On the Inhibition of the Mitochondrial Inner Membrane Anion Uniporter by Cationic Amphiphiles and Other Drugs*

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Depleting the mitochondrial matrix of divalent cations with the ionophore A23187 activates a pH-sensitive, anion uniport pathway which can transport many anions normally regarded as impermeant (Beavis, A. D., and Garlid, K. D. (1987) J. Biol. Chem. 262, 15085-15093). Addition of valinomycin to respiring mitochondria of two also induce the uptake of the wide variety of anions; however, the mechanism of anion transport during this "respiration-induced" swelling is less certain. In this paper, I demonstrate that both of these processes are inhibited by a variety of cationic amphiphiles including propranolol, quinine, amiodarone, imipramine and amitriptyline, and the benzodiazepine R05-4864. Although the IC_{50} values for the two processes are not equal, the ratio of IC_{50} values for the two processes appears to be the same for all drugs. Measurements of net transmembrane proton fluxes that occur during the assays reveal that respiration-induced swelling is associated with extensive proton ejection, the peak of which coincides with the maximum rate of anion transport. Moreover, from measurements of matrix buffering power, it is estimated that the matrix pH is 3 units more alkaline during respiration-induced swelling than during A23187-induced swelling. It is also shown that the IC_{50} for A23187-induced transport is pH-dependent in a manner consistent with modulation of drug binding by protonation of two sites. These findings allow the difference in IC_{50} values for the two types of assay to be explained by the pH dependence of the binding constant for the drug. Furthermore, the pH gradient generated during respiration-induced swelling is so large that the electrical component of the proton-motive force will be negligible. Thus, despite the fact that the mitochondria are "energized," rapid electrophoretic anion influx is possible. These data provide evidence that the transport of anions in these two types of assay occurs via the same pathway.

The inner membrane of isolated mitochondria normally exhibits a very low electrophoretic permeability to most anions including Cl^{-} and substrate anions. More than 20 years ago, however, Azzi and Azzone (1, 2) showed that mitochondria become permeable to anions when the pH of the medium is raised to 8 or above. Soon afterwards, Brierley (3, 4) showed that the permeability is also increased at neutral pH when valinomycin is added to respiring mitochondria. This led him to propose that it is the pH of the matrix which regulates permeability to anions. More recently, Beavis and Garlid (5) demonstrated that depletion of matrix divalent cations with A23187 also opens an anion uniport pathway. Furthermore, they proposed that the same pathway, namely the inner membrane anion uniporter, is responsible for the transport observed under all three of these conditions. Evidence for this conclusion includes the finding that the pathway observed in divalent cation-depleted mitochondria is regulated by protons (5) and the finding that transport under all three conditions is irreversibly inhibited by N,N'-dicyclohexylcarbodiimide (6, 7).

In addition to this divalent cation and proton regulated pathway, there are three other putative pathways which are able to translocate anions. The two most widely studied require Ca^{2+} for activation and can also conduct cations and nonelectrolytes. One is thought to result from the accumulation of lysophosphatidylethanolamine secondary to activation of phospholipase A_{2} by Ca^{2+} (8-11). The other is thought to be a pore which is activated directly by matrix Ca^{2+} (12-15). The exact nature of and relationship between these pathways is unknown; however, it is possible that they are identical. The third pathway has been revealed by patch-clamp studies of the inner mitochondrial membrane (16) and does not appear to require Ca^{2+} for activity. As Selwyn (17) has stated, the situation is confused but exciting, and further characterization of these pathways will be necessary before their separate existences can be established.

To extend my characterization of the inner membrane anion uniporter, I have sought additional inhibitors of this transport. Garlid and Beavis (18) have reported that the cationic amphiphiles quinine and propranolol, inhibitors of the K^{+}/H^{+} antiporter (19), are able to block this uniporter. Furthermore, Selwyn et al. (20) have reported that local anesthetics, which are also cationic amphiphiles, block the "pH-dependent anion-conducting pore," a finding that is consistent with the identity of the pH- and divalent cation-regulated pathways. It should be pointed out, however, that cationic amphiphiles also prevent the Ca^{2+}-induced increase in mitochondrial permeability discussed above. This effect has been attributed to their ability to inhibit phospholipase A_{2} (10, 21-25). In contrast, Sorgato et al. (16) report that quinine has no effect on their voltage-dependent channel. This class of compounds also interacts with a number of other membrane proteins including porin or VDAC (26, 27), the F_{1}-ATPase (28-30) and the adenine nucleotide translocator (31) in mitochondria and Ca^{2+} and Mg^{2+} transport systems in sarcoplasmic reticulum (30, 32). In addition, they are also reported to inhibit ruthenium red insensitive Ca^{2+} efflux from mitochondria (33, 34) and cation transport mediated via ion-
in the medium in the absence of mitochondria, were 49, 52, and 69 nmol of H+/ApH-mg, respectively. Values of the matrix buffering power were 19, 20, and 36 nmol of H+/ApH-mg, respectively, at each pH. These values are similar in magnitude to those obtained by Mitchell and Moyle (46, 47).

**RESULTS**

Inhibition of A23187-induced Anion Uniport—The LS traces contained in Fig. 1 show the effect of various doses of propranolol on the passive swelling of mitochondria suspended in a KCl medium. A23187 and EDTA were added from zero time to deplete matrix divalent cations. As we have shown previously (5), this leads to activation of a uniport pathway which can carry a wide variety of anions including Cl-. Thus, in the presence of sufficient valinomycin to ensure that K+ transport is not limiting, the rate of KCl influx and consequent swelling should be limited by the ratio of anion uniport. As shown in curves b-f of Fig. 1, addition of propranolol induces immediate inhibition. Rates of Cl- transport determined from these and other traces (see "Experimental Procedures") were used to construct dose-response curves and Hill plots (see Fig. 2). From this analysis it is evident that inhibition approaches 100% and that the IC50 for propranolol is 25 μM.

Since swelling requires the transport of both K+ and Cl-, it could be argued that the observed inhibition results from inhibition of either one of these processes. This is not a trivial consideration, since Azzi and Scarpa (35) and Johnson and Schwartz (36) have shown that dibucaine, which is structurally similar to propranolol, is able to inhibit K+ transport catalyzed by valinomycin. This does not, however, appear to be the explanation for our observations, since we find that

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**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 (44, 45). Using this technique I generate a light-scattering variable, β, which normalizes reciprocal absorbance for mitochondrial protein concentration, P (mg/ml), according to the formula:

\[ \beta = \frac{P}{P_s} (A^+ - a) \]

where a is a machine constant and \( P_s \) (equals 1 mg/ml) is a constant introduced to make β dimensionless.

The rate of salt transport is calculated from the rate of change of β according to the formula (45):

\[ J_t = \frac{\Delta S_t}{nb} \frac{\Delta t}{\Delta t} \]

where \( \phi_s \) is the medium osmolality (110 mosm in most studies reported here), \( S_s \) is the solute concentration of the stock preparation of mitochondria (190 mosmol/mg), b (15 mosm) is the slope of the equilibrium absorbance osmotic curve (44) and n is the number of moles of osmotically active particles which make up 1 mol of the transported salt. At \( \phi = 110 \) mosm, \( \Delta S_t/b \) is about 1400 nmol/mg.

To determine rates of solute transport, I use a Brinkmann Probe Colorimeter (model PC700) with a 1-cm probe (2-cm light path). With this probe, for optimum sensitivity I normally use mitochondria at a concentration between 0.1 and 0.2 mg/ml. However, for studies in which H+ fluxes are examined, it is necessary to use mitochondria at about 2 mg/ml. To follow light-scattering (LS)\(^1\) changes in these suspensions we employ a probe with a variable path length and adjust the gap to about 2.5 mm. It should be noted, however, that these data cannot be compared quantitatively with those obtained with the 1-cm probe. Although β is normalized for mitochondrial concentration, it remains dependent on the length of the light path and the system used to determine light-scattering changes. Thus, to determine mitochondrial volume and solute fluxes, the absorbance osmotic curve must be determined with the same apparatus. For this reason in this study the LS data obtained with the variable path length probe are simply presented in terms of \( A^{+1} \).

**Assay of Proton Fluxes**—Proton fluxes were followed using an Orion combination glass pH electrode (model 81-02) connected to an Orion pH meter (model 701A) connected to a strip chart recorder. Proton fluxes were quantitated by internal calibration of each trace by adding 5-μl volumes of standard HCl (0.100 M).

**Determination of Mitochondrial Buffering Power**—The buffering power of mitochondria was determined as described by Mitchell (46, 47), except that K+/H+ exchange was equilibrated with nigericin rather than valinomycin plus CCCP. Mitochondria (2 mg/ml) were suspended in unbuffered KCl (150 mM) and the pH was adjusted to the desired value. Small pulses of HCl (100 nmol) were then added and the change in pH was recorded (approximately 0.1 unit). KOH was then added to adjust the pH back to the starting value and another addition of HCl was made. After 4-5 measurements were made, nigericin (1 nmol/mg) was added and the process repeated. The buffering power was calculated as \( \Delta H^+/ApH \) mg and the matrix buffering power was estimated from the difference between the values obtained with and without nigericin. Values of total buffering power at pH values of 8.4, 7.3, and 6.5, corrected for the buffering power of

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1 The abbreviations used are: LS, light-scattering; EGTA, [ethylenebis(oxyethylenenitri1o)]tetraacetic acid; CCCP, carbonylcyanide m-chlorophenylhydrazone; TES, N-tris(hydroxymethyl)methyl-2-amino-ethanesulfonic acid; IC50, concentration which gives 50% inhibition; VDAC, voltage-dependent anion conducting channel.
inhibition is not diminished if we raise the concentration of valinomycin. Furthermore, the values for the IC_{50} obtained when nigericin and CCCP are substituted for valinomycin and when ammonium chloride is used with CCCP are very similar to those obtained with valinomycin (results not shown). In view of these findings, we conclude that propranolol inhibits the anion uniport pathway.

Similar results were obtained with a number of different drugs. Typical dose-response curves and Hill plots for some of these are shown in Fig. 2, A and B, respectively. The IC_{50} values and Hill slopes for all drugs investigated are presented in Table I. Most of the drugs investigated are cationic amphiphiles, including the \( \beta \)-blockers propranolol, pindolol, and timolol, the tri cyclic antidepressants amitriptyline and imipramine, and the antimalarial quinine. I have also investigated the local anesthetics lidocaine and benzocaine and the benzodiazepines clonazepam. All the drugs tested, with the exceptions of lidocaine and benzocaine, were found to inhibit and, as found with propranolol, inhibition appears to approach 100%. The IC_{50} values range from 0.6 \( \mu \)M for amiodarone to 1930 \( \mu \)M for timolol.

**Inhibition of Respiration-induced Anion Uniport**—As first demonstrated by Brierley (3, 4) and confirmed by others (6, 18), mitochondria respiring in KCl swell rapidly when valinomycin is added. It has been proposed that the CI^- transport responsible for this swelling occurs via the divalent cation-dependent pathway (6, 18). To examine this proposal further, I have investigated whether the inhibitors mentioned above also inhibit this respiration-induced transport.

Trace a of Fig. 3A shows a typical LS trace obtained when valinomycin is added to respiring mitochondria suspended in KCl. Note that there is a short lag before the rate of swelling becomes maximal. Addition of propranolol inhibits this swelling and inhibition is immediate when it is added during the swelling phase.

**Fig. 2.** Dose-response curves and Hill plots for inhibition of CI^- uniport by various drugs. Rates of CI^- uniport were determined as described for Fig. 1 in the presence of various concentrations of the following drugs: \( \bullet \), amiodarone; \( \bigcirc \), imipramine; \( \Delta \), propranolol; \( \Delta \), quinine. Panel A contains the dose-response curves and Panel B the Hill plots, where \( J \) and \( J_0 \) are the rates in the presence and absence of the drug respectively. The IC_{50} values and Hill slopes are shown in Table I.

**Table I.**

<table>
<thead>
<tr>
<th>Drug</th>
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<th>Respiration-induced transport</th>
</tr>
</thead>
<tbody>
<tr>
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<td>IC_{50} ( \mu )M</td>
<td>Hill slope</td>
</tr>
<tr>
<td>Amiodarone</td>
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</tr>
<tr>
<td>Amifostyline</td>
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<tr>
<td>Imipramine</td>
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</tr>
<tr>
<td>Propranolol</td>
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</tr>
<tr>
<td>R05-4864</td>
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</tr>
<tr>
<td>Quinine</td>
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</tr>
<tr>
<td>Clonazepam</td>
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<td>1.89</td>
</tr>
<tr>
<td>Pindolol</td>
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</tr>
<tr>
<td>Timolol</td>
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<td>1.14</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>Benzocaine</td>
<td>(-)</td>
<td>(-)</td>
</tr>
</tbody>
</table>

* a See text.
* b ND, not determined.
* c No inhibition detected up to 1 mM lidocaine.
* d No inhibition was observed up to a concentration of 0.5 mM benzocaine.

To determine whether this inhibition reflects inhibition of the anion uniport mechanism or inhibition of respiration and/ or valinomycin-catalyzed K^+ uptake, I carried out a similar experiment in which phosphate was added to the medium (see Fig. 3B). Under these conditions, swelling can take place as a result of respiration-driven K^+ uptake coupled to passive accumulation of phosphate and succinate via the classical phosphate and dicarboxylate carriers (6). Comparison of traces a and b of Fig. 3B reveals that in this experiment propranolol had only a very small effect on the rate of swelling, indicating that neither respiration nor valinomycin-catalyzed K^+ uptake are significantly blocked by this drug. Furthermore, in this experiment, there is essentially no lag in the swelling trace, which suggests that the lag observed in Fig. 3A reflects a delay in the activation of CI^- transport.

Similar results were obtained with all the other drugs tested with the exception of quinine, which was found to inhibit swelling also under these conditions. To determine whether this effect was mediated via inhibition of the phosphate carrier or inhibition of respiration or uncoupling, we compared the effects of propranolol and quinine on respiration. The results are shown in Fig. 4. Propranolol up to a concentration of 0.5 mM has no effect on uncoupled respiration, whereas quinine inhibits with an IC_{50} of 490 \( \mu \)M and a Hill slope of 1.3. Both drugs lead to a slight stimulation of state 4 respiration.

Fig. 5, A and B, shows dose-response curves and Hill plots, respectively, for some of the most potent inhibitors of the A23187-induced swelling. In these experiments the drug was added at the end of the lag phase, as shown in Fig. 3. Inhibition approached 100% with all of these drugs and the Hill slopes are about the same in the two assays; however, the IC_{50} values for respiration-induced swelling are found to be considerably higher than for A23187-induced swelling (see Table I). Although this finding suggests that the transport pathways involved might be different, further examination of the data reveals that there is a linear relationship between the IC_{50} values with a slope of 0.16 (see Fig. 6). However, for this data to be consistent with the inhibition of a single pathway, it is
Anion Uniport in Mitochondria

**FIG. 3.** The effect of propranolol on swelling induced by valinomycin in respiring mitochondria. LS kinetics of respiring mitochondria (0.1 mg/ml) suspended in a KCl assay medium are shown. A, in the absence of a permeant acid, addition of valinomycin (0.5 nmol/mg) induces rapid swelling following a short lag (trace a). Addition of propranolol (0.33 mM) inhibits the rate of swelling by 60% (trace b). B, in the same medium supplemented with phosphate (2 mM), addition of valinomycin induces immediate swelling (trace a); however, in this case, addition of propranolol (0.33 mM) has a negligible effect (trace b). The basic assay medium contained the K⁺ salts of Cl⁻ (55 mM), TES (5 mM), succinate (5 mM), and EGTA (0.1 mM) plus rotenone (2 μg/mg) and was maintained at pH 7.4 and 25 °C.

**FIG. 4.** Comparison of the effects of propranolol and quinine on mitochondrial respiration. The rates of state 4 respiration and uncoupled respiration determined with an oxygen electrode are plotted versus the concentration of drug. Mitochondria (2 mg/ml) were suspended in an assay medium containing the K⁺ salts of Cl⁻ (120 mM), TES (5 mM), succinate (5 mM), and EGTA (0.1 mM) plus MgCl₂ (3 mM) and rotenone (0.8 μg/ml). After 0.5 min the indicated dose of quinine or propranolol was added and the subsequent rate of oxygen consumption was determined. After 25% of the oxygen had been consumed, CCCP (0.25 nmol/ml) was added and the subsequent rate of oxygen consumption determined. State 4 rates: O, quinine; □, propranolol. Rates with uncoupler: ●, quinine; ■, propranolol.

**FIG. 5.** Dose-response curves for inhibition of respiration-induced swelling by various drugs. Rates of Cl⁻ transport were determined from the LS kinetics of swelling in experiments carried out as described for Fig. 3A. In each case the drug was added after the short lag following addition of valinomycin. The curves shown are: ●, amiodarone; ○, imipramine; ▲, propranolol; △, quinine. Panel A shows the dose-response curves and panel B the Hill plots, where J and J₀ are the rates in the presence and absence of drug, respectively. The IC₅₀ values and Hill slopes are shown in Table I.

**FIG. 6.** Relationship between IC₅₀ for A23187-induced and respiration-induced anion transport. The IC₅₀ (μM) values for five of the most potent inhibitors of A23187-induced Cl⁻ transport ([(IC₅₀)ₐ]) are plotted against the values obtained for inhibition of respiration-induced swelling ([(IC₅₀)ₐ]). The slope of the line fitted to these data by linear regression is 0.163.

necessary to account for this difference in the IC₅₀ values. The only data which did not fit on this curve were those obtained with R05-4864. This drug is not very soluble in ethanol, and at the high doses required to block respiration-induced swelling, effects of the high concentration of ethanol made it difficult to determine the true IC₅₀.

Proton Fluxes Associated with Induction of Anion Uniport—One factor which could affect the IC₅₀ is the matrix pH, which one can predict should be very different in the two assays. To investigate this possibility, I have attempted to determine the magnitude of this difference. Since it is difficult to determine non-steady-state pH gradients using the distribution of weak acids or bases, I have estimated changes in matrix pH from
measurements of the net $H^+$ fluxes across the inner membrane and the matrix buffering power. The proton fluxes were followed with a pH electrode using the same conditions as the swelling assays except for the concentration of mitochondria which was raised to 2 mg/ml to increase the sensitivity. The buffering power of the mitochondria was determined as described under "Experimental Procedures" and found to equal 19, 20, and 36 nmol of $H^+/\Delta p$H mg at pH values of 8.1, 7.3, and 6.6 respectively. Changes in pH were calculated from these values using the assumption that the buffering power changed linearly with pH between these values (see Refs. 46, 47).

When mitochondria were added to the assay medium containing rotenone, there appeared to be a slow efflux of protons which ceased after about 1 min. Subsequent addition of nigericin to equilibrate $K^+/H^+$ antiport led to a rapid influx of protons (see Table II). The extent of this flux declined as the pH of the medium was raised but did not reach zero. If it is assumed that $K^+$ constitutes 73% of the osmotically active ions in the matrix, it follows that $[K_in]/[K_out]$ equals 1.6 and, therefore, since $[H^+]/[H^+]_{out} = [K_in]/[K_out]$ in the presence of nigericin, $\Delta p$H must be buffered at a value of 0.2. Assuming this value for the $\Delta p$H, we can calculate the matrix pH prior to addition of nigericin from the magnitude of the nigericin-induced $H^+$ flux and the buffering power of the matrix (see Table II). The results suggest that in the absence of nigericin the matrix pH changes as the medium pH is altered but that the $\Delta p$H is not constant. These values for matrix pH are similar to those I have previously determined from distribution of acetate under similar conditions (results not shown).

Addition of A23187 to these mitochondria should lead to an efflux of endogenous Mg$^{2+}$ and an influx of protons. The magnitude of this proton influx was estimated using atomic absorption spectroscopy to determine the amount of Mg$^{2+}$ lost from mitochondria upon addition of A23187 (see "Experimental Procedures"). Dilution of the stock mitochondrial suspension into the EDTA-containing medium led to an immediate release of 8.2 ± 0.9 nmol of Mg$^{2+}$/mg leaving 28.2 ± 1.2 nmol of Mg$^{2+}$/mg in the mitochondrial pellet. Inclusion of nigericin had no effect on this release. When A23187 is added to the medium, 38.6 ± 1.3 nmol of Mg$^{2+}$/mg are found in the supernatant, leaving only about 0.5 nmol of Mg$^{2+}$/mg detectable in the pellet. Thus, addition of A23187 appears to lead to the release of 28.2 nmol of Mg$^{2+}$/mg and this should be associated with an influx of 56.4 nmol of H$^+/mg$. In view of the magnitude of matrix buffering power, this $H^+$ flux would lead to a decrease in matrix pH of about 2 units. In the presence of nigericin, however, the net proton flux was found to be negligible at all pH values with values ranging from -3 to +1 nmol of H$^+/mg$ (see Table II). Thus, these data indicate that nigericin-mediated $K^+/H^+$ exchange effectively buffers the $\Delta p$H.

During respiration-induced swelling there is rapid and extensive $H^+$ ejection. Fig. 7 shows the results of an experiment in which LS and medium pH were followed simultaneously. The changes in LS were followed using a colorimeter probe with a short path length to compensate for the elevated buffering power of the mitochondria (see "Experimental Procedures"). The data obtained have several interesting features which support Brierley’s proposal (4) that it is the matrix pH which regulates anion uniport. First, the rapid phase of swelling only begins when $H^+$ ejection approaches its peak. Second, as the pH gradient collapses to a steady-state level, the rate of swelling declines markedly. In fact, in agreement with the findings of Brierley (3, 4), I find that, if the trace is followed over a longer time period, swelling ceases and the mitochondria begin to shrink (not shown). Upon anaerobiosis or addition of CCCP during the steady-state phase there is a rapid backflux of protons (see Fig. 7). Thus, the steady-state pH gradient is maintained by respiration. If CCCP is added after the shrinkage phase has begun, then shrinkage ceases and the mitochondria begin to swell slowly again (results not shown). Thus, the shrinkage phase is dependent on respiratory energy.

The extent of $H^+$ ejection at the peak is between 50 and 60 nmol of $H^+/mg$, therefore, since the buffering power of the matrix is of the order of 18 nmol of $H^+/\Delta p$H mg, this $H^+$ ejection must lead to a 2.5-3.0 unit increase in matrix pH. In

![Fig. 7. Examination of the proton fluxes which accompany respiration-induced swelling. Simultaneous recordings of medium pH (trace a) and LS kinetics (trace b) are shown for mitochondria (21.7 mg) suspended in 10 ml of KCl medium. Valinomycin (25, 0.5 nmol/mg) and CCCP (10 nmol/mg) were added where indicated. At the end of the experiment, two 5-ml aliquots of 0.100 M HCl were added to calibrate the pH trace. LS kinetics were followed using a variable path length colorimeter probe with a gap set to about 2.5 mm (see "Experimental Procedures"). Apart from the concentration of mitochondria, the assay medium was the same as that described for Fig. 3. The extent of $H^+$ ejection was 54.3 nmol of $H^+/mg$ at the peak and 32.0 nmol of $H^+/mg$ in the subsequent steady state.]

<table>
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<tr>
<th>Medium</th>
<th>$\Delta p$H</th>
<th>A23187</th>
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<td>213</td>
<td>0.9</td>
<td>8.7</td>
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</table>

*No mitochondria.
contrast, during A23187-induced swelling, the matrix should be 0.2 units more acid than the medium; therefore, there is a substantial difference in matrix pH in these two assays. These findings lead to two obvious explanations for the difference in IC50 values obtained in the two types of assay. First, since the cationic form of a weak base such as propranolol distributes according to the proton gradient, the matrix concentration of propranolol should be much lower during respiration-induced swelling. Thus, if the inhibitory site were in the matrix, the IC50 (based on external concentration) would be very much higher. Second, the binding of the drug might be independent of the pH gradient but dependent on the matrix pH per se. To distinguish these two possible explanations, I have investigated the pH dependence of the IC50 for A23187-induced swelling.

Inhibition of A23187-induced Transport by Propranolol Is pH-dependent—The data contained in Fig. 8 show that the IC50 for inhibition of Cl– transport by propranolol is strongly pH-dependent, rising from about 24 µM at pH 7.2 to about 280 µM at pH 8.7. Since the pH gradient is constant under these conditions, this pH dependence cannot be attributed to a change in the distribution of propranolol. Consequently, these data suggest that the binding constant itself is pH-dependent. Similar results were obtained with quinine (not shown).

Two simple models in which protonation of the uniporter regulates drug binding are: 1) The drug is able to bind and inhibit both protonated and nonprotonated forms of the carrier but with different affinity. 2) The drug is only able to bind and inhibit the uniporter if a proton is bound to a specific site. These two models can be distinguished by plotting the IC50 versus 1/[H+]. Model 1 should yield a curve which intercepts the ordinate at the K1 for the protonated form (K1I) and rises asymptotically to the K1 of the unprotonated form (K12) as 1/[H+] increases. In contrast, model 2 should yield a linear relationship with intercepts equal to the binding constant for the H+ and the K1 for the drug. I have attempted to fit the data to both models. With the exception of the high IC50 observed at pH 8.7, the data fit model 1 quite well. Curve a of Fig. 8B was drawn with the values of K1, and K12 set at 210 and 4 µM, respectively, and the pK of the protonation site set at 6.32. The data also fit model 2 quite well if the K1 is set at 47 µM and the pK set at 8.0 (curve b, Fig. 8B); however, in this case the model does not explain the decline of the IC50 to almost half the K1 value at low pH. The best fit is obtained by combining the two models to produce model 3, in which two protonation sites affect drug binding. In this model the following equilibria are considered:

$$K_1 = \frac{[C][H^+]}{[CH_1]}$$

$$K_2 = \frac{[C][H^+]}{[CH_1][H^+]}$$

$$K_{12} = \frac{[CH_1][D]}{[CH_1][H_2]}$$

where C is the active free carrier, CH1, CH2, and CH1D are protonated forms which may or may not be active, and CH1D and CH1H2D are inactive forms with the drug bound. On the basis of these equations an expression relating the IC50 to the H+ concentration can be derived:

$$IC_{50} = \frac{1 + \frac{K_1}{[H^+]} + \frac{K_1}{K_1 + [H^+]} \frac{K_1}{K_{12}}}{K_{12}}$$

Curves c of Fig. 8B was drawn on the basis of this model in which protonation of “site 1” (pK1 = 8.38) allows propranolol to bind with a K1 of 100 µM (K1) and then protonation of “site 2” (pK2 = 6.32) lowers this K1 to 4 µM (K12).

**Discussion**

In this paper I have presented evidence that a variety of cationic amphiphiles reversibly inhibit anion uniport through the inner membrane of mitochondria. The potency of the inhibitory drugs varies widely, with amiodarone having the lowest IC50 (0.6 µM) and timolol having the highest (1.9 mM). Most of these drugs have two or more hydrophobic rings and a protonated secondary or tertiary amino group. Note that lidocaine and benzocaine, which only have a single ring, do not inhibit; however, RO5-4864 and clonazepam, which have multiple rings but no charge, do inhibit. In view of the lipophilic nature of these drugs, it is likely that inhibition of transport results from interaction of the drug with the uniporter from within the lipid phase of the membrane. Although the concentration of these drugs in the membrane will far exceed those in the aqueous phase, I have expressed the IC50 values in terms of molarity for the following reason: The partition coefficients for most of the drugs tested are less than 1000 and therefore, since the ratio of the lipid:aqueous phase volumes is less than 10^-4, most of the drug will be in the aqueous phase. Much of the difference in the IC50 values observed may be due to the difference in partition coefficients; however, it does not appear to be the only factor. For example, the ratio of partition coefficients for propranolol and quinine
is about 100, whereas the ratio of the IC₅₀ values is only about 0.5.

Although there are reports that cationic amphiphiles, e.g. dibucaine, inhibit cation transport mediated by ionophores (35–39), no evidence of this was apparent in my experiments. This finding is not consistent with the previous reports, since in my experiments I employed a high K⁺ concentration (57 mM) and it has been reported (35) that inhibition of ionophore mediated K⁺ transport is competitive with respect to the concentration of K⁺.

Although my working hypothesis is that anion uniport is catalyzed by a specific membrane protein, this has not yet been proved. The drugs I have used in this study are not highly specific inhibitors of anion uniport and they have many effects on different membranes. For example, they are able to bind to lipid bilayers (40–42) and they inhibit phospholipase A₂ (21, 48, 49), the activity of which has been associated with changes in the permeability of mitochondria (6–11, 22–25, 49). Local anesthetics also exert effects on the F,Fₐ-ATPase (28–30), Ca²⁺ and Mg²⁺ transport in sarcoplasmic reticulum (30, 32), Ca²⁺ transport in mitochondria (33, 34), ionophore-mediated transport (35–39, 50), ATP transport (51) and bind to VDAC or porin (26, 27). Despite the diverse effects reported for this class of drugs, I find that when used to investigate anion uniport in isolated mitochondria their effects can be quite specific. For example, out of the six most potent inhibitors of anion uniport, only one, quinine, has an appreciable effect on respiration. Furthermore, they appear to have a negligible effect on phosphate and succinate transport via the classical phosphate and dicarboxylate carriers. Many of these drugs, however, with the notable exception of amiodarone, have been shown to inhibit the K⁺/H⁺ antiporter (18). It is unlikely that the effects I have observed are mediated via phospholipase A₂, since the inhibitory effect exerted by these drugs is immediate and they inhibit in the absence of divalent cations which are necessary for activation of phospholipase A₂.

The drug which I have investigated most extensively is propranolol. Since this drug is both a β-blocker and a local anesthetic, I also investigated the β-blockers timolol and pindolol and the local anesthetics lidocaine and benzocaine. All of these drugs proved to be very poor inhibitors of anion uniport. Thus, the ability of propranolol to block anion uniport is probably not closely related to its β-blocking or local anesthetic properties.

In view of the fact that there could be a link between the outer membrane pore (VDAC or porin) and the anion uniporter (18), I also investigated the benzodiazepines R05-4864 and clonazepam. R05-4864 binds to the so-called peripheral receptors, which have been tentatively identified as the mitochondrial VDAC, whereas clonazepam binds to the central receptors (28). Both of these drugs inhibited anion uniport; however, it is noteworthy that the peripheral receptor-specific drug R05-4864 was 6.5 times more potent than clonazepam. This inhibition, however, does not appear to be related to binding to VDAC, since the Kᵦ for that process is reported to be about 1 nM (26), at least 2 orders of magnitude lower than the IC₅₀ for anion uniport. The tricyclic antidepressants amitriptyline and imipramine were chosen for investigation because they are water-soluble and possess three hydrophobic rings. On this basis I predicted that they would be more potent than propranolol, which is water-soluble but only has two hydrophobic rings. This proved to be the case and is consistent with the idea that polycyclic amphiphilic drugs in general will inhibit the anion uniporter.

Transport of many normally impermeant anions can be induced in three different ways: 1) by incubating the mitochondria at alkaline pH, as first demonstrated by Azzi and Azone (1, 2); 2) by depleting the matrix of divalent cations as demonstrated by Beavis and Garlid (6); and 3) by allowing mitochondria to respire in the presence of K⁺ and valinomycin, as first demonstrated by Brierley (3, 4). It has been proposed that a common pathway is responsible for the anion transport observed under each of these conditions (5, 18). In a previous paper (6), it was demonstrated that respiration-induced transport and A23187-induced transport are inhibited by DCCD with similar kinetics. I have now presented evidence that both A23187-induced and respiration-induced transport of anions is inhibited by a variety of different drugs. I have not investigated the effects of all these drugs on anion transport induced by alkaline pH; however, Selwyn et al. (20) have reported that local anesthetics inhibit the "pH-dependent anion-conducting pore" and have found that dibucaine, which is similar in structure to propranolol and quinine, is the most potent, with an IC₅₀ of 0.2 mM.

In addition to the showing that these drugs inhibit both A23187-induced and respiration-induced swelling, in this study I have made the important finding that the IC₅₀ values are not equal for the two processes. This could indicate that different pathways are active in the two assays; for example respiration-induced swelling could easily be explained on the basis of an electroneutral Cl⁻/OH⁻ exchange mechanism. This mechanism would remove the need for the anion to enter against the electrical gradient generated by respiration. The finding that the IC₅₀ values for the two processes appear to be related (Fig. 6), however, suggests that the transport processes are in fact related. Furthermore, the matrix pH differs substantially in the two assays and this provides an explanation for the difference. Thus, if the binding of the drug were dependent on matrix pH, different IC₅₀ values would be expected for the two assays. This conclusion is supported by the finding that the IC₅₀ for A23187-induced transport is strongly dependent on pH under conditions where the matrix pH parallels the medium pH. In fact, on the basis of this pH dependence and the finding that the difference in matrix pH between the two assays is 2–3 units, a much larger difference in IC₅₀ values might be expected. The effect of alkaline matrix pH on the binding of these drugs may, however, be compensated by endogenous Mg²⁺ present during the respiration-induced swelling experiments. This explanation is supported by two findings. First, matrix Mg²⁺ lowers the propranolol IC₅₀ for A23187-induced transport and second, depletion of matrix Mg²⁺ raises the IC₅₀ for respiration-induced swelling.² The interaction between the binding of Mg²⁺ and these drugs is currently under further investigation.

Analysis of the pH dependence of the IC₅₀ suggests that two protonation sites are involved in modulation of drug binding, one with a pKᵦ of about 8.4 and one with a pKᵦ of about 8.3. Since there is evidence that protonation of a site in the matrix with a high pKᵦ regulates the activity of the uniporter (5), the possibility that inhibition of transport and regulation of drug binding result from protonation of the same high pK site cannot be ruled out at present.

As mentioned above, the precise mechanism of anion transport during respiration-induced swelling is uncertain. It could occur via electrochemical unipor or electroneutral anion/OH⁻ antiport. The pH gradient clearly favors entry via an antiporter mechanism, whereas the membrane potential, negative inside, opposes influx via a unipor mechanism. Consequently, it would seem difficult to rationalize entry via unipor in energized mitochondria; however, the large extent of H⁺ ejection

² A. D. Beavis, unpublished observations.
observed during respiration-induced swelling solves this problem. The rate of swelling appears to peak when the extent of H⁺ ejection is maximal. On the basis of the matrix buffering power, the ΔpH at this point must be about 3 units (or 180 mV). Since, at the rate of respiration under these conditions, ΔpH cannot be much higher than 180 mV (51–53), the membrane potential must be close to zero. Thus, during this brief phase, the electrophoretic influx of anions is unopposed by the membrane potential. The subsequent decline in ΔpH to the steady-state value probably reflects activation of the K⁺/H⁺ antiporter. Although this should lead to a decrease in the overall ΔpH, a small increase in the membrane potential as the pH gradient decreases would be sufficient to explain the sharp decrease in the rate of uptake and the subsequent spontaneous contraction which I and others (1–4, 54) have observed.

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