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Minireview

Properties of the Inner Membrane Anion Channel in Intact Mitochondria

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The mitochondrial inner membrane possesses an anion channel (IMAC) which mediates the electrophoretic transport of a wide variety of anions and is believed to be an important component of the volume homeostatic mechanism. IMAC is regulated by matrix Mg^{2+} (IC_{50} = 38 μM at pH 7.4) and by matrix H^+ (pIC_{50} = 7.7). Moreover, inhibition by Mg^{2+} is pH-dependent. IMAC is also reversibly inhibited by many cationic amphiphilic drugs, including propranolol, and irreversibly inhibited by *N,N'*-dicyclohexylcarbodiimide. Mercurials have two effects on its activity: (1) they increase the IC_{50} values for Mg^{2+}, H^+, and propranolol, and (2) they inhibit transport. The most potent inhibitor of IMAC is tributyltin, which blocks anion uniport in liver mitochondria at about 1 nmol/mg. The inhibitory dose is increased by mercurials; however, this effect appears to be unrelated to the other mercurial effects. IMAC also appears to be present in plant mitochondria; however, it is insensitive to inhibition by Mg^{2+}, mercurials, and *N,N'*-dicyclohexylcarbodiimide. Some inhibitors of the adenine nucleotide translocase also inhibit IMAC, including Cibacron Blue, agaric acid, and palmitoyl CoA; however, atractyloside has no effect.

KEY WORDS: Anion channel; anion uniport; transport in mitochondria; volume homeostasis.

INTRODUCTION

It is now generally accepted that the large protonmotive force ($\Delta\tilde{\mu}_{H^+}$) generated by the proton pumps of the mitochondrial respiratory chain is used by the F_1F_0ATPase to drive ATP synthesis. This chemiosmotic mechanism of coupling has the advantage that the energy from respiration can be directly used to drive other processes in addition to ATP synthesis. This is achieved by exploiting transport processes in which a net translocation of electric charge takes place. Such transport is referred to as "electrophoretic" transport and is driven by the difference in electrochemical potential ($\Delta\tilde{\mu}$) of the "reactants" and "products." Examples include antiport of ATP^{4-} for ADP^{3-}, antiport of asparate$^-$ for glutamate$^-$ + H^+, uniport of Ca^{2+}, and reduction of $NADP^+$ by the transhydrogenase. Since all these processes are dependent on the maintenance of a high membrane potential, an efficient volume homeostatic mechanism is required to preserve the integrity of the inner membrane. This mechanism also appears to use the protonmotive force itself as a source of energy.

Mitochondria are near perfect osmometers [see Beavis *et al.* (1985) and references therein]; therefore, their volume has to be regulated by controlling the net salt flux across the inner membrane. Respiration can drive both influx and efflux of salts. The influx mechanism, shown in Fig. 1A, employs electrophoretic cation uniport and electroneutral anion-H^+ symport pathways. K^+ influx, driven by the high negative inside membrane potential generated by the proton pumps of the respiratory chain, leads to generation of a pH gradient which drives the uptake of Pi via the classical phosphate carrier as well as the secondary uptake of dicarboxylates and tricarboxylates via the classical anion exchange carriers [see LaNoue and

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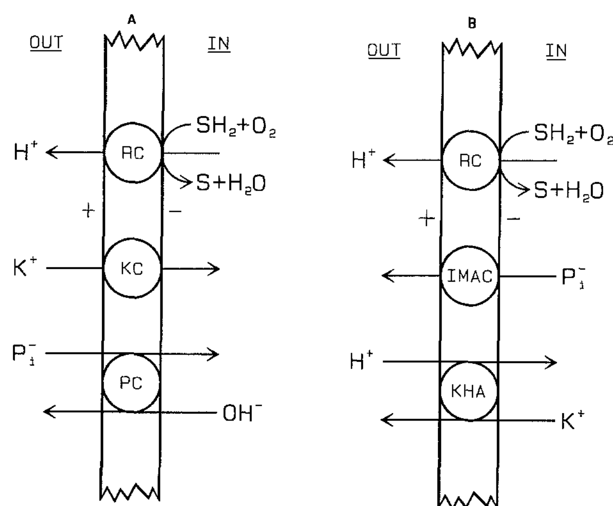


Fig. 1. Respiration-driven salt fluxes in mitochondria. The proton pumps of the respiratory chain (RC) generate an electrochemical proton gradient ($\Delta\tilde{\mu}_H$). A. Salt influx. The membrane potential drives the electrophoretic influx of K^+ via a K^+ channel (KC) or leak pathway, and the pH gradient drives the electroneutral influx of phosphate via the phosphate carrier (PC). B. Salt efflux. The membrane potential drives the electrophoretic efflux of anions via IMAC, and the pH gradient drives the electroneutral efflux of K^+ via the K^+/H^+ antiporter (KHA) or the ionophore nigericin.

Schoolwerth (1979)]. In contrast, the efflux mechanism, shown in Fig. 1B, employs electrophoretic anion transport and electroneutral cation/proton antiport pathways. In this case, the membrane potential drives the efflux of anions, and the pH gradient drives the efflux of K^+ via the K^+/H^+ antiporter. Thus, the matrix volume can be regulated by modulating one or more of the four types of passive ion flux involved. Note that when these fluxes occur simultaneously the system can also be viewed as two energy-consuming futile cycles, one involving K^+ and one involving anions.

Regulation of electroneutral anion transport pathways does not appear to be, nor is it expected to be, important in volume homeostasis, since these pathways are important in many metabolic pathways including ATP synthesis. Regulation of the anion uniport pathways and the K^+/H^+ antiporter and possibly the K^+ uniport pathway is, however, thought to be very important. The properties of the K^+/H^+ antiporter (Garlid, 1988; Brierley and Jung, 1988) and K^+ uniporter or channel (Diwan, 1988) have been reviewed recently. Also, Garlid and Beavis (1986) have reviewed much of the pioneering work on the anion uniport pathway carried out by the groups of Azzone, Brierley, and Selwyn and have proposed that a specific anion channel is responsible for anion uniport in mitochondria. This pathway, now referred to as the inner membrane anion channel or IMAC,[2] and its properties, many of which are summarized by the cartoon shown in Fig. 2, are the subject of the present review.

ASSAY OF IMAC IN INTACT MITOCHONDRIA

IMAC differs from most other inner membrane anion transporters in that it is regulated. In fact, in normal mitochondria isolated from heart and liver, it has negligible activity due to inhibition by matrix H^+ and matrix Mg^{2+} and this inhibition must be relieved before IMAC can be assayed. In each of the assays described below a different procedure is used to activate IMAC; however, in all three, influx of anions driven by the anion concentration gradient is assayed using light scattering to follow changes in mitochondrial volume secondary to net salt influx. If the anion is transported electrophoretically, net salt influx only takes place if there is a mechanism by which cation entry can balance the electric charge entering with the anion. For example, when K^+ salts are used, the K^+ ionophore valinomycin may be added; however, net K^+ transport can also be achieved by coupling electroneutral K^+/H^+ antiport mediated by nigericin to the electrophoretic transport of H^+ mediated by a protonophore such as CCCP.

Most of the studies discussed in this review have employed an assay developed by Beavis and Garlid (1987) in which IMAC is activated by depleting matrix divalent cations with the ionophore A23187. Typical data obtained with KCl are shown in Fig. 3A. Addition of nigericin alone does not induce swelling (an increase in β), showing that no Cl^-/OH^- exchange mechanism exists. Subsequent addition of A23187, which catalyzes $Mg^{2+}/2H^+$ exchange, induces the efflux of about 28 nmol Mg^{2+}/mg an a consequent small increase in volume as the osmotically inactive

[2]Abbreviations: IMAC, inner membrane anion channel; EGTA, [ethylene bis (oxyethylenenitrilo)]tetraacetic acid; p-CMS, *p*-chloromercuribenzenesulfonate; NEM, *N*-ethylmaleimide; TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; LS, light scattering; DCCD, *N,N'*-dicyclohexylcarbodiimide; TBT, tributyltin; TϕT, triphenyltin; TMT, trimethyltin; TET, triethyltin; TPT, tripropyltin; Val, valinomycin; Nig, nigericin; TMPD, tetramethyl-*p*-phenylenediamine.

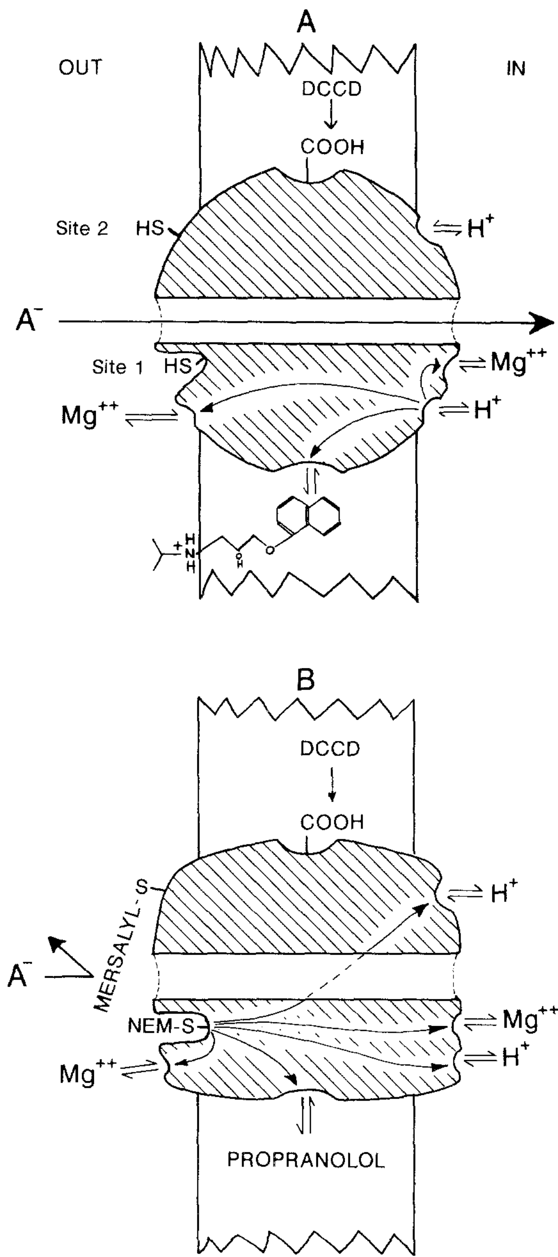
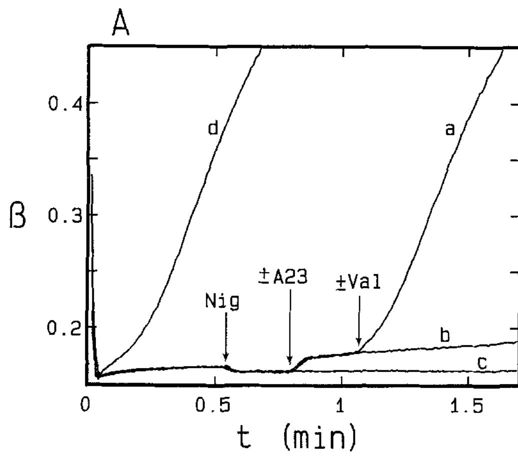


Fig. 2. Hypothetical model summarizing some properties of IMAC. A. IMAC is an anion uniport pathway which is inhibited by reaction with DCCD, matrix H^+, matrix Mg^{2+}, cationic amphiphiles, and cytosolic Mg^{2+}. A second H^+ binding site in the matrix decreases the IC_{50} for cationic amphiphiles, matrix Mg^{2+}, and cytosolic Mg^{2+}. Two thiol groups also exist on the outside; one (site 1) lies in a cleft, whereas the other is more exposed and close to the channel mouth or substrate binding site. B. Reaction of NEM (or mersalyl) at site 1 induces a conformational change which leads to an increase in IC_{50} for matrix H^+, matrix Mg^{2+}, cationic amphiphiles, and cytosolic Mg^{2+}. It may also decrease the affinity of the regulatory protonation site. Site 2 reacts with mersalyl but not NEM and due to a steric effect inhibits the transport of most anions. (Reproduced from Beavis, 1991.)

Mg^{2+} is exchanged for K^+ which enters via nigericin (Beavis and Garlid, 1990). Rapid swelling is not observed until valinomycin (or CCCP) is added (Fig. 3A, trace a). Similar rates of swelling are obtained if all three ionophores are added to the medium at zero time (trace d). Because K^+ is the most abundant endogenous cation in the mitochondrial matrix, use of K^+ salts in this assay has two advantages. First, the membrane potential will be close to zero due to the presence of valinomycin, especially if respiration is inhibited. Second, the transmembrane ΔpH will be minimized and buffered by nigericin-mediated K^+/H^+ antiport.

A second assay procedure, used extensively by Selwyn's group, does not require depletion of matrix Mg^{2+} to unmask the activity of IMAC. Instead, NH_4^+ salts are used at pH 8.0. Since the inner membrane is freely permeable to NH_3, the entry of NH_3 alkalinizes the matrix and this activates IMAC (Beavis and Garlid, 1987). Net salt transport is made possible by simply including a protonophore in the assay medium. Typical data are shown in Fig. 3C (trace a). The disadvantage of this assay is that the rate of swelling is dependent on the time of addition of CCCP. For example, if the addition of CCCP is delayed for 1 min, the rate of transport is decreased by 40% (trace b). This is a result of a decline in the pH gradient as matrix K^+ is lost via the K^+/H^+ antiporter (Beavis and Garlid, 1987). Nigericin can be added to equilibrate K^+/H^+ antiport; however, as shown in Fig. 3C (trace c), even with the medium at pH 8 this leads to very low transport rates.

A third assay procedure which unmasks the activity of IMAC was first demonstrated by Brierley (1970) using heart mitochondria. He showed that when mitochondria are allowed to respire in KCl in the absence of permeant acids, rapid swelling is induced by the addition of valinomycin, even at pH 7.0. Results of a similar experiment with liver mitochondria are shown in Fig. 3E (trace a). The mechanism of salt transport under these conditions can be best understood after examination of a simultaneous recording of the medium pH (trace b). Although addition of valinomycin induces immediate and rapid H^+ ejection by the respiratory chain, the rate of swelling does not become maximal until the extent of H^+ ejection reaches a maximum of about 54 nmol H^+/mg. From the buffering power of the matrix, Beavis (1989a) has calculated that at the peak the transmembrane ΔpH approaches 3 units. At this point, the membrane potential will be close to zero.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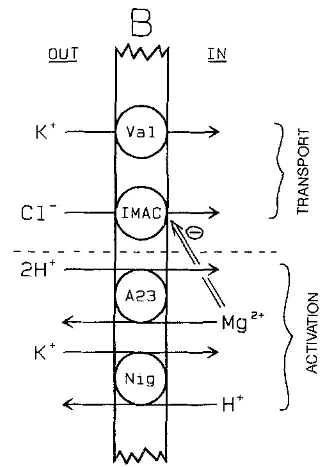
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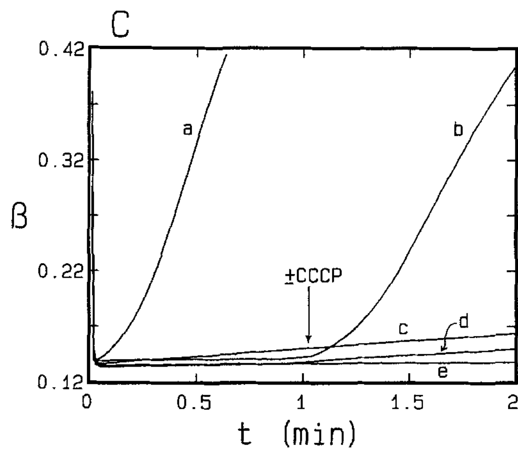
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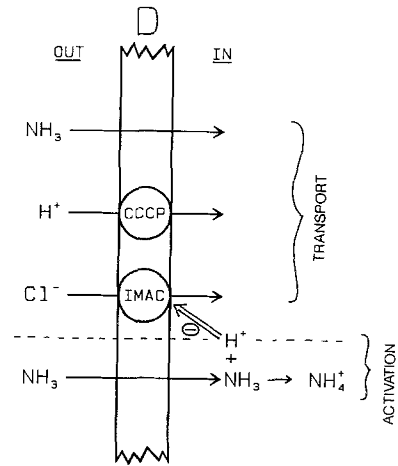
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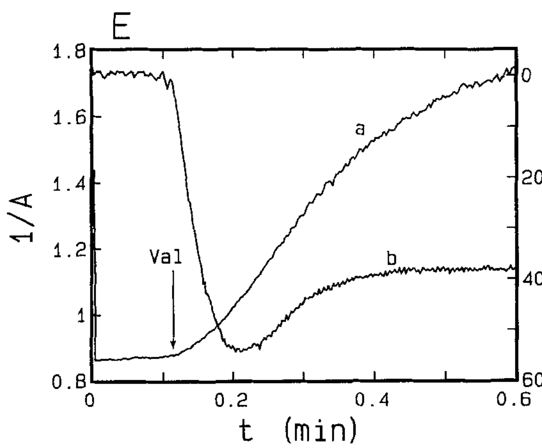
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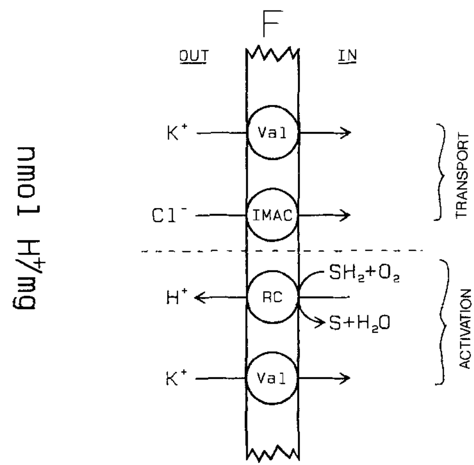
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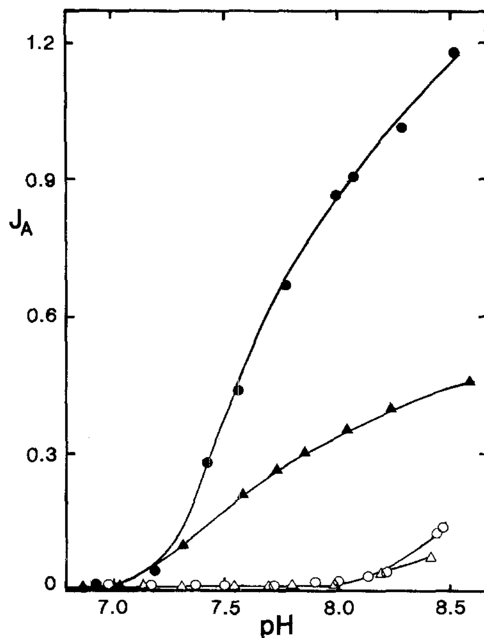

Fig. 4. The pH dependence of Cl^- and malonate uniport via IMAC. The rate of anion transport (J_A, μmol/min · mg) determined using the Beavis assay is plotted versus the pH of the assay medium. (●) Cl^- transport, plus A23187; (○) Cl^- transport, minus A23187; (▲) malonate transport, plus A23187; (△) malonate transport, minus A23187. (Reproduced from Beavis and Garlid, 1987.)

Thus, respiration does not drive transport, as it does when permeant acids are present, but simply activates IMAC by alkalinizing the matrix (cf. Figs. 1A and 3F). Addition of CCCP or nigericin to this assay prevents the generation of a pH gradient and inhibits swelling 98%. For convenience, the assay types shown in Figs. 3A, 3C, and 3E will be referred to as the Beavis assay, the Selwyn assay, and the Brierley assay, respectively, throughout the remainder of this review.

REGULATION OF IMAC BY PROTONS

From the preceding discussion, it is evident that IMAC is regulated by protons. In fact, when Selwyn *et al.* (1979) first proposed that a specific protein must be responsible for this electrophoretic transport, he called it the pH-dependent anion-conducting pore. From the effect of nigericin on the rate of swelling in NH_4^+ salts (Fig. 3C), and the activation of IMAC by respiration (Fig. 3E), it has been concluded that the regulatory site lies on the matrix side of IMAC (Brierley, 1970; Selwyn *et al.*, 1979; Beavis and Garlid, 1987).

The pH dependence of IMAC in Mg^{2+}-depleted mitochondria has been studied using the Beavis assay (Beavis and Garlid, 1987). As indicated in Fig. 4, the rate is negligible at pH 7.0 but rises rapidly as the pH is raised above 7.2. Extrapoltion to a J_{max} allows Hill plots to be drawn and pIC_{50} values of 7.8 and 7.7 to be estimated for Cl^- and malonate, respectively. The J_{max} ($[H^+] \to 0$) for Cl^- is 1.35 μmol/min · mg, which probably makes IMAC the fastest anion-transport pathway in mitochondria. If nigericin is omitted, transport rates are much less sensitive to changes in medium pH, consistent with location of the inhibitory site in the matrix. Figure 4 also shows that in the absence of A23187, transport rates are much slower even at the highest pH values employed. It is also noteworthy that IMAC can be inhibited after swelling has reached its maximum rate by addition of acid, indicating that activation is reversible and that the effect is direct.

Fig. 3. Assay of IMAC in intact mitochondria. LS kinetics of mitochondria (0.1 mg/ml) suspended in Cl^- salts at 25°C are shown. β is a LS parameter which normalizes reciprocal absorbance for mitochondrial concentration, and is a function of mitochondrial volume; see Beavis *et al.* (1985) and Garlid and Beavis (1985). A. Beavis assay. Addition of nigericin (Nig, 1 nmol/mg), A23187 (A23, 10 nmol/mg), and valinomycin (Val, 0.5 nmol/mg) induces swelling in KCl at pH 7.4. The rates of Cl^- transport (nmol/min · mg) determined from the traces shown are: trace a, 418; trace b (no Val), 10; trace c (no Val, no A23), 0; trace d (ionophores added before the mitochondria), 445. The assay medium contained the K^+ salts of Cl^- (55 mM), TES (5 mM), EDTA (0.1 mM), EGTA (0.1 mM), and rotenone 2 μg/mg. B. Fluxes involved in panel A. A23187 and Nig allow net exchange of matrix Mg^{2+} for medium K^+. IMAC and valinomycin permit net influx of KCl. C. Selwyn assay. Addition of CCCP (10 μM) induces swelling in NH_4Cl at pH 8.0. The rates of Cl^- transport are: trace a (CCCP added at $t = 0$) 730; trace b (CCCP added at $t = 1$) 420; trace c (CCCP and Nig added at $t = 0$) 17; trace d (Nig at $t = 0$, CCCP at $t = 1$) 12; trace e (Nig at $t = 0$, no CCCP). The assay medium contained the NH_4^+ salts of Cl^- (100 mM) and TES (5 mM). D. Fluxes involved in panel C. Influx of NH_3 alkalinizes the matrix which activates IMAC. Addition of CCCP then allows net influx of NH_4Cl. E. Brierley assay. Addition of valinomycin induces swelling in respiring mitochondria suspended in KCl at pH 7.0. Simultaneous recordings of LS (trace a) and medium pH (trace b) are shown. Valinomycin (Val, 0.5 nmol/mg) was added where indicated [see Beavis (1989a) for further details]. The maximum rate of Cl^- transport is 700 nmol/min · mg. The assay medium was similar to that described in A with the addition of succinate (5 mM); also, TES was decreased to 2 mM and the mitochondrial concentration was increased to 1.8 mg/ml to allow H^+ fluxes to be followed. F. Fluxes involved in panel E. Respiratory chain H^+ ejection drives sufficient Val-mediated K^+ uptake to alkalinize the matrix and activate IMAC. Val and IMAC then mediate net influx of KCl.

REGULATION OF IMAC BY DIVALENT CATIONS

Inhibition of IMAC by endogenous matrix divalent cations is evidenced by the effect of A23187 on anion uniport (Fig. 3A). Moreover, using a modification of the Beavis assay, it can be readily shown that exogenous Mg^{2+} can inhibit IMAC. Dose–response curves obtained with this assay at pH 7.4 are shown in Fig. 5A and yield an IC_{50} value of 38 μM (Beavis and Powers, 1989). Similar experiments carried out with Ca^{2+} yield an IC_{50} of 16 μM. These findings clearly demonstrate that matrix Mg^{2+} inhibits IMAC; however, they do not indicate whether external Mg^{2+} can also inhibit. Beavis and Powers (1989) used several approaches to answer this question. These include investigation of the effect of Mg^{2+} on mitochondria which have been depleted of endogenous Mg^{2+} by a long pretreatment with a very low dose of A23187 followed by a wash to remove the A23187, and also an investigation of the effect of Mg^{2+} on IMAC in the absence of A23187 using the Brierley assay. Inhibition of IMAC by Mg^{2+} could not be eliminated; however, the IC_{50} under these conditions is 10-fold higher. Consequently, it is believed that there is regulatory site on the cytosolic side which is different from that in the matrix. This finding also suggests that IMAC is inserted asymmetrically in the membrane.

In view of the low IC_{50} value obtained for Mg^{2+}, an explanation is required for why such rapid transport can be observed in the absence of A23187 in the Brierley and Selwyn assays. The answer is suggested by the data contained in Fig. 5B which show that the IC_{50} for Mg^{2+} is pH dependent, rising from 38 μM at pH 7.4 to about 270 μM at pH 8.4. Analysis of the data reveals that the relationship is consistent with a model in which Mg^{2+} only binds to a protonated form of the channel with $K_{Mg^{2+}}$ and pK_H values of less than 10 μM and 7.0, respectively. This pK value is too low for this protonation site to be the inhibitory site discussed above; therefore another protonation site is believed to be responsible. In order to explain the rapid swelling in the Selwyn assay and the Brierley assay, Beavis and Powers (1989) propose that this second protonation site is also located in the matrix. If this is the case, then on the basis of the data in Fig. 5B, one can calculate that if the matrix pH were 10 during respiration-induced swelling, the IC_{50} should be 10.6 mM. Consistent with this prediction, addition of A23187 to the Brierley assay has only a small stimulatory effect. Examination of the effect of pH on inhibition by external Mg^{2+} reveals that this also is dependent on pH.

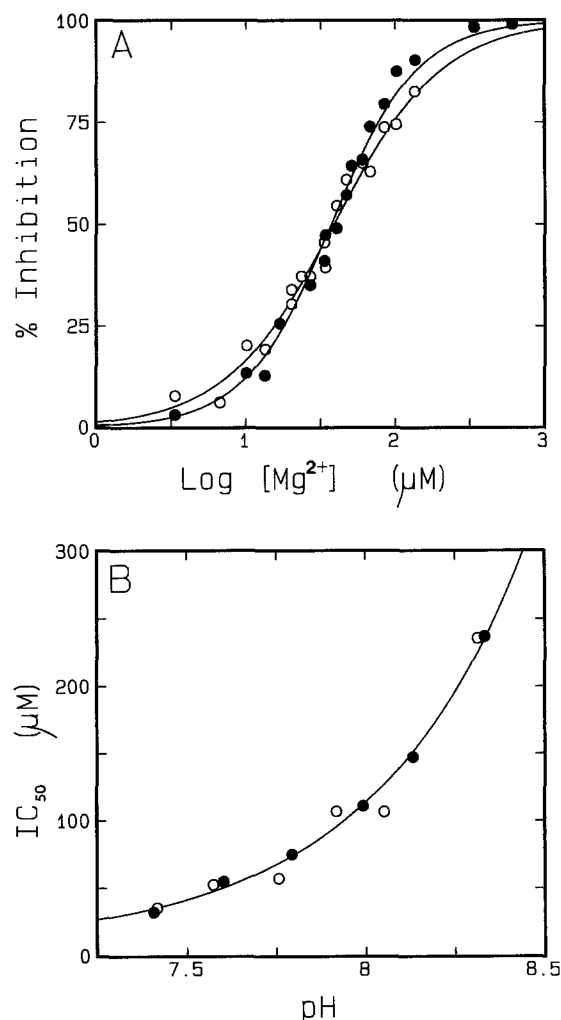

Fig. 5. Inhibition of IMAC by Mg^{2+}. A. Percent inhibition of Cl^- uniport is plotted versus $\log_{10}$ of the medium Mg^{2+} concentration. Two procedures were used: •, Mg^{2+}, A23187 (10 nmol/mg), nigericin (1 nmol/mg), and rotenone (2 μg/mg) were added to the assay medium at zero time, before addition of the mitochondria. ○, mitochondria were pretreated with A23187 (10 nmol/mg) and EDTA, and then transferred to the assay medium which contained Mg^{2+}, nigericin, and rotenone. In both experiments, valinomycin was added at 0.2 min to initiate KCl transport. The control rates were determined in the absence of added Mg^{2+}. •, IC_{50} = 37.2 μM, Hill slope = 1.50; ○, IC_{50} = 38.8, Hill slope = 1.19. **B.** The Mg^{2+} IC_{50} determined as above is plotted versus the pH of the assay medium. (Reproduced from Beavis and Powers, 1989.)

Selwyn *et al.* (1990) have recently suggested that IMAC may be activated by low levels of matrix Ca^{2+}. Using the Selwyn assay, they find that swelling rates are increased up to 2.5-fold by addition of Ca^{2+} to the assay medium provided the mitochondria have been

allowed to respire in the stock suspension. The effect is maximal at 5 μM Ca^{2+} and Selwyn *et al.* (1990) have attributed it to inhibition of the loss of endogenous Ca^{2+} accumulted in the stock suspension. However, in view of the apparent sensitivity of anion uniport to changes in matrix pH, the stimulation could be indirect and result from a decrease in CCCP-mediated influx of protons coupled to the Ca^{2+} efflux or elevation of matrix pH by inhibition of K^+ loss via the K^+/H^+ antiporter. There is also a possibility that this change in permeability may be related to the Ca^{2+}-induced permeability transition (see Gunter and Pfeiffer, 1990). Thus, this interesting and potentially important finding deserves further investigation.

Table I. Substrates for IMAC in Liver Mitochondria[a]

Anion	J	Anion	J
SCN^-	1618	malate^{2-}	134
NO_3^-	808	1,2,4, BTCA^{3-b}	110
ClO_4^-	789	α-ketoglutarate[b]	106
HCO_3^-	630	1,2,4,5 BTCA^{4-b}	75
Cl^-	418	fumarate^{2-}	70
SO_4^{2-}	387	1,3,5 BTCA^{3-b}	52
$Pi^{1 1/2-}$	267	ATP^{4-b}	50
1,2,3-BTCA^{3-}	262	p-aminohippurate$^-$	26
$Fe(CN)_6^{3-}$	259	adipate^{2-}	12
maleate^{2-b}	230	gluconate$^-$	< 1
butylmalonate^{2-}	212	glucuronate$^-$	< 1
oxaloacetate^{2-b}	190	$EDTA^{3-}$	< 1
$Fe(CN)_6^{4-}$	178	$EGTA^{2-}$	< 1
citrate^{3-}	164	TES	< 1
malonate^{2-}	140		

[a]Rates of anion transport (nmol/min · mg) determined using the Beavis assay are shown. For the measurements of HCO_3^- transport, acetazolamide (40 μM) was added to the medium to block carbonic anhydrase. For measurements of Pi transport the mitochondria were pretreated with *N*-ethylmaleimide (40 nmol/mg) to block the electroneutral Pi^--H^+ symporter. For measurements of ferrocyanide transport 0.5 mM CN^- was added to prevent oxidation of ferrocyanide and 0.5 nM ascorbate was added to reduce any ferricyanide formed. ATP transport was determined in mitochondria treated with oligomycin (1 nmol/mg) and carboxyatractyloside (0.5 nmol/mg) and was completely blocked by DCCD, mersalyl, TBT, and Cibacron Blue. All media were 110 millosmolal (equiosmolal with 60 mM KCl).

[b]A. D. Beavis, unpublished data; all other data are from Beavis and Garlid (1987).

SELECTIVITY OF IMAC

IMAC transports a wide variety of physiological and nonphysiological anions, in fact, it is difficult to find an anion which is not transported. However, as shown by the data in Table I, all anions are not transported at equal rates. Small monovalent anions such as Cl^- and NO_3^- are transported most rapidly, while large monovalent anions such as glucuronate are transported slowly. Multivalent anions such as 1,2,3-benzenetricarboxylate and ferrocyanide are also transported rapidly. For multivalent anions the rate appears to depend more on the charge distribution in the molecule than on its size. Thus, butylmalonate is transported about 18-fold faster than adipate, 1,2,3-benzenetricarboxylate is transported 5-fold faster than 1,3,5-benzenetricarboxylate, and maleate is transported 3-fold faster than fumarate. One of the largest anions which can be transported via IMAC is ATP (A. D. Beavis, unpublished data).

IMAC IS INHIBITED BY *N,N'*-DICYCLOHEXYLCARBODIIMIDE (DCCD)

DCCD is a hydrophobic alkylating agent which reacts with a number of different mitochondrial membrane proteins [see Azzi *et al.* (1984) for review], including the K^+/H^+ antiporter (Martin *et al.*, 1984) and putative K^+ uniporter (Gauthier *et al.*, 1979; Jung *et al.*, 1980; Hegazy *et al.*, 1991). It is usually thought to react with carboxyl groups located within the lipid bilayer; however, it can also react with other amino acid residues. Warhurst *et al.* (1982) were the first to show that DCCD inhibits anion uniport activated by alkaline pH, and Beavis and Garlid (1983, 1988) showed that it could completely block the Cl^- flux induced by depletion of matrix divalent cations. Unlike the K^+/H^+ antiporter, IMAC is blocked by DCCD without requiring depletion of matrix divalent cations, without lowering medium osmolality, and without raising medium pH. Inhibition is also observed using the Brierley assay. These findings provide evidence that the same pathway is responsible for anion tansport in each of the three assays. DCCD appears to have no effect on the Pi, dicarboxylate, or tricarboxylate carriers, suggesting that anion uniport is not mediated by one of these proteins.

INHIBITION OF IMAC BY CATIONIC AMPHIPHILES

Quite a large variety of drugs inhibit IMAC, including the β-blocker propranolol, the antiarrhythmic amiodarone, the tricyclic antidepressants amitrip-

Table II. IC_{50} Values and Hill Slopes for Inhibition of Cl^- Transport by Various Drugs[a]

	A23187-induced transport, Beavis assay		Respiration-induced transport, Brierley assay	
Drug	IC_{50} (μM)	Hill slope	IC_{50} (μM)	Hill Slope
Amiodarone	0.6	1.51	1.41	1.10
Amitriptyline	13	1.53	53	1.68
Imipramine	15	1.30	90	1.37
Dibucaine[b]	20	1.40	N.D.	N.D.
Propranolol	25	1.21	174	1.06
RO5-4864	34	1.72	N.D.[c]	N.D.
Quinine	63	1.26	371	1.15
Clonazepam	220	1.89	N.D.	N.D.
Bupivacaine[b]	440	1.15	N.D.	N.D.
Etidocaine[b]	470	1.40	N.D.	N.D.
Pindolol	629	1.26	N.D.	N.D.
Timolol	1930	1.14	N.D.	N.D.
Lidocaine[b]	[d]	—	N.D.	N.D.
Benzocaine	[e]	—	[e]	—

[a] IC_{50} values (μM) and respective Hill slopes are shown for inhibition of A23187-induced and respiration-induced Cl^- transport. The data were collected and plotted as described in Beavis (1989a).
[b] A. D. Beavis, unpublished data. All other data are from Beavis (1989a).
[c] N.D., not determined.
[d] 25% inhibition was observed with 1.5 mM lidocaine.
[e] No inhibition was observed up to a concentration of 0.5 mM benzocaine.

tyline and imipramine, the antimalarial quinine, and the local anesthetics dibucaine and bupivacaine (see Table II). These drugs are all cationic amphiphiles and possess a hydrophobic moiety which is composed of 1–3 rings. The potency appears to increase with the number of rings in the hydrophobic moiety. The charge does not appear to be essential for inhibition since benzodiazepines such as Ro5-4864 and clonazepam also inhibit. Due to these broad structural requirements, Beavis (1989a) has proposed that inhibition results from interaction with IMAC at a site located in the lipid bilayer. Consistent with this conclusion is the recent report by Zernig *et al.* (1990) that many Ca^{2+} antagonists, including dihydropyridines, phenylalkylamines, and benzothiazepines inhibit IMAC in guinea pig liver mitochondria.

Inhibition by these agents appears to be reversible, rapid, and complete, i.e., analysis of the dose–response curves indicates that inhibition approaches 100%. When the effect of these agents is investigated using the Brierley assay, it is found that the IC_{50} values are about 6-fold higher (Table I). This can be explained by a dependence of the IC_{50} on matrix pH, since the IC_{50} is found to be pH-dependent using the Beavis assay (see Fig. 7). The relationship is quite complex; however, two different models (Beavis, 1989a, 1991) suggest that drug binding is dependent on the protonation of a site with a p*K* between 6 and 7. Since Mg^{2+}-binding also appears to require protonation of a site with similar p*K*, as suggested in Fig. 2, the same regulatory protonation site may be involved. This pH dependence also explains the high IC_{50} reported by Selwyn *et al.* (1978) for dibucaine using the Selwyn assay.

EFFECTS OF MERCURIALS AND *N*-ETHYLMALEIMIDE (NEM) ON IMAC

Mercurials react with and inhibit many transport proteins in the inner mitochondrial membrane including the phosphate, dicarboxylate, tricarboxylate, oxoglutarate, and pyruvate carriers [see Fonyo (1979) for review]. Thus, it is not surprising that IMAC too is affected by mercurials. On the basis of studies which showed that Pi could be transported via IMAC in NEM-treated mitochondria and reports which suggested that Pi transport could take place

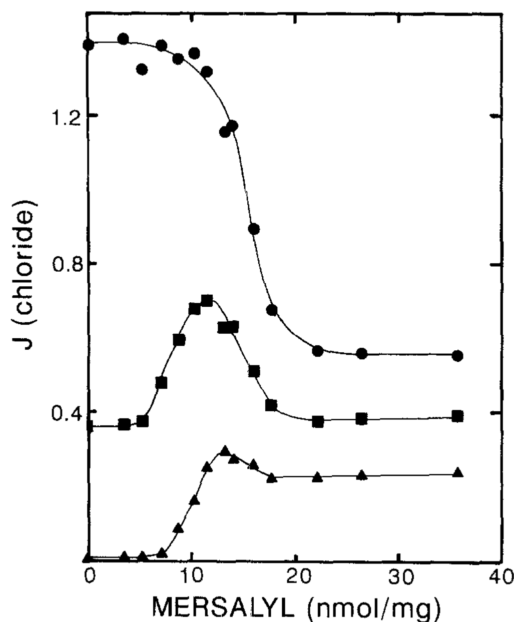

Fig. 6. Inhibition of Cl^- transport by mersalyl. Rates of Cl^- transport (J, μmol/min · mg) assayed at pH values of 8.4 (●), 7.4 (■), and 6.9 (▲) using the Beavis assay are plotted versus the dose of mersalyl. The mitochondria were pretreated with mersalyl under identical conditions before being transferred to the assay medium. (Reproduced from Beavis, 1989b.)

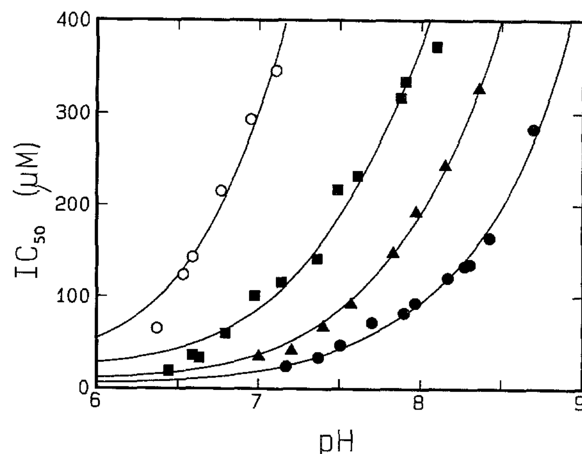

Fig. 7. Effect of NEM and mersalyl on pH dependence of propranolol IC_{50} for inhibition of IMAC. IC_{50} values for inhibition of Cl^- uniport are plotted versus the pH of the assay medium. ●, control mitochondria; ▲, NEM-pretreated (50 nmol/mg) mitochondria; ■, mersalyl-pretreated (25 nmol/mg) mitochondria assayed with 1 mM cysteine in the assay medium; ○, mersalyl-pretreated (25 nmol/mg) mitochondria. (Reproduced from Beavis, 1991.)

in the presence of mercurials (Scott *et al.*, 1970; Reynafarje and Lehninger, 1978; Bogucka and Wojtczak, 1979), Garlid and Beavis (1986) suggested that mercurials may activate IMAC. Subsequent investigation, however, has revealed that mersalyl is in fact a potent inhibitor of IMAC-mediated transport of anions including malonate, Pi, ferrocyanide, and ATP (Beavis, 1989b). Inhibition is essentially complete at 20 nmol/mg, which is the same dose required to block the dicarboxylate carrier, suggesting that a group with similar affinity, probably a thiol, is involved. Furthermore, this inhibition is reversed by addition of cysteine, thioglycolate, etc. to the assay medium.

Surprisingly, when the effect of mersalyl on the transport of Cl^-, NO_3^-, and HCO_3^- was first examined at pH 7.4, no inhibition was observed (Beavis, 1989b). However, when IMAC is stimulated by raising the assay pH to 8.4, Cl^- transport can be inhibited 60% by mersalyl (see Fig. 6). In addition, the apparant lack of inhibition at pH 7.4 was found to result from a combintion of two effects—stimulation and inhibition —with the stimulatory effect occurring at slightly lower doses than the inhibitory effect. The stimulatory effect is most apparent when Cl^- transport is assayed at pH 6.9 where IMAC is normally inhibited by protons. Two mercurial reactive sites appear to be involved, since both stimultion and inhibition can be observed at the same pH (Fig. 6). Moreover, unlike the inhibitory effect, the stimulatory effect is not reversed by thioglycolate or other thiols. This latter finding allowed Beavis (1989b) to demontrate that pretreatment with mersalyl could also "stimulate" the transport at low pH of other substrates of IMAC such as malonate. Beavis (1991) went on to show that *N*-ethylmaleimide, which does not inhibit IMAC, also stimulates both Cl^- and malonate uniport at low pH. Investigation of the pH dependence of anion uniport in mersalyl- and NEM-treated mitochondria reveals that the stimulation is not due to an increase in J_{max} ($[H^+] \rightarrow 0$), but to a decrease in the pIC_{50} for H^+. These findings make it unnecessary to postulate the existence of a separate mersalyl-activated transport pathway for Cl^-, NO_3^-, and HCO_3^-.

In addition to increasing the IC_{50} for H^+, treatment of mitochondria with mercurials and NEM has other effects on IMAC, including increasing the IC_{50} values for cationic amphiphiles and Mg^{2+}. The data in Fig. 7 show the effect on the IC_{50} for propranolol, when Cl^- transport is assayed. Addition of cysteine to the assay partially reverses the effect of mersalyl, suggesting that reaction at another site may also influence propranolol binding. With both NEM and mersalyl, the IC_{50} remains sensitive to pH. NEM

also increases the IC_{50} for inhibition by Mg^{2+}. At pH 7.4 it is increased from 37 to 86 μM, while at pH 8.3 it is increased from 220 μM to 1.9 mM. Thus, the IC_{50} remains sensitive to pH. NEM also appears to increase the IC_{50} for inhibition of IMAC by cytosolic Mg^{2+} (Beavis, 1991). Since all three of these effects of NEM and mercurials are exerted by equal doses and all are irreversible, Beavis (1991) has proposed that they are mediated by modification of the same site (see Fig. 2).

Since the regulatory site and the inhibitory site are both readily accessible to membrane impermeant mercurials, these sites, designated site 1 and site 2, respectively, in Fig. 2, are believed to be on the outer surface of IMAC. Consequently, to explain the diversity of effects including modulation of inhibition by Mg^{2+} and H^+ which are thought to bind to the inner surface, Beavis (1989b) has suggested that site 1 is located in a cleft and that reaction at this site induces a large conformational change in IMAC. This location and maybe also the conformational change itself could prevent access of exogenous aqueous thiols to the Hg–S bond and explain the irreversibility of the effects (see Fig. 2). Note that reaction at this site has no effect on the kinetics of reaction with DCCD.

To explain the dependence of the maximum extent of inhibiton on the size of the transported anion, Beavis (1989b) has proposed that the putative thiol at site 2 does not play a vital role in transport, but that inhibition is due to a steric effect of the R-group blocking the channel entrance or anion binding site (see Fig. 2). Consistent with this model is the finding that another mercurial, *p*-chloromercuribenzenesulfonate, which has a smaller R group, has essentially no effect on Cl^- transport and does not block malonate transport completely. This site is freely accessible to aqueous thiols and does not react with NEM, since NEM is unable to protect against inhibition.

INHIBITION OF IMAC BY TRIORGANOTINS

Triorganotin compounds have several properties in common with organomercurials. Both of these classes of compounds are electrophiles and both are able to mediate halide/OH^- exchange across lipid membranes when the organic groups are uncharged (Selwyn *et al.*, 1970; Watling and Selwyn, 1970; Selwyn, 1972, 1976). Unlike mercurials, however, triorganotins are not reported to react with many mitochondrial proteins. The notable exception is the F_1F_0ATPase which is potently inhibited by triorganotins (Aldridge and Street, 1970; Stockdale *et al.*, 1970). The only other effect reported is the stimulation of K^+ uniport (Diwan, 1982; Diwan *et al.*, 1983). Thus, Powers and Beavis (1991) investigated the effect of these agents on IMAC in an attempt to identify a more selective inhibitor. This study showed that triorganotins do indeed inhibit IMAC; in fact, tributyltin (TBT) is the most potent inhibitor of IMAC identified to date. Complete inhibition of malonate uniport via IMAC is achieved with about 1 nmol TBT/mg, which is only slightly higher than the dose of 0.8 nmol/mg required to block the ATPase.

Although the finding that TBT could inhibit IMAC was consistent with naive expectations, it does not in fact appear to be related to reaction at mercurial site 2. Unlike mercurials, TBT is found to block completely the transport of even small anions such as Cl^- and NO_3^-. Also, the inhibition cannot be prevented or reversed by thioglycolate or mercaptoethanol. The only compounds found to reverse inhibition by TBT are dithiols such as dithiothreitol and 2,3-dimercaptopropanol and sodium sulfide (100 μM), with the latter being the most effective (Powers and Beavis, 1991).

To investigate directly the possibility that mercurials and tin compounds inhibit by binding to a common site, Powers and Beavis (1991) exploited the fact that although *p*-CMS binds to IMAC, evidenced by the inhibition of malonate transport, it does not block the transport of NO_3^-. Thus, if *p*-CMS could protect the site or displace TBT, transport should be observed. This turned out to be the case; in fact, *p*-CMS is the most potent agent for reversing TBT inhibition of NO_3^- transport! Moreover, the dose–response of this effect was found to be the same as that for inhibition of malonate transport by *p*-CMS. Despite this finding, the protective effect does not appear to result from reaction at mercurial binding site 2, because addition of thioglycolate to the assay, which reverses inhibition of IMAC by *p*-CMS, does not reverse the protective effect of *p*-CMS. The mechanism of this protective effect is unknown. *p*-CMS may interact at the TBT-binding site; however, IMAC can still be inhibited by TBT, TϕT, and TET if the doses are increased 10-, 24-, and 24-fold, respectively. This increase in IC_{50} could reflect the existence of a second inhibitory site or a *p*-CMS-induced increase in the K_I of the first site. NEM has no effect on inhibition by TBT; moreover, NEM does not protect against the

effect of *p*-CMS, and therefore this mercurial effect does not appear to be related to modification of site 1. Thus, Powers and Beavis (1991) have attributed the effect of mercurials on inhibition by TBT to modification of a third mercurial reactive site—site 3.

In addition to mercurials, pH also affects the IC_{50} for trialkyltins. The effect, however, is less dramatic. Using TMT, which is the least potent and most water soluble trialkyltin, Powers and Beavis (1991) have shown that the IC_{50} increases 4-fold when the pH is raised from 7.4 to 8.4. This pH dependence is lost following pretreatment with *p*-CMS, suggesting that the mercurial may react with the protonation site responsible for this pH effect and, therefore, that it may be on the outside. Consistent with this is the finding that malonate uniport in the Brierley assay can be blocked by doses of TBT which are no more than 2-fold higher than those required in the Beavis Assay (A. D. Beavis, unpublished data).

IS THERE A RELATIONSHIP BETWEEN IMAC AND THE ADENINE NUCLEOTIDE TRANSLOCATOR FAMILY OF CARRIERS?

Sequence analysis of the purified mitochondrial adenine nucleotide translocator, phosphate carrier, and uncoupling protein has led Aquila *et al.* (1987) to propose that these proteins belong to a homologous protein family. Recently, Runswick *et al.* (1990) have obtained evidence that the oxoglutarate carrier is also a member of this family. The molecular identity of IMAC is not yet known; however, on the basis of its regulatory properties, Selwyn's group has proposed that it may also be a member of the same family (Murray *et al.*, 1988).

Selwyn's group has investigated the effects of a number of different inhibitors of the translocase on IMAC. They find that palmitoyl CoA (Halle-Smith *et al.*, 1988), agaric acid, bongkrekic acid, and Cibacron Blue 3GA (Murray *et al.*, 1988) all inhibit IMAC, whereas atractyloside does not. In these studies, Cl^- transport was followed using the Selwyn assay, and in all cases inhibition was incomplete. For example, the maximum inhibition obtained with palmitoyl CoA was 57%. Halle-Smith *et al.* (1988) tentatively attributed this incomplete inhibition to a detergent effect. Powers and Beavis (1990, unpublished data) have confirmed the inhibitory effect of palmitoyl CoA, agaric acid, and Cibacron Blue using the Beavis assay, including the finding that inhibition of Cl^- transport is incomplete. However, they also find that inhibition of malonate transport approaches 100%, which suggests that incomplete inhibition is not due to a nonspecific increase in membrane permeability. In this respect, the inhibitory properties of these agents resemble those of mercurials. In these assays, the IC_{50} values for Cl^- transport are 1, 1.6, and 5.2 μM for palmitoyl CoA, agaric acid, and Cibacron Blue, respectively, which compare with the values of 2-3, 3, and 4 μM obtained by Selwyn's group.

Cibacron Blue is an analog of AMP and is able to inhibit many enzymes which use nucleotides containing this moiety (Neslund *et al.*, 1984). Many other dyes fall into this class, and Powers and Beavis (1990) have demonstrated that a number of them inhibit IMAC. The most potent are fluorescein derivatives including erythrosin B (IC_{50} for $Cl^- = 5.9\,\mu$M), and rose bengal (IC_{50} for $Cl^- = 1.4\,\mu$M). Other analogs such as bromcresol green (IC_{50} for $Cl^- = 21\,\mu$M) and alizarin red S (IC_{50} for $Cl^- = 114\,\mu$M) are less potent. The maximum extent of inhibition of Cl^- transport also varies, with erythrosin B being the most efficacious with a maximum inhibition of 95%. All these compounds appear capable of blocking the transport of malonate and larger anions completely, and the IC_{50} values are about half those obtained with Cl^-. Unlike inhibition by Mg^{2+}, cationic amphiphiles, and triorganotins, the IC_{50} values for these nucleotide analogs are not increased by pH. Although these inhibitors are nucleotide analogs, there is no evidence that nucleotides, such as NAD^+, $NADP^+$, ATP, or ADP, inhibit IMAC.

Although it is interesting to compare the properties of IMAC with those of the adenine nucleotide translocase family of carriers, it will not be possible to determine whether IMAC is a member of this family until it has been identified and sequenced.

DOES IMAC EXIST IN MITOCHONDRIA FROM OTHER TISSUES?

Mitochondria from different tissues have evolved to play roles in metabolic pathways peculiar to those tissues. This has led to some diversity in the expression of the various substrate porters in these mitochondria [see LaNoue and Schoolwerth (1979) and Douce and Neuberger (1989)]. Volume homeostasis, however, should be important in all mitochondria and, therefore, IMAC should be present in all mitochondria. Most studies of IMAC to date have used heart and

liver mitochondria; however, Jezek *et al.* (1989) have demonstrated that IMAC is also present in mitochondria from brown adipose tissue and that it is distinct from the uncoupling protein.

Even before Azzi and Azzone (1966, 1967) demonstrated that liver mitochondria become permeable to Cl^- at alkaline pH, it was recognized that plant mitochondria from many sources are permeable to Cl^- at neutral pH [see Hanson and Hodges (1967), Hanson and Koeppe (1975), and Day and Wiskich (1984) for reviews]. The rate of Cl^- transport does, however, increase with pH (Stoner and Hanson, 1966; Yoshida, 1968), suggesting that the transport mechanism may be via a channel similar to IMAC. If this is the case, then plant mitochondria should be permeable to other anions in addition to Cl^-. Examination of the literature reveals evidence that uniport of other anions in addition to halides also occurs in plant mitochondria. Hensley and Hanson (1975) have studied swelling and contraction cycles in corn mitochondria and have explained contraction which occurs after inhibition of the phosphate carrier on the basis of efflux of Pi by Pi^- uniport. Further evidence for Pi uniport and also SO_4^{2-} uniport in plant mitochondria was provided by Huber and Moreland (1979). More recently, Zoglowek *et al.* 1988 have shown that plant mitochondria mediate electrophoretic transport of anions such as malate, oxaloacetate, maleate, fumarate, succinate, malonate, butylmalonate, and 1,2,3-benzenetricarboxylate but not α-ketoglutarate. Moreover, these authors suggest that this transport pathway may be involved in the malate/oxaloacetate shuttle in plant mitochondria. It is unlikely that these anions could be transported rapidly through the lipid bilayer in the absence of a very large membrane potential; consequently, a uniporter or channel is probably involved.

Using the large number of inhibitors of IMAC which have been identified, Beavis and Vercesi (1992) have now examined anion uniport in potato tuber mitochondria to determine whether they contain a similar transport pathway. These mitochondria are permeable to Cl^- and, as in animal mitochondria, transport appears to be modulated by matrix protons. In addition, other substrates of IMAC are transported via this pathway, including anions such as Pi, 1,2,3-benzenetricarboxylate, ferrocyanide, α-ketoglutarate, malate, and malonate. Other common properties include inhibition by propranolol ($IC_{50} = 14\,\mu M$ at pH 7.4), Cibacron Blue ($IC_{50} = 23\,\mu M$), and TBT ($IC_{50} = 4$ nmol/mg); however, TBT and Cibacron Blue are less potent in potato, while propranolol appears to be slightly more potent in potato.

The most interesting feature of the anion channel in plant mitochondria is that is unnecessary to use A23187 to unmask its activity. Moreover, in potato mitochondria it is esentially insensitive to exogenous Mg^{2+} (+A23187). Since Cl^- transport has been observed in all plant mitochondria studied (Hanson and Koeppe, 1975), insensitivity to Mg^{2+} may be a common property of IMAC from plants. This is not the only property which differs between the two channels. The channel in potato mitochondria also appears to be insensitive to inhibition by DCCD and mercurials (Beavis and Vercesi, 1992).

In view of the the similarities in anion uniport in animal and plant mitochondria, it is likely that plant mitochondria possess a specific inner membrane anion channel closely related to IMAC. However, since there apper to be significant differences between these channels from plants and animals, we now refer to the channel in plant mitochondria as PIMAC. Furthermore, it is interesting to speculate whether a similar channel is present in chloroplasts and whether this channel is responsible for the KCl fluxes and consequent volume changes observed upon energization and deenergization of chloroplasts (Nobel, 1975; Heber and Heldt, 1981) or whether it is involved in the transfer of reducing equivalents between chloroplast and mitochondria (see Kromer and Heldt, 1991).

THE PHYSIOLOGICAL ROLE OF IMAC

As discussed in the Introduction, IMAC is believed to be important in volume homeostasis to allow respiration to drive anion efflux. However, in most of the studies described in this review, anion influx has been assayed under nonphysiological conditions, i.e., in the absence of a membrane potential. Brierley *et al.* (1977) have carried out many studies demonstrating that heart mitochondria can undergo energized contraction. Cycles of swelling and contraction have also been demonstrated in both liver mitochondria (Azzi and Azzone, 1966, 1967) and plant mitochondria (Hanson and Koeppe, 1975), Jung and Brierley, 1979). To date, however, there is little direct evidence that the anion efflux which occurs under these conditions is mediated by IMAC.

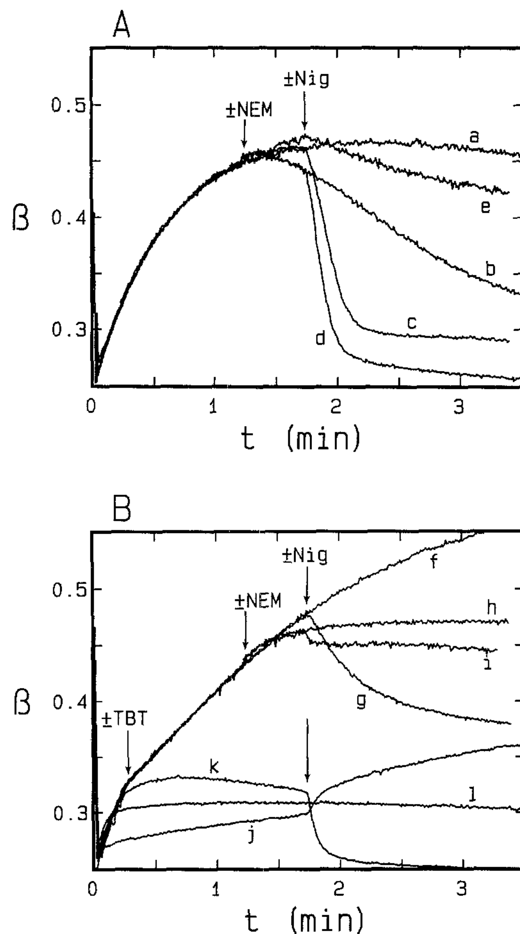

Fig. 8. IMAC is involved in energy-dependent contraction and volume homeostasis. LS kinetics of beef heart mitochondria (0.1 mg/ml) suspended in a medium containing the K^+ salts of Pi and NO_3^- at pH 7.4 and respiring on ascorbate (2.5 mM), TMPD (0.27 mM), and cytochrome *c* (10 μM) are shown. A. Trace a, control. Trace b, NEM (0.5 mM) blocks electroneutral Pi influx and induces contraction. Trace c, nigericin (1 nmol/mg) stimulates K^+/H^+ antiport and induces contraction. Trace d, NEM and nigericin together produce the maximum rate of contraction. Trace e, CCCP (+NEM +Nig) inhibits contraction 95%. B. Trace f, TBT (6 nmol/mg) blocks IMAC but does not block swelling and eliminates spontaneous contraction. Trace g, TBT inhibits Nig-induced TBT contraction. Trace h, TBT and NEM together block contraction completely even with nigericin added (trace i). Omission of ascorbate/TMPD (trace j) or addition of NEM (trace k) or Nig (trace l) at zero time prevents swelling, indicating that swelling is dependent on energy, the phosphate carrier, and K^+ uniport, respectively.

The data in Fig. 8 demonstrate that IMAC can in fact mediate energy-dependent anion efflux and mitochondrial contraction in beef heart mitochondria. In this experiment, the assay medium containied the K^+ salts of Pi and NO_3^- with ascorbate, TMPD and cytochrome *c* added as substrates for respiration. Pi was used because it is a physiological anion and can be transported both electroneutrally by the classical phosphate carrier and electrophoretically via IMAC. More importantly, the Pi carrier can be selectively inhibited by *N*-ethylmaleimide while IMAC can be selectively blocked by tributyltin and DCCD. Mitochondria added to this medium swell in an energy-dependent manner. Nigericin and NEM block this swelling but TBT and DCCD do not; consequently, it is attributed to activity of an endogenous K^+ channel or uniport pathway and the phosphate carrier as depicted in Fig. 1A. After about 1 min, the volume reaches a maximum and a slow spontaneous contraction begins (trace a). According to our model for volume homeostasis, in the steady state the rate of KPi influx mediated by K^+ uniport and Pi^-/OH^- exchange must equal the rate of efflux mediated by the K^+/H^+ antiporter and IMAC. Since three of these transport processes can be modulated, this hypothesis can be tested. Thus, when NEM is added to block the influx pathway for Pi, contraction is observed (trace b). If nigericin is added to accelerate K^+/H^+ antiport, there is a rapid contraction (trace c). This nigericin-induced contraction is not due to efflux of phosphate via the phosphate carrier, since it is not blocked, but further stimulated, by the addition of NEM (trace d). Moreover, addition of CCCP also inhibits the contraction, showing that it is energy dependent (trace e).

The effects of TBT are opposite to those of NEM. Thus, addition of TBT does not block swelling, and, in the absence of nigericin, the volume does not reach a maximum and no spontaneous contraction is observed (cf. traces f and a). Furthermore, the contraction induced by nigericin is substantially inhibited (cf. traces g and c), suggesting that it occurs via IMAC. The remaining contraction appears to represent Pi efflux via the Pi carrier, since addition of both TBT and NEM blocks contraction completely (traces h and i). Pretreatment of mitochondria with DCCD to block IMAC has similar effects to the addition of TBT (not shown). These data illustrate how it might be possible to regulate matrix volume by modulating the activities of IMAC, the K^+/H^+ antiporter, and the K^+ channel. Although the conditions used for this illustrative experiment are not physiological, it should be noted that it was not necessary to use nonphysiological pH values, nor was it necessary to deplete matrix Mg^{2+} to observe these effects. In fact, addition of 3 mM Mg^{2+} to the medium has essentially no effect on the contraction observed.

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