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Uncouplers and the molecular mechanism of uncoupling in mitochondria

(cyclical cation transport/ionophoric synergism/protonophores)

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ABSTRACT Uncouplers are molecules with protonophoric and ionophoric capabilities that mediate coupled cyclical transport of cations—a transport that takes precedence over all other coupled processes. Uncouplers form cation-containing complexes with electrogenic ionophores that potentiate cyclical transport of cations. The molecular mechanism of uncoupling sheds strong light on the mechanism of coupling.

Electron flow in mitochondria can drive synthesis of ATP, accumulation of cations (K^+, Mg^{2+}, Ca^{2+}), or transfer of hydrogen from NADPH to NAD^+. Uncouplers sever the link between the driving reaction and the driven reactions without suppressing the rate of electron flow (1–3). The same effect of uncoupler on coupled reactions is observed when the driving reaction is hydrolysis of ATP rather than electron flow. This phenomenon of uncoupling is the most characteristic feature of mitochondrial energy coupling, and, although discovered some 30 years ago (3), its molecular mechanism has been an enigma. One valuable hint about the action of uncouplers has come to light. Under the conditions in which uncoupling takes place, all protonic and cationic gradients are abolished (4, 5)—an indication that uncouplers are carriers of both protons and cations.

There are 20 to 30 different molecular species that have in common the property of suppressing all coupled processes whether driven by electron flow or hydrolysis of ATP and the property of being equally effective in these two respects regardless of the substrate used for electron flow or the nature of the coupling site (6, 7). They differ only in respect to the concentration at which uncoupling is maximal. The most efficient uncouplers such as SF6847 can uncouple in the nanomolar range, whereas the less efficient uncouplers such as pentachlorophenol and 2,4-dinitrophenol are effective only in the millimolar range.

Uncoupling by the combination of valinomycin and nigericin in presence of K^+

It has been known for some time that the combination of two ionophores (valinomycin and nigericin) can uncouple oxidative phosphorylation in the same fashion as do classical uncouplers but only in the presence of K^+ (8, 9). We considered this correspondence of action as an invaluable clue since it has already been established that the combination of valinomycin and nigericin induces cyclical transport of K^+ coupled to electron flow (Fig. 1). The critical question was whether this combination of ionophores could duplicate all the known properties of uncouplers. The data of Table 1 clearly show that the correspondence is complete in all respects.

On the basis of this correspondence we formulated the hypothesis that uncouplers are inducers of coupled cyclical transport of cations and that this particular coupled process takes precedence over all other coupled processes such as coupled ATP synthesis, active transport of cations, and energized transhydrogenation. It would be predictable that, in order to mediate cyclical transport of cations: (*i*) uncouplers must be both ionophores and protonophores; (*ii*) uncoupling should depend upon the availability of cations for cyclical transport; and (*iii*) the concentration of uncoupler required for maximal uncoupling should be at least equal to the concentration of the complexes of the electron transfer chain [this equality has already been demonstrated for valinomycin (16) in the release of respiration of Complex IV by the combination of valinomycin, nigericin, and K^+]. One of the key predictions of the cyclical cation transport hypothesis—the elimination of all gradients—has already been demonstrated (see Table 1).

Uncouplers as protonophores and ionophores

The prevailing dogma that uncouplers uncouple by virtue of collapsing a proton gradient has spawned a vast literature attesting to the protonophoric capability of uncouplers (17–19), and hence this capability has been most thoroughly documented and verified. All uncouplers, without exception, can dissociate a proton in their uncharged state and can take up a proton in their charged state in the pH range of uncoupler action. In general, the best uncouplers have a pK above pH 6 and the less efficient uncouplers have a pK below pH 6.

Elsewhere we have presented evidence for the ionophoric capability of all uncouplers for both monovalent and divalent cations (6). Although there can be no question from the available data that uncouplers are bona fide ionophores, the conditions under which this capability is demonstrable rule out the possibility that uncouplers can function as ionophores in the physiological range of pH. It is only when the pH of the aqueous phase in a two-phase system is in the range of 8–10 that the ionophoric capability of uncouplers is significant. The important point to be emphasized, however, is that the capability is present.

Requirement of cations for the action of uncouplers

The general impression in the literature has been that uncoupling is not a cation-requiring process, although there have been occasional reports to the contrary (20, 21). What was not appreciated by other investigators of the uncoupling phenomenon and what we had to learn the hard way was that well-coupled mitochondria contain bound Ca^{2+} and Mg^{2+} in more than sufficient amounts to mediate the action of uncouplers. Table 2 contains data on the average values for bound Ca^{2+} and Mg^{2+} in well-coupled beef heart mitochondria and the degree to which the complement of bound divalent ions can be depleted by various reagents and experimental conditions. We have found that the acidic ionophore A-23187 is the reagent *par excellence* for depletion of coupled mitochondria to the point at which a cation requirement for uncoupling is demonstrable (22). The action of A-23187 can be duplicated by EDTA (23)

Table 1. Parallelism of the action of uncoupler and the combination of valinomycin and nigericin in presence of K^+ on coupled mitochondrial processes

Mitochondrial process	Units of change*	Control	Plus uncoupler	Plus valinomycin and nigericin
Release of respiratory control	ng atoms O	79	424	394
Release of latent ATPase activity	nmol ATP hydrolyzed	62	830	800
Coupled ATP synthesis	nmol Pi esterified	612	23	23
Active transport of Ca^{2+}	ng atoms Ca^{2+} transported	732	5	20
Energized transhydrogenation	nmol NADPH formed	76	0	0
Reversed electron flow	nmol NADH formed	135	10	6
Energized release of protons	ng atoms H^+ released at equilibrium	33	0	0

The various measurements were carried out at 30° with beef heart mitochondria according to the following methods: release of respiratory control, Hunter *et al.* (10); release of latent ATPase activity, Sadler *et al.* (11); coupled ATP synthesis, Southard and Green (12); active transport of Ca^{2+}, Southard and Green (12); energized release of protons, Southard *et al.* (13); energized transhydrogenation, MacLennan *et al.* (14); reversed electron flow, Ernster and Lee (15).

* All are per min/mg of mitochondrial protein, except energized release of protons.

(at pH 8.2 and 30°) and by other suitable ionophores. It cannot be a happenstance that uncouplers generally can induce the partial release of bound divalent cations from mitochondria (6)—a token that these bound cations can be mobilized by and made available to the uncouplers.

Table 3 shows that the respiration of A-23187-treated mitochondria suspended in a low-cation medium is increased only marginally by uncoupler and that monovalent cations at relatively high levels can restore the full extent of uncoupler action. A 10-fold increase in the uncoupler-dependent rate of respiration on addition of high levels of monovalent cations to A-23187-treated mitochondria was routine in multiple experiments that we have carried out. We have been unable to decrease the bound divalent cation level below 1-2 nmol/mg, and this residual level undoubtedly accounts for the low but significant respiratory rate induced by uncoupler in absence of added monovalent cation. The same pattern as that shown in the experiments of Table 3 was observed no matter which uncoupler replaced mClCCP. In other words, the requirement of monovalent cations for uncoupler action in A-23187-treated mitochondria is general for all uncouplers tested.

When mitochondria were exposed to 3.5 mM EDTA at pH 7.9 for 10 min at 25° and then washed free of the original medium, they showed a minimal response to the addition of uncoupler, but addition of 1 mM $CaCl_2$ or $MgCl_2$ prior to the addition of uncoupler induced a massive uncoupler response (Table 4). Several important conclusions may be drawn from experiments of this kind. First, uncoupler-mediated release of respiration can be potentiated by either monovalent or divalent cations. Second, the level of divalent cations required for this potentiation is ≤1% of the level of monovalent cations, indicating a clear preference for participation of divalent cations in uncoupling. Third, the uncoupler response of untreated mitochondria suspended in 1 mM Tris-HCl is 50% of that shown by EDTA-treated mitochondria in the presence of 1 mM Ca^{2+}. This discrepancy would suggest that, even in untreated mitochondria, augmentation of uncoupler action by addition of cation should be demonstrable (at least a 2-fold increase in the rate of respiration by either low levels of divalent cations or high levels of monovalent cations). Augmentation to this predicted level was indeed observed.

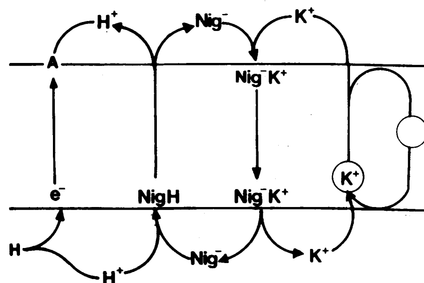

FIG. 1. Cyclical transport of K^+ by the combination of valinomycin and nigericin. ○, valinomycin; Nig^- charged form of nigericin; NigH, protonated form of nigericin; H, hydrogen originating from the substrate for electron transfer complex; A, final acceptor of the electron transfer chain. Charge separation in the electron transfer complex (separation of H^+ and e^-) is paired to the valinomycin-mediated separation of Nig-K^+ into K^+ and Nig^-. Charge elimination in the electron transfer complex (combination of e^- and H^+ with A) is paired to the combination of K^+ with Nig^-.

Synergism of uncoupler and intrinsic ionophore

Whenever a requirement of cations for uncoupler action was demonstrable, there was generally potentiation of the respiration *in the absence of added uncoupler*. We took this to mean that addition of salts of monovalent or divalent cations induced active transport of that cation and that there was a parallelism between the extent of active transport as measured by the rate of respiration in the absence of uncoupler and the magnitude of the uncoupler response. This parallelism was suggestive of a cooperative relationship between the uncoupler and the electrogenic ionophores that mediated active transport or other coupled processes.

Table 2. Bound Ca^{2+} and Mg^{2+} in different states of beef heart mitochondria

State of mitochondria	Bound divalent metal, nmol/mg of mitochondrial protein	
	Ca^{2+}	Mg^{2+}
Aggregated	30	40
Orthodox	0–2	3.0
A-23187-treated	0–2	4.3
Exposed to EDTA	19.5	5.4

The mitochondrial suspensions were finally washed in 0.25 M sucrose containing 1 mM Tris-HCl at pH 7.4 before analyses by atomic absorption by the method of Southard and Green (12). For details of the preparation of mitochondria in the various states, see Hunter *et al.* (10) for the preparation of orthodox mitochondria, legend of Table 3 for the preparation of A-23187-treated mitochondria, and legend of Table 4 for the mitochondria exposed to EDTA.

Table 3. Cation-dependence of uncoupler action in beef heart mitochondria depleted of divalent metals with A-23187

Addition to mitochondria	Rate of oxidation of durohydroquinone, ng atoms O/min per mg of protein		
	Without uncoupler	With uncoupler	Δ
None	223	345	122
KCl (10 mM)	264	416	152
KCl (100 mM)	619	1379	760
NaCl (100 mM)	619	1480	861
Tris · HCl (100 mM)	219	1366	1147

Heavy beef heart mitochondria were exposed to A-23187 (0.5 nmol/mg of protein) for 30 min at 30° in 0.25 M sucrose/10 mM Tris·HCl, pH 7.6/1 mM EDTA. The mitochondria were then washed twice in 0.25 M sucrose/10 mM Tris·HCl, pH 7.6, and resuspended in 0.25 M sucrose/1 mM Tris·HCl, pH 7.5. In the measurement of respiration by the oxygen electrode method, the substrate was durohydroquinone (1.3 mM) and the uncoupler was mClCCP (2 μM). The concentration of mitochondrial protein was 0.5 mg/ml. The experiment was carried out at 30°.

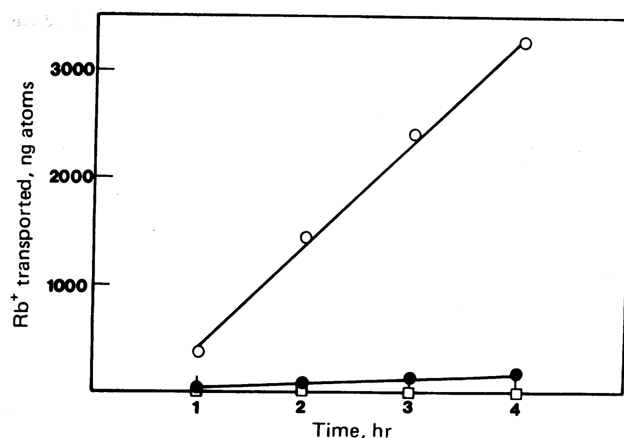

FIG. 2. Synergism of valinomycin and SF-6847 in the transport of Rb^+ in a Pressman cell. The donor aqueous compartment (2 ml) was 5 mM in $^{86}Rb_2SO_4$ and 25 mM in tetramethylammonium hydroxide (TMA)/*N*-tris(hydroxymethyl)methylglycine (tricine) (pH 8.0). The receiver aqueous compartment (2 ml) was 25 mM in TMA citrate (pH 4.0). The organic phase was chloroform/*n*-butanol, 70:30 (vol/vol). The concentration of valinomycin and SF-6847 in the organic phase was 2 mM. The samples taken from the receiver compartment for determination of $^{86}Rb^+$ were 20 μl. ○, valinomycin + SF-6847; ●, valinomycin; □, SF-6847.

Valinomycin can potentiate active transport of K^+ in mitochondria, and this active transport is suppressed by addition of uncoupler (24, 25). We could then use valinomycin as a model for the interaction of uncouplers with intrinsic electrogenic ionophores. The combination of valinomycin and uncoupler (SF-6847) increased the partition of Rb^+ from an aqueous to an organic phase (toluene/butanol, 70:30) by a factor of 27 or more as compared to the partition induced by valinomycin or uncoupler alone. A Pressman cell experiment (26) confirmed that the combination of valinomycin and uncoupler mediated transport of Rb^+ through an organic phase under conditions in which neither valinomycin nor SF-6847 alone could mediate a significant degree of transport (Fig. 2). We have tested synthetic neutral ionophores that can induce active transport of monovalent cations (crown ether and nactins) and found that these show synergistic action with uncouplers comparable to that of valinomycin (5 to 10-fold increase in the degree of partition or the rate of transport of Rb^+). Furthermore, we have found that SF-6847 can be replaced by other uncouplers in these synergistic reactions.

It is a reasonable inference that underlying the synergistic action is a 1:1:1 complex of electrogenic ionophore (EI), monovalent cation (Me^+), and uncoupler (U^-), represented by EI-Me^+-U^-. The cation is coordinated with polar groups in both the electrogenic ionophore and the uncoupler. A simple experiment provided strong support for this postulated composition of the complex. The combination of valinomycin and uncoupler, each at a concentration of 100 nmol/ml of the aqueous phase, induced the partition of 87 nmol of Rb^+ in the organic phase. When the concentration of valinomycin, but not of uncoupler, was increased or when the concentration of uncoupler, but not of valinomycin, was increased, the partition of Rb^+ remained at the value corresponding to equimolar concentrations of the two reactants.

Does the same synergism apply to electrogenic ionophores for divalent cations? To test this possibility it was first necessary to find ionophores that could induce active transport of divalent cations in mitochondria. We have found that beauvericin and Triton X-100 could function in that capacity in mitochondria exposed to a level of ruthenium red that inactivates the intrinsic electrogenic ionophore for Ca^{2+}. Under these conditions, beauvericin and Triton X-100, each at about 0.02 mM concentration, induced active transport of Ca^{2+} in presence of appropriate anions such as inorganic phosphate. The synergistic effect of beauvericin and uncoupler in mediating transport of Ca^{2+} was demonstrable in a Pressman cell experiment and was

Table 4. Restoration of uncoupler-mediated release of respiration by addition of Mg^{2+} or Ca^{2+} to heavy beef heart mitochondria depleted of divalent metal cations by exposure to EDTA

Type of mitochondria	Additions	Rate of respiration, ng atoms O/min per mg of protein		
		Without uncoupler	With uncoupler	Δ
Untreated	0	377	1640	1263
Exposed to EDTA	0	311	590	279
Exposed to EDTA	$CaCl_2$ (1 mM)	754	3440	2686
Exposed to EDTA	$MgCl_2$ (1 mM)	622	2790	2168

Heavy beef heart mitochondria suspended in 0.25 M sucrose/1 mM Tris·HCl at a final protein concentration of 10 mg/ml were supplemented with the sodium salt of EDTA (3.5 mM) and the pH was adjusted to 7.9. The suspension was incubated for 10 min at 25° and then cooled on ice before centrifugation. The pellet was resuspended in the original medium (no EDTA added). Durohydroquinone (1.3 mM) was used as substrate; mClCCP (2 μM) was the uncoupler.

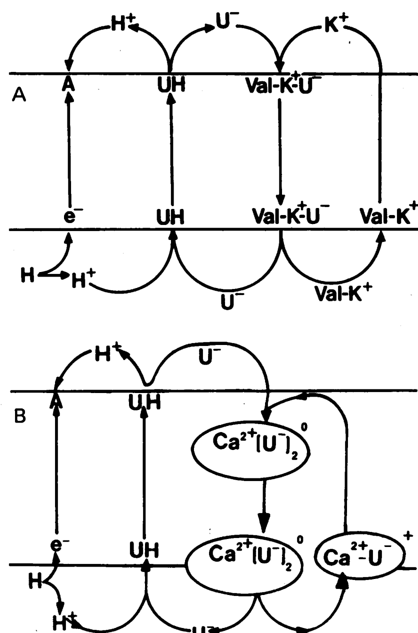

FIG. 3. (*A*) Cyclical transport of a monovalent cation (K^+) mediated by the combination of an electrogenicionophore and an uncoupler (U^-). The charged form of the uncoupler is represented as U^- and the protonated form, as UH. Val represents the neutral form of the intrinsic ionophore, Val-K^+ as the electrogenic form. The movement of e^- through the electron transfer chain is coupled to the movement of Val-K^+ across the membrane. (*B*) Cyclical transport of a divalent cation (Ca^{2+}) mediated by the combination of an electrogenic ionophore and an uncoupler (U^-). $\bigcirc^+$ represents the electrogenic ionophore in its positively charged form and $\bigcirc^0$, the electrogenic ionophore in its fully neutralized form.

indistinguishable from that shown in Fig. 2 for the valinomycin-uncoupler synergism. Thus, we may conclude that the synergistic effect of uncoupler and electrogenic ionophore is general whether the electrogenic ionophore acts on monovalent or divalent cations [see (27) for the electrogenic nature of beauvericin].

Molecular mechanism of uncoupler-induced cyclical transport of cations

Fig. 3 shows formulations in terms of the paired moving charge model of the mechanism of uncoupler-induced cyclical transport of monovalent and divalent cations. Strictly speaking, both the cation and the uncoupler are cyclically transported according to the formulations. In cyclical transport of monovalent cations, the cycling of the uncoupler is nonelectrogenic but in cyclical transport of divalent cations, the cycling of the uncoupler has both an electrogenic and a nonelectrogenic step. It has been established by Moyle and Mitchell (28) and others (29) that the electron drives the transport of one calcium ion across the membrane. This 1:1 stoichiometry would require the cotransport of a singly charged negative species with Ca^{2+} in order to satisfy the requirements of charge neutrality.

The molar relationship between the concentration of uncoupler and the concentration of the electron transfer complex

According to the formulations shown in Fig. 3, there should be a 1:1 molar relationship between the electron and the uncoupler in cyclical transport of monovalent cations and a 1:2 molar relationship in cyclical transport of divalent cations. In untreated mitochondria suspended in 0.25 M sucrose buffered with 1–10 mM Tris·HCl, the uncoupler-mediated release of respiration

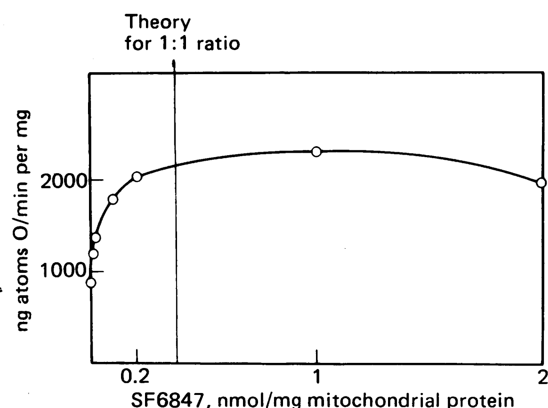

FIG. 4. The molar relation between the concentration of uncoupler (SF-6847) required for release of respiratory control and the concentration of the electron transfer complex in beef heart mitochondria. Oxygen consumption was measured at 30° with an oxygen electrode with durohydroquinone as substrate in a medium 0.25 M in sucrose, 100 mM in Tris·HCl (pH 7.5) and 1.3 mM in durohydroquinone. The concentration of uncoupler in the mitochondrion is assumed to be equal to the concentration added to the suspension. The concentration of SF-6847 is expressed as nmol per mg mitochondrial protein. Note that in this medium with a high cation content the respiration of the mitochondrial suspension is partially released in absence of added uncoupler by virtue of active transport. In presence of uncoupler this active transport would be suppressed.

was entirely dependent on bound divalent metals. The stoichiometry for the electron and the uncoupler was approached at the point of maximal release of respiration (Fig. 4). Because both monovalent and divalent cations are available to the uncoupler, the theoretical ratio should be between 1:1 and 1:2. The uncouplers tested that showed close to theoretical stoichiometry (SF-6847 and mClCCP) were among the most efficient uncouplers. Such titrations are difficult to carry out with the weaker uncouplers because in general their partition is greatly in favor of the aqueous phase. The antimycin titer is taken as a measure of the concentration of each of the electron transfer complexes (30). This comes to a value of 0.21 nmol/mg of protein. Because the oxidation of durohydroquinone by molecular oxygen involves two complexes (III and IV), the concentration of the electron transfer complexes reacting with uncoupler is assumed to be 0.42 nmol/mg of protein. There have been reports in the literature that less uncoupler is required than is stoichiometric with the electron (31). In such determinations, the substrates selected for driving electron transfer did not fully exploit the capacity of the electron transfer chain for electron flow.

Uncoupler-mediated release of ATPase activity

All the effects that have been documented for uncoupler-mediated release of electron flow apply as well to the effects of uncouplers on ATP-driven coupled reactions. These effects include the cation requirement for uncoupling and the 1:1 or 1:2 molar relationship between ATPase and uncoupler at the point of maximal release of ATP activity.

Synergism of intrinsic electrogenic ionophore and uncoupler

Blondin *et al.* (32, 33) have isolated from beef heart mitochondria a neutral peptidic ionophore that can induce active transport of monovalent cation in untreated mitochondria. The electrogenic influx rate induced by the isolated peptidic ionophore is a small fraction (<10%) of the nonelectrogenic efflux rate observed when active transport is terminated by addition

of uncoupler. This 10-fold or more augmentation in the efflux rate of K^+ induced by the combination of uncoupler and electrogenic ionophore is another manifestation of the synergistic action of these two species. That this is a synergistic action is confirmed by the efflux rate when active transport is inhibited by addition of antimycin. In this instance, the efflux rate is equal to the influx rate.

Direct demonstration of uncoupler-induced cyclical cation transport

When beef heart mitochondria under anaerobic conditions in a medium containing catalase, K^+ (2 mM), and durohydroquinone as electron transfer substrate were pulsed with oxygen, a spike of proton release followed by proton uptake and an inverse spike of K^+ uptake followed by K^+ release were observed with a pH- and a K^+-sensitive electrode, respectively, measured simultaneously. The proton and K^+ spikes lasted as long as the available oxygen introduced in the pulse (20–30 sec). When uncoupler was added to such a test system in increasing amounts, the spikes became sharper and of shorter duration (1–3 sec at 30°). The duration of such proton and K^+ spikes induced by oxygen pulsing in presence of uncoupler was far less than the time required for complete utilization of oxygen (a 20- to 50-fold discrepancy). What this indicates is that, even in presence of excess uncoupler, there is a temporary imbalance between the influx and efflux rates for protons and K^+. This imbalance visualized by the spikes constitutes direct evidence for cyclical cation transport mediated by uncoupler.

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