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The Mitochondrial Inner Membrane Anion Channel Is Inhibited by DIDS

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The mitochondrial inner membrane anion channel (IMAC) is a channel, identified by flux studies in intact mitochondria, which has a broad anion selectivity and is maintained closed or inactive by matrix Mg^{2+} and H^+. We now present evidence that this channel, like many other chloride/anion channels, is reversibly blocked/inhibited by stilbene-2,2′-disulfonates. Inhibition of malonate transport approaches 100% with IC_{50} values of 26, 44, and 88 μM for DIDS, H_2-DIDS, and SITS respectively and Hill coefficients ≤ 1. In contrast, inhibition of Cl^- transport is incomplete, reaching a maximum of about 30% at pH 7.4 and 65% at pH 8.4 with an IC_{50} which is severalfold higher than that for malonate. The IC_{50} for malonate transport is decreased about 50% by pretreatment of the mitochondria with *N*-ethylmaleimide. Raising the assay pH from 7.4 to 8.4 increases the IC_{50} by about 50%, but under conditions where only the matrix pH is made alkaline the IC_{50} is decreased slightly. These properties and competition studies suggest that DIDS inhibits by binding to the same site as Cibacron blue 3GA. In contrast, DIDS does not appear to compete with the fluorescein derivative Erythrosin B for inhibition. These findings not only provide further evidence that IMAC may be more closely related to other "Cl^-" channels than previously thought, but also suggest that other Cl^- channels may be sensitive to some of the many regulators of IMAC which have been identified.

KEY WORDS: Anion channel; anion uniport; transport in mitochondria; stilbene-2,2′-disulfonates.

INTRODUCTION

In general, anion channels have been less well characterized than cation channels, and although a wide variety have been identified by the patch-clamp technique, the precise physiological function of many of them is uncertain. The mitochondrial inner membrane contains an anion uniport pathway which has been characterized by flux measurements in intact mitochondria and has been proposed to be involved in volume homeostasis. These studies have led us to postulate the existence of an anion channel which we refer to as the inner membrane anion channel or IMAC [see Beavis (1992) for review].

The activity or open probability of IMAC is decreased by matrix Mg^{2+}, matrix H^+, and also a wide variety of nonphysiological agents. For a long time, we believed that one of the unique features of this channel was its ability to conduct a wide variety of anions ranging from Cl^- and HCO_3^- to multivalent anions as large as ATP (Powers *et al.*, 1994). It is becoming increasingly evident, however, that many so-called Cl^- channels which have been characterized electrophysiologically are also able to conduct larger anions. One notable example is the cystic fibrosis transmembrane regulator (CFTR) channel, which has recently been reported to conduct HCO_3^- (Poulsen *et al.*, 1994) and ATP (Reisin *et al.*, 1994) in addition to

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[2] The abbreviations used are: EGTA, [ethylene bis(oxyethylenenitrilo)]tetraacetic acid; NEM, *N*-ethylmaleimide; IMAC, inner membrane anion channel; TES, *N*-tris[hydroxymethyl]methyl-2-amino-ethanesulfonic acid; LS, light scattering; DMSO, dimethylsulfoxide; DIDS, 4,4′-diisothiocyanato-stilbene-2,2′-disulfonate; DNDS, 4,4′-dinitro-stilbene-2,2′-disulfonate; SITS, 4 acetamido-4′-isothiocyanato-stilbene-2,2′-disulfonate; ANDS, 4-amino-4′-nitrostilbene-2,2′-disulfonate.

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Cl^-. Another example is a high-conductance channel found in frog skeletal muscle by Woll *et al.* (1987) which also transports ions ranging from Cl^- to glucuronate and including citrate. Thus, it is becoming apparent that IMAC may have more properties in common with other Cl^- channels than previously believed. Selwyn *et al.* (1993) have recently pointed out that Zn^{2+}, which has been shown to inhibit a number of Cl^- channels (Kokubun, 1991; Woll *et al.*, 1987; Groschner and Kukovetz, 1992; Harrison and Gibbons, 1994) is a very potent inhibitor of IMAC.

Among the most frequently tested potential inhibitors of Cl^-/anion channels are the stilbene-2,2′-disulfonates DIDS, H_2-DIDS, and SITS. These compounds were first investigated because they had been shown to be very potent irreversible inhibitors of the erythrocyte Cl^-/HCO_3^- exchanger [see Cabantchik and Greger (1992) for a review]. Examples of channels blocked by DIDS include the double-barrelled Cl^- channel from the electric organ of *Torpedo californica* (White and Miller, 1979); the outwardly rectifying Cl^- channels (ORCC) from many secretory epithelia (Bridges *et al.*, 1989; Tilmann *et al.*, 1991; Kubo and Okada, 1992); and Cl^- channels in mast cells (Dietrich and Lindau, 1994), vascular smooth muscle (Kokubun *et al.*, 1991), and vascular endothelia (Groschner and Kukovetz, 1992). The IC_{50} values range from 2–100 μM and in many cases inhibition is reversible. Some anion channels are not blocked by DIDS, including the cystic fibrosis transport regulator channel (Tabcharani *et al.*, 1991), and a small conductance Cl^- channel in pancreatic duct cells (Gray *et al.*, 1990).

In this paper, we present an investigation of the effects of stilbene-2,2′-disulfonates on the activity of IMAC. Transport of malonate is blocked completely with an IC_{50} in the same range found for many other anion channels. We also find that Cl^- transport is only partially blocked and conclude that DIDS blocks IMAC via the same mechanism as Cibacron blue 3GA (Powers *et al.*, 1994). These data provide further evidence that IMAC has many properties in common with anion channels in other membranes.

EXPERIMENTAL PROCEDURES

Assay of Anion Transport

Anion transport was assayed by following swelling which accompanies net salt transport, using the light scattering technique as described in detail elsewhere (Beavis *et al.*, 1985; Garlid and Beavis, 1985; Powers *et al.*, 1994). Using this technique, we generate a light scattering variable, β, which normalizes reciprocal absorbance for mitochondrial protein concentration. The rate of salt transport is calculated from the rate of change of β as described by Garlid and Beavis (1985).

Pretreatment of Mitochondria with NEM

The normal mitochondrial stock suspensions (50 mg protein/ml) were diluted 1:1 in 0.25 M sucrose containing K^+ salts of TES (12 mM) and EGTA (0.5 mM) adjusted to pH 6.7 (at 25°C) and maintained at 0°C. The desired dose of NEM was then added and at least 10 min was allowed to elapse after mixing before the mitochondria were transferred to the various assay media.

Assay Media for Anion Transport

The potassium chloride and malonate media for light scattering studies contained the K^+ salts of Cl^- (55 mM) or malonate (36.7 mM) and EDTA (0.1 mM), EGTA (0.1 mM), and TES (5 mM) plus rotenone (2 μg/ml). The pH was adjusted to 7.4 unless indicated otherwise and the temperature was maintained at 25°C. For most experiments mitochondria (0.1 mg/ml), A23187 (10 nmol/mg), nigericin (1 nmol/mg), and valinomycin (0.5 nmol/mg) were added separately to each assay.

Drugs and Reagents

Most drugs were obtained from the Sigma Chemical Company. H_2-DIDS and DNDS were obtained from Molecular Probes. The ionophores and rotenone were dissolved in ethanol. Thioglycolate, mersalyl, and *p*-CMS were dissolved in water. Cibacron blue 3GA was dissolved in water. DIDS, SITS, H_2-DIDS, DNDS, and Erythrosin B were dissolved in DMSO at concentrations ranging from 20–100 mM and were freshly prepared for each experiment, stored in tubes wrapped in aluminum foil, and used within 6 hours. Rat liver mitochondria were prepared as described previously (Beavis *et al.*, 1985).

RESULTS

Stilbene-2,2′-disulfonates Inhibit IMAC

In isolated mitochondria, the inner membrane anion channel (IMAC) can be activated in several ways. One of these is depletion of endogenous divalent cations by addition of the ionophore A23187 and EDTA. As shown by trace a in Fig. 1A, this treatment allows net salt transport to take place on subsequent addition of the K^+ ionophore valinomycin. The anion, malonate in this experiment, enters electrophoretically via IMAC, and K^+ enters electrophoretically via valinomycin to compensate for the charge movement (Beavis, 1992). Net salt influx leads to swelling and a consequent decrease in light scattering and increase in the parameter β (see Experimental Procedures). As shown by traces b–e of Fig. 1A, addition of DIDS prior to valinomycin leads to a dose-dependent inhibition of transport. Similar results were obtained if both DIDS and valinomycin were added to the medium at zero time. Inhibition is also observed if IMAC is activated by addition of valinomycin to respiring mitochondria in what we term the "Brierley assay" (Beavis, 1992). In this assay, IMAC is activated by alkalinization of the matrix rather than by depletion of Mg^{2+}.

Surprisingly, when KCl is used in the assay medium in place of potassium malonate, DIDS proves to be a very poor inhibitor of IMAC. Figure 1B shows light scattering traces obtained in KCl assay medium at pH 7.4 using DIDS up to a concentration of 0.66 mM. Not only is the IC_{50} severalfold higher in KCl, but also inhibition does not appear to approach 100%.

Figure 2 contains typical Hill plots for inhibition of malonate transport via IMAC by DIDS, H_2-DIDS, and SITS. The closed circles represent typical data obtained with DIDS. The IC_{50} is 25.7 μM and the Hill coefficient is 0.86. In almost all our experiments using this assay, we obtain Hill coefficients for DIDS ranging from 0.8–0.9. This is not sufficiently low to produce a nonlinear Dixon plot; however, the intercept on the ordinate of a Dixon plot predicts a lower J_0 (the rate of transport in the absence of inhibitor) than actually obtained, and consequently, the IC_{50} obtained from the intercept on the abscissa is about 25% higher than that obtained from the Hill plot. For H_2-DIDS this phenomenon is more pronounced (closed triangles), and the Dixon plot is clearly nonlinear (not shown).

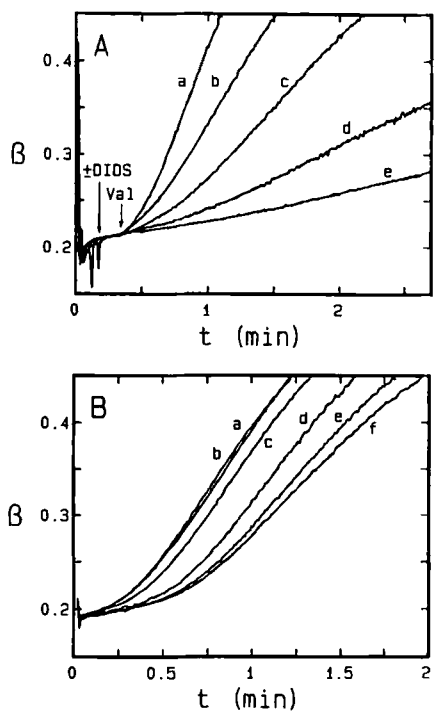

Fig. 1. DIDS inhibits malonate uniport. Light scattering kinetics of mitochondria (0.12 mg/ml) suspended in potassium malonate and KCl assay media are shown. (A) Malonate. Nigericin (0.5 nmol/mg), rotenone (2 μg/mg), an A23187 (10 nmol/mg) were added at zero time. Valinomycin (0.5 nmol/mg) was added at 0.35 min. The following doses of DIDS (μM) were added at about 0.15 min: trace a, 0; b, 8.3; c, 24.8; d, 82.8; e, 207. (B) KCl. Nigericin (0.5 nmol/mg), rotenone (2 μg/mg), A23187 (10 nmol/mg), valinomycin (.5 nmol/mg), and various doses of DIDS were added to the assay medium at zero time. DIDS (μM) added: trace a, 0; b, 6.6; c, 26.4; d, 198; e, 330; f, 660. The experiment was carried out at pH 7.4 and 25°C. The assay media are described under Experimental Procedures.

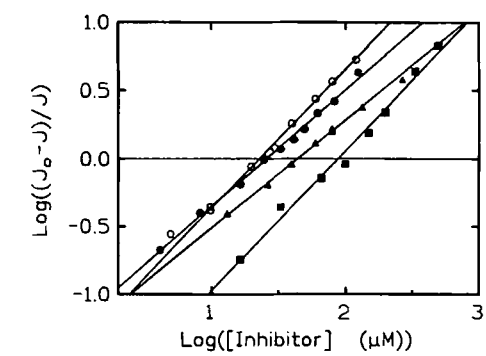

Fig. 2. Inhibition of IMAC by stilbene disulfonates. Hill plots for inhibition of malonate uniport are shown (J_0 represents the rate of transport observed in the absence of inhibitor). The data were collected as described in Fig. 1. Simple linear regression was used to determine IC_{50} values and Hill coefficients (h). ●, DIDS IC_{50} = 25.7 μM, h = 0.9; ▲, H_2-DIDS IC_{50} = 44 μM, h = 0.8; ■, SITS IC_{50} = 87.7 μM, h = 1.0; ○, DIDS (data obtained using the "Brierley assay" as described in the text) IC_{50} = 23 μM, h = 1.0.

This phenomenon is reproducible and is not dependent on the batch of mitochondria used, since in a parallel experiment with the same preparation of mitochondria, we have found that inhibition by SITS (Fig. 2, closed squares) is well behaved, yielding an IC_{50} of 88 μM with a Hill coefficient of 1.0. When the Brierley assay is used, the IC_{50} is slightly (≈10–20%) lower than for the A23187 assay and the Hill coefficient is very close to unity (open circles, Fig. 2). This difference may be related to the fact that transport rates are much faster in the Brierley assay due to the extreme alkalinization of the mitochondrial matrix. This finding also indicates that inhibition by DIDS, unlike inhibition by Mg^{2+} and cationic amphiphiles [see Beavis (1992) for review], is not sensitive to matrix pH.

We have also examined the effect of 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS) and 4-amino-4′-nitrostilbene-2,2′-disulfonate (ANDS) on IMAC. These agents are relatively poor inhibitors, with 80 μM DNDS inhibiting by less than 20% and 165 μM ANDS by less than 15% (data not shown).

The IC_{50} values reported above are those obtained using fresh solutions of the inhibitors dissolved in DMSO prepared on the day of the experiment. We have observed, however, that when older solutions are used, the IC_{50} values for DIDS can decrease by as much as 50–60%. We have not examined this phenomenon systematically; however, it may be related to the light-dependent conversion of the *trans* form to the *cis* form (see Cabantchik and Greger (1992)).

Inhibition of Cl^- Transport by DIDS Is Only Partial

In view of the relatively small effect of DIDS on Cl^- transport, we also examined the effect of DIDS on Cl^- transport at pH 8.4 at which transport rates are 7-fold higher. The data contained in Fig. 3 show the results obtained at both pH values. The curves were fitted to the data by nonlinear regression and, at pH 8.4, this analysis gave an IC_{50} of 107 μM with a maximum inhibition of 64% and a Hill coefficient of 1.17. The data at pH 7.4 are consistent with these values and, if it is assumed that the values of the IC_{50} and Hill coefficients are the same at both pH values, nonlinear regression predicts a maximum percent inhibition of about 30%.

The Effect of *N*-Ethylmaleimide and pH on Inhibition by DIDS

Many of the findings described above are strongly reminiscent of the behavior of Cibacron blue (Powers *et al.*, 1994). Moreover, since Cibacron blue, like the stilbene-disulfonates, is a multi-ringed aromatic compound containing sulfonate groups, it is quite possible that they inhibit by binding to a common site. To investigate this hypothesis further, we examined the effect of *N*-ethylmaleimide and pH on inhibition by DIDS. NEM was found to decrease the IC_{50} for DIDS from 23.5 to 13.9 μM, while raising the pH to 8.4 increased the IC_{50} from 30 μM to 50 μM. It should be pointed out, however, that the increase in pH not only increases the rate of transport but also increases the Hill coefficient from a value of 0.8 to a value close to 1 (data not shown). These effects of pH are much smaller than those on inhibition by Mg^{2+} and propranolol (Beavis, 1992), but are similar to the effects on inhibition by Cibacron blue (Powers *et al.*, 1994).

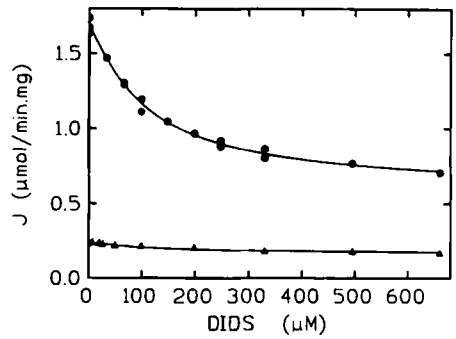

Fig. 3. Dose–response curves for DIDS inhibition of Cl^- transport. The rates of C^- transport (μmol/mg·min) at pH 8.4 (●) and pH 7.4 (▲) are plotted versus the concentration of DIDS (μM). The curve for the data at pH 8.4 was fitted to the data using nonlinear regression with the formula

$$J = (J_0 - J_\infty)/(1 + ([\text{DIDS}]/IC_{50})^h) + J_\infty$$

yielding $J_0 = 1.68$, $J_\infty = 0.60$, $h = 1.17$, and $IC_{50} = 107$ μM. For the data obtained at pH 7.4, the values of IC_{50} and h were assumed to be unchanged and constant and J_0 and J_∞ determined by nonlinear regression ($J_0 = 0.24$, $J_\infty = 0.17$). The experiment was carried out as described in Fig. 1.

Mutual Effects on Inhibition by DIDS and Cibacron Blue

If the binding of these two inhibitors is mutually exclusive, e.g., if they both bind to the same site, partial inhibition by one inhibitor should raise the IC_{50} for the second without changing the slope of the Dixon plots. Figure 4A contains Dixon plots for inhibition by DIDS in the presence and absence of 2 μM Cibacron blue, which was determined to be the IC_{50} in a parallel

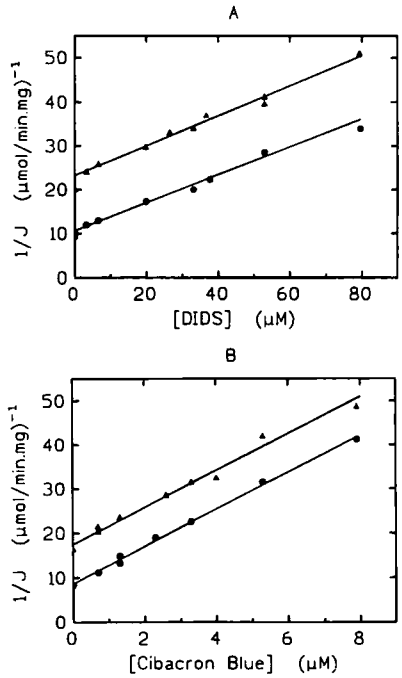

Fig. 4. DIDS and Cibacron blue bind to the same site. The reciprocal rate of malonate uniport is plotted versus the concentration of inhibitor (μM). (A) Inhibition by DIDS. • Control, IC_{50} = 33.3 μM; ▲ + 2 μM Cibacron blue, IC_{50} = 68 μM. (B) Inhibition by Cibacron blue. • Control, IC_{50} = 2.1 μM; ▲ + 20 μM DIDS, IC_{50} = 41.5 μM. The experiments were carried out at pH 7.4 and 25°C as described in Fig. 1. The composition of the assay medium is described under Experimental Procedures.

experiment (Fig. 4B). Parallel curves are obtained and the apparent IC_{50}, determined from the intercept on the abscissa, is increased from 33 μM to 68 μM. The actual IC_{50} value determined from Hill plots also increases twofold from 23 μM to 50 μM. As discussed above, the difference in the absolute values obtained in the different plots reflects the fact that the true J_0 is greater than that predicted by the linear Dixon plot. Figure 4B shows the corresponding Dixon plots for inhibition of malonate transport by Cibacron blue in the presence and absence of 20 μM DIDS. Again the IC_{50} is increased 2-fold from 2.1 to 4.2 μM and the curves are parallel. These findings suggest that DIDS and Cibacron blue inhibit by binding to a common site.

Does Erythrosin B Bind to the DIDS Site?

In a previous study (Powers *et al.*, 1994), we demonstrated that in addition to Cibacron blue, halogenated fluorescein derivatives such as Erythrosin B also inhibit IMAC. In view of the fact that these compounds are classified as nucleotide analogs, we tentatively assumed that they were probably acting at the same site. We could not, however, examine the effect of both these dyes together using the LS assay, since one is blue and the other red. In contrast, DIDS has very little color and can be used with both Cibacron blue and Erythrosin B.

Figure 5 shows data from an experiment in which we examined the mutual effects of DIDS and Erythro-

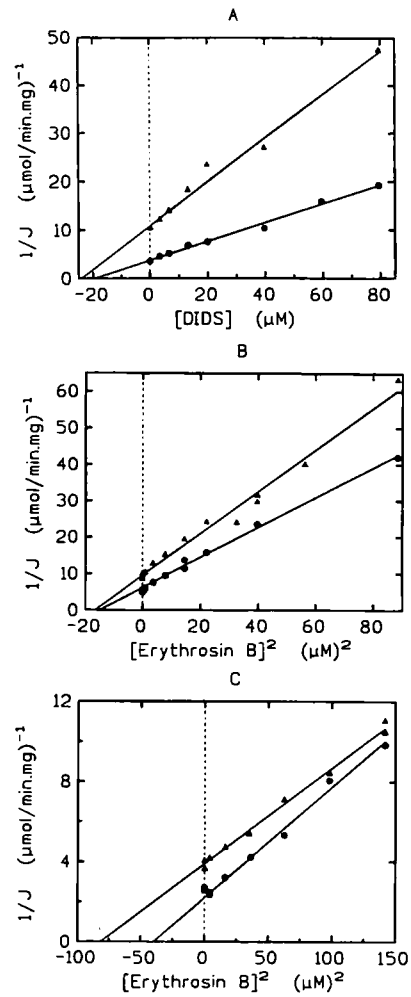

Fig. 5. DIDS and Erythrosin B do not bind to the same site. The reciprocal rate of malonate transport (Panels A and B) and Cl^- transport (Panel C) is plotted versus the concentration of inhibitor. (A) Inhibition of malonate transport by DIDS. • control, IC_{50} = 19.6 μM; ▲ + 3.1 μM Erythrosin B, IC_{50} = 24 μM. (B) Inhibition of malonate transport by Erythrosin B. In this case, $1/J$ is plotted versus $[Erythrosin]^h$, where we have assumed a value of 2 for the Hill coefficient (h). • Control, IC_{50} = 3.9 μM; ▲ + 19 μM DIDS, IC_{50} = 4.1 μM. (C) Inhibition of Cl^- transport by Erythrosin B. The data are plotted versus $[Erythrosin]^h$, where we have assumed a value of 2 for the Hill coefficient (h). • Control, IC_{50} = 6.3 μM; ▲ + 200 μM DIDS, IC_{50} = 9.0 μM. The experiments were carried out at pH 7.4 and 25°C as described in Fig. 1 and under Experimental Procedures.

sin B. Figure 5A contains Dixon plots for inhibition by DIDS in the presence and absence of 3.2 μM Erythrosin B. In contrast to Cibacron blue, despite inducing greater than 50% inhibition, Erythrosin B only increased the IC_{50} for DIDS from 19.6 μM to 24 μM. Moreover, since the plots are not parallel, it is unlikely that Erythrosin inhibits by binding to the DIDS site. Figure 5B contains Dixon plots for inhibition of IMAC by Erythrosin in the presence and absence of 19 μM DIDS. The rates are plotted as function of the square of the Erythrosin B concentration since the Hill coefficient for this inhibitor is close to 2.0. In this case, DIDS only increases the Erythrosin B IC_{50} from 3.9 μM to 4.1 μM, and again the plots are not parallel. These findings strongly suggest that DIDS and Erythrosin do not inhibit IMAC by binding to a common site.

To further examine the relationship between the inhibition of IMAC by DIDS and Erythrosin B, we have exploited the fact that DIDS can only inhibit the transport of Cl^- by 30%, whereas Erythrosin B can inhibit transport by greater than 95% (Powers *et al.*, 1994). Thus, we were able to examine the effect of a high concentration of DIDS (200 μM) on the IC_{50} for Erythrosin B in KCl. The Dixon plots contained in Fig. 5C reveal that this dose of DIDS only increases the Erythrosin B IC_{50} from 6.3 μM to 9.0 μM. This indicates that if DIDS binds at the Erythrosin B inhibitory site, the binding constant must be greater than 200 μM, which suggests that it is not the primary DIDS inhibitory site. The Dixon plots obtained in this type of experiment are found to converge and actually intersect at positive Erythrosin B concentrations. This finding suggests that, although DIDS may bind weakly to the Erythrosin site, when it does so, it blocks transport to a lesser extent than Erythrosin B. This conclusion is consistent with the dose–response curves for DIDS in KCl presented in Fig. 3.

DISCUSSION

In this paper, we have presented evidence that stilbene-2,2′-disulfonates are reversible inhibitors of IMAC. DIDS appears to be the most potent with an IC_{50} for inhibition of malonate transport of around 27 μM. The characteristics of this inhibition are very similar to those described previously for Cibacron blue (Powers *et al.*, 1994). Thus, the Hill coefficient is close to unity, the IC_{50} is essentially insensitive to matrix pH and is decreased by *N*-ethylmaleimide, and inhibition of Cl^- transport is only partial. In addition, DIDS and Cibacron blue appear to compete for the same binding site. It is possible that this inhibitory site is also responsible for the relatively weak inhibition of IMAC induced by a number of buffers containing sulfonate groups reported by Ng *et al.* (1993a).

In an earlier study of the inhibition of IMAC by Cibacron blue and other nucleotide analogs, Powers *et al.* (1994) demonstrated that Cibacron blue and agaric acid and palmitoyl CoA appear to bind to the same site, but did not examine the relationship between these inhibitors and those related to fluorescein and phenolphthalein. The results presented in the present paper strongly suggest that Erythrosin B does not inhibit by binding to the Cibacron blue/DIDS binding site. Other differences consistent with this conclusion are the finding that the Hill coefficients for DIDS, Cibacron blue, palmitoyl CoA, and agaric acid are close to unity while that for Erythrosin B is close to 2 and the finding that inhibition of Cl^- transport is incomplete for the first group while it is essentially complete for Erythrosin and bromcresol green.

The inhibition of Cl^- transport by DIDS is complex and difficult to characterize since it is incomplete. One possible interpretation is that the partial inhibition of Cl^- transport observed at high pH is a consequence of weak binding of DIDS to one of the Erythrosin B binding sites and that binding of DIDS to the site responsible for inhibition of the malonate flux has a minimal effect on Cl^- transport. This could explain the small increase in Erythrosin B IC_{50} and the decrease in the slope of the Dixon plot induced by 200 μM DIDS. A second possibility is that the binding constant for DIDS is different in the two media. Further work is necessary to distinguish these possibilities.

In a previous publication (Beavis, 1994), a channel hypothesis for IMAC was proposed in which inhibitors such as H^+, Mg^{2+}, and propranolol inhibit flux by decreasing the open probability of the channel, while Cibacron blue inhibits flux by binding to the open state of the channel and decreasing its conductance. The exact mechanism by which DIDS blocks anion channels has not been established; however, Bridges *et al.* (1989) (see also Venglarik *et al.*, 1994) have presented evidence which indicates that DIDS is an open channel blocker of the outwardly rectifying Cl^- channel of rat colonic enterocytes. Since it is an anion itself, it is not unreasonable to suggest that it may block anion channels by occupying an anion binding site in the channel. Such a mechanism for IMAC would be consistent with our finding that the extent of inhibition depends on the nature of the substrate

anion. IMAC differs from many other anion channels in that it is able to conduct many multivalent anions including those as large as ATP^{4-}. Thus, to explain the relatively easy passage of multivalent anions, it is not unreasonable to assume that a site or sites must exist along the transport pathway which favor the binding of an anion with several charges. If this were the case, an anion such as DIDS could bind to these sites and prevent the entry of a large multivalent substrate anion while leaving a sufficient number of charged or polar groups available to facilitate the passage of small singly charged anions such as Cl^-, NO_3^-, and HCO_3^-. In the absence of the inhibitor, it is quite possible that several Cl^- ions may have to enter the channel to permit rapid transport. This type of behavior is characteristic of so-called multi-ion pores (Hille, 1992). Recently, it has been demonstrated that the cystic fibrosis transmembrane regulator (CFTR) channel is a multi-ion pore for conduction of Cl^- (Tabcharani *et al.*, 1993) and that this pore can also conduct ATP (Reisin *et al.*, 1994). It does not, however, appear to be blocked by DIDS (Tabcharani *et al.*, 1991).

The potencies of the stilbene-disulfonates tested in this study fall in the order DIDS > H_2-DIDS > SITS > DNDS > ANDS. Thus, it is evident that the isothiocyanate groups play an important role in binding. We have no evidence, however, that inhibition is irreversible. Different potency sequences have been reported for other channels. Tilmann *et al.* (1991), find that the intermediate-conductance outwardly rectifying epithelial Cl^- channel is inhibited most potently by DNDS (8 μM IC_{50}) while SITS and DIDS have IC_{50} values of 100 and 80 μM respectively. For the large-conductance Cl^- channel of vascular smooth muscle, Kokubun *et al.* (1991) report that DIDS (IC_{50} = 10 μM) is 10-fold more potent than SITS and also apparently more potent than DNDS. The large-conductance Cl^- channel in vascular endothelial cells (Groschner and Kukovetz, 1992), however, has an IC_{50} for DIDS of about 100 μM. Inhibition of the K_{ATP} channel by stilbene disulfonates follows a potency sequence, albeit with higher values, similar to the one we observe here, with IC_{50} values of 71 μM DIDS, 390 μM for SITS, and 590 μM for DNDS (Furukawa *et al.*, 1993). In a recent study, Venglarik *et al.* (1994) have investigated inhibition of an outwardly rectifying chloride channel by DNDS, ANDS, and DADS, which unlike DIDS are reversible blockers of this channel, and find that the electron-withdrawing–NO_2 groups are very important. From this small sampling of data from the literature, it is evident that both the absolute and relative potencies of the stilbene disulfonates vary from one channel type to another, as one might expect for binding to different sites on different proteins. It is also clear, however, that the doses necessary to block IMAC fall well within the range observed for other anion channels. Thus, if our conclusion that DIDS probably inhibits IMAC by binding to the same site as Cibacron blue is correct, it becomes quite possible that many other channels which are reversibly blocked by DIDS may also be inhibited by Cibacron blue, palmitoyl CoA, or agaric acid.

The findings in this paper provide further weight to the argument that IMAC may not be very different from many anion channels found in other membranes which have been characterized mainly by electrophysiological means. We should point out, however, that the effects of stilbene disulfonates are not limited to anion transport pathways. As mentioned above, recently it has been reported that a K_{ATP} channel is inhibited by DIDS (Furukawa *et al.*, 1993). Other transport pathways reported to be blocked by DIDS include a pyruvate/lactate transport system in cardiac myocytes (Wang *et al.*, 1993), a CoA transport pathway in heart mitochondria (Tahiliani *et al.*, 1992), as well as an ATP transporter in yeast endoplasmic reticulum (Mayinger and Meyer, 1993). In contrast, two anion channels from the mitochondrial inner membrane which have been found using electrophysiological methods, the MCS or 107 pS anion channel first described by Sorgato [see Sorgato and Moran (1993) for a review] and an anion channel studied by Hayman *et al.* (1993), are reported not to be sensitive to inhibition by DIDS. While we were preparing this manuscript, a report appeared presenting evidence that in the presence of Ca^{2+} the Ca^{2+}-dependent permeability transition pore of the inner mitochondrial membrane is activated by DIDS (75 μM) (Bernardes *et al.*, 1994). We do not expect this pore, which is able to transport cations, anions, and nonelectrolytes such as sucrose, to be active under the conditions of our experiments, since our media contain EGTA, the mitochondria are deenergized, and measurements are made on a relatively short time scale.

In addition to inhibition by DIDS, IMAC has other properties shared by other anion channels (see Hille, 1992). These include a broad selectivity and a finite conductance to cations especially in the presence of certain anions (Beavis and Garlid, 1986). Other common properties which have been suggested include inhibition by Zn^{2+} (e.g., Kokubun *et al.*, 1991; Woll *et al.*, 1987; Groschner and Kukovetz, 1992; Harrison

and Gibbons, 1994) which Selwyn *et al.* (1993) have demonstrated is a very potent inhibitor of IMAC. Interestingly, Selwyn's group (Ng *et al.*, 1993b) has also reported that matrix phosphate is an activator of IMAC, and recently Carson *et al.* (1994) have reported that the open probability of CFTR Cl^- channels is also increased by phosphate.

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