made quantitative analysis of these bilayers impossible, but qualitatively the results were similar to those of Fig. 3: the appearance 5–30 s after DIDS addition of "isolated protochannels," followed by the eventual elimination of all channel-mediated currents.

Analysis of Isolated Protochannels. In only five membranes did we succeed in performing the experiment of Fig. 3 with just a single Cl^- channel present in the bilayer. In three of these we were able to collect enough data on the isolated protochannel to carry out a quantitative analysis of its behavior, with the intention of comparing its properties with those inferred from the unmodified Cl^- channel complex. As shown previously (4, 5), we can use the dimeric model to extract the properties of the individual protochannel from the observable characteristics of the normal multistate channel. In particular, the probability of the protochannel's being in its open state, p, is given in terms of the measured frequencies f_i of the U and M states:

$$p = f_U + f_M/2. \quad [1]$$

Likewise, the protochannel's mean open and closed times τ_o and τ_c are given by the mean dwell times in the substates:

$$\tau_o = 2\tau_U, \quad [2a]$$

and

$$\tau_c = 2\tau_D. \quad [2b]$$

Finally, the conductance of the isolated protochannel, γ, is obviously

$$\gamma = \gamma_M = \gamma_U/2. \quad [3]$$

Table 1 compares the protochannel parameters derived from the control channel fluctuations with those directly measured after half-inhibition by DIDS. The main conclusion is that the parameters deduced in these two ways agree very well. The protochannel conductance is exactly equal to the conductance of the M state (and half that of the U state). The probability of the protochannel's being open, and the mean open and closed times, agree respectably as well. This result provides further confirmation of the idea that the three-state substructure of the active Cl^- channel represents the parallel, independent gating of two identical protochannels associated in a complex. The easiest way to imagine such a complex is as a dimer of two identical protein subunits.

Table 1. Effect of DIDS on protochannel characteristics

		p		τ_o, ms		τ_c, ms		τ_I, ms		γ, pS	
V, mV	pH	Control	DIDS	Control	DIDS	Control	DIDS	Control	DIDS	Control	DIDS
−55	7.4	0.94	0.83	*	*	*	*	***	***	10.8	10.0
−80	7.2	0.84	0.77	80	51	16	17	330	460	11.5	11.2
−80	7.4	0.63	0.51	40	29	28	29	***	***	10.9	10.1

Data from three experiments carried out as in Fig. 2 were analyzed to calculate the channel parameters shown. Each row represents a separate bilayer with indicated conditions of voltage and pH. Control records were collected before addition of 10 μM DIDS, and protochannel parameters were calculated from Eqs. 1–3. Channel fluctuation parameters after the first hit by DIDS were calculated directly from the records. Each analysis was carried out on approximately 100 transitions. Approximate standard errors on parameters are: ±4% for γ, ±20% for τ_I, ±10% for p. Asterisks mark measurements for which not enough data were available for accurate determination.

We should also note from Table 1 two observations that we regard as additional, second-order effects of DIDS. First, after DIDS the protochannel opening probability is slightly but systematically smaller than that in the control; this effect is due to a lowered mean open time rather than to an increased mean closed time. Furthermore, DIDS causes a clear increase in the inactivated state's mean dwell-time, τ_I. Thus, while the inhibitor's overall effect is quite clean, its detailed mechanism is likely to turn out to be complicated.

DISCUSSION

The important conclusion to be drawn from this work is that the *Torpedo* Cl^- channel functions as a complex of two identical protochannels, each opening and closing independently on a time scale of milliseconds, while together entering and leaving an inactivated state on a slower time scale. Both the rapid opening and slow inactivation reactions are strongly influenced by voltage and by the solution pH on the *cis* side of the membrane (1–5). A plausible cartoon of such a structure is drawn in Fig. 4. There are several characteristics of this cartoon which we take seriously enough to discuss here.

First, we propose that the channel is constructed as a true dimer of identical protein subunits. Though not required by any of the data here, this picture is the simplest way to imagine a complex of two Cl^- diffusion pathways which are identical and independent in conduction and gating characteristics. Following the arguments offered by Kyte (11) and by Klingenberg (12) concerning the stability of membrane proteins, we suggest that the dimeric complex should possess a dyadic axis of symmetry normal to the membrane, at least near the interface between the two subunits. Since DIDS inhibits only from the *cis* side and since the voltage and pH dependences of the protochannels are identical, the protochannels must be arranged in a parallel rather than antiparallel orientation. We picture this as an extremely tight or possibly covalent dimeric complex because the single channels are studied at infinite dilution in the vast excess lipid of the planar bilayer.

The cartoon emphasizes a second conclusion from this work: that the inactivation process is a result of a change in quaternary structure of the dimer—i.e., in the interaction between the subunits. In contrast, the opening of the individual protochannel is viewed as a conformational change in tertiary structure within the monomer itself. This picture provides a natural and economical explanation of the fact that both protochannels always enter and leave the inactivated state together, while opening and closing independently of one another. This suggestion may be subjected to future experimental tests; if the protochannel monomer can be isolated biochemically and reconstituted in an active state, we would expect to observe opening and closing phenomena but no inactivation or bursting.

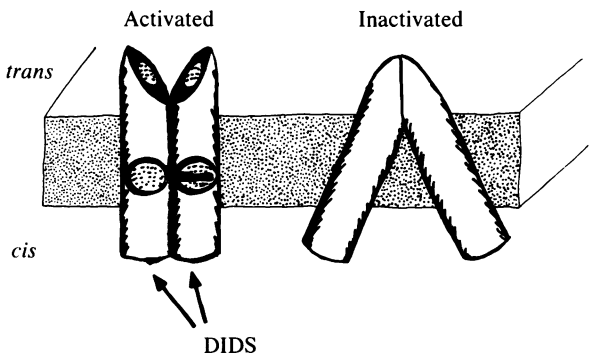

FIG. 4. Cartoon of dimeric Cl^- channel complex. This cartoon is drawn to visualize in as simple a way as possible the main conclusions concerning the *Torpedo* Cl^- channel structure. The conducting unit is viewed as a dimer of identical protein subunits, or protochannels, in close association spanning the membrane. Each protochannel carries its own "gate" (shown in cutaway view), which opens and closes on a time scale of milliseconds. A DIDS-reactive inhibitory site on each protochannel is exposed to the *cis*-facing aqueous phase. The dimeric complex can exist in two quaternary structures as shown, activated and inactivated. The association of the two subunits in the inactivated conformation leads to occlusion of both protochannels simultaneously.

A third point to be noted is that DIDS inhibits the protochannel in the inactive as well as in the active state. This means that the DIDS-reactive site must be exposed to the *cis* solution in both active and inactivated configurations of the channel complex. To visualize this point in a simple way, we have drawn the inactivation machinery on the *trans* side of the membrane, so as to occlude conduction of Cl^- through the protochannel without preventing access of DIDS to its target site.

Finally, we note that this is the first demonstrated example of a membrane protein functioning as a dimer of identical subunits. Many membrane transport proteins have been proposed to operate as oligomers (12), and very good evidence exists for the dimeric structure of the erythrocyte Cl^- exchanger (13) in particular; but the functional significance of these proposed oligomeric structures remains in doubt (11). In addition to establishing the Cl^- channel as a functional dimer, the results here lead to the immediate conclusion that this complex can form a "mixed-state dimer"; the M state of the channel represents a structure in which one of the protochannels is in its conducting conformation, while the other is in its nonconducting conformation. A mixed-state configuration of this type strictly breaks the protein symmetry alluded to above, but if the conformational changes involved in opening and closing are small or far removed from the subunit–subunit interface, this symmetry-breaking would not greatly destabilize the dimeric complex (11). Symmetry violations of this type are *a priori* forbidden in certain theories of quaternary structure of proteins, most notably the Monod–Wyman–Changeux model of cooperative allosteric interactions (14) but are permitted in others, such as Koshland's "induced-fit" theory (15).

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