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Uncouplers of Oxidative Phosphorylation

by Hiroshi Terada*

Uncouplers of oxidative phosphorylation in mitochondria inhibit the coupling between the electron transport and phosphorylation reactions and thus inhibit ATP synthesis without affecting the respiratory chain and ATP synthase (H^+–ATPase). Miscellaneous compounds are known to be uncouplers, but weakly acidic uncouplers are representative because they show very potent activities. The most potent uncouplers discovered so far are the hindered phenol SF 6847, and hydrophobic salicylanilide S-13, which are active *in vitro* at concentrations in the 10 nM range. For induction of uncoupling, an acid dissociable group, bulky hydrophobic moiety and strong electron-withdrawing group are required. Weakly acidic uncouplers are considered to produce uncoupling by their protonophoric action in the H^+–impermeable mitochondrial membrane. For exerting these effects, the stability of the respective uncoupler anions in the hydrophobic membrane is very important. High stability is achieved by delocalization of the polar ionic charge through uncoupler (chemical)-specific mechanisms. Such an action of weakly acidic uncouplers is characteristic of the highly efficient membrane targeting action of a nonsite-specific type of bioactive compound.

Introduction

The common bioenergy currency, ATP, is synthesized in energy-transducing membranes such as those of mitochondria, chloroplasts, and various microorganisms. The energy to drive the uphill reaction (phosphorylation) for synthesis of ATP from Pi (orthophosphate) and ADP by ATP synthase (ATPase) is supplied by sequential oxidation-reduction chain reactions in electron transporting systems. During photophosphorylation in chloroplasts, this energy is supplied by the photosynthetic electron transport chain, whereas during oxidative phosphorylation in mitochondria and prokaryotic cells, it is supplied by the respiratory chain. Thus ATP is synthesized by coupling two reactions, electron transport and phosphorylation. Uncouplers inhibit ATP synthesis by preventing this coupling reaction in such a fashion that the energy produced by redox reactions cannot be used for phosphorylation. Thus, in the presence of an uncoupler, the activities of electron flow and ATPase are not inhibited, but ATP synthesis cannot take place (Fig. 1) (*1*).

A wide variety of compounds are known to be uncouplers of oxidative phosphorylation in mitochondria. Most of them are hydrophobic weak acids that possess protonophoric activities; i.e., activities for transporting H^+ through an H^+–impermeable membrane. According to the chemiosmotic theory of Mitchell (*1*), direct energy for ATP synthesis in the form of the chemical potential of H^+ (proton motive force) across H^+–impermeable energy-transducing membranes is supplied by redox reactions. ATP is synthesized from ADP and Pi when H^+ enters the mitochondria via H^+–ATPase (F_oF_1–ATPase), which consists of the catalytic site F_1 projecting from the membrane and a connecting hydrophobic protein F_o buried in the membrane. Thus a protonophoric action to collapse the H^+ chemical potential by transport of H^+ into the mitochondria via the membrane is regarded as essential for uncoupling action.

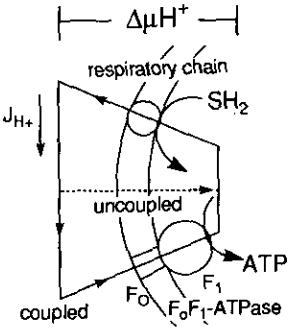

FIGURE 1. Coupling and uncoupling of the two reactions of electron transport and phosphorylation based on the H^+ motive force.

Furthermore, since the proton motive force across membranes consists of a pH difference (ΔpH) and a membrane potential ($\Delta\psi$), any compound or physical force such as osmotic shock and aging that dissipates the pH difference and membrane potential can cause uncoupling. Because weakly acidic uncouplers are representative of various types of uncouplers, this paper focuses mainly on the features of the uncoupling actions of weakly acidic uncouplers in mitochondria and their structural requirements for uncoupling activity.

*Faculty of Pharmaceutical Sciences, University of Tokushima, Shomachi-1, Tokushima 770, Japan.

Biological Responses Induced by Uncoupling in Mitochondria

The rate of mitochondrial respiration (Fig. 2) in the presence of a respiratory substrate such as succinate is low (state 4 respiration). However, respiration increases about 5-fold on addition of exogenous ADP when the incubation medium contains Pi (state 3 respiration). This increase is the result of phosphorylation, and the respiratory rate returns to the original state 4 level when sufficient ADP is phosphorylated to ATP. On the addition of an uncoupler, the respiratory rate increases abruptly in a dose-dependent fashion. If the uncoupler produces no inhibitory effect on the respiratory chain, the maximum respiratory rate attained is more than 6-fold or 7-fold that of state 4. Since this respiratory rate exceeds that in state 3, the state 3 respiration is also stimulated. State 3 respiration is inhibited to the level of state 4 by an addition of phosphoryl transfer inhibitors (i.e., oligomycin) because of their inhibition of H^+ entry through the H^+–channel in the F_o portion of the H^+–ATPase. Uncouplers release this inhibited respiration. Uncouplers, however, cannot stimulate respiration that has been inhibited by respiratory inhibitors such as antimycin and KCN. These effects of uncouplers on respiration provide simple methods for determining whether or not a given compound has uncoupling activity.

In general, weakly acidic uncouplers activate ATPase more than 6-fold when ATPase is bound to the mitochondrial membrane (F_oF_1-ATPase), but they do not activate isolated F_1-ATPase. This suggests that these uncouplers act on membranes and not directly on the F_oF_1–ATPase protein. However, some uncouplers such as DNP[1] activate isolated ATPase (*2*).

General Structural Features of Weakly Acidic Uncouplers

Phenols, benzimidazoles, *N*-phenylanthranilates, salicylanilides, phenylhydrazones, salicylic acids, acyldithiocarbazates, cumarines, and aromatic amines are known to induce uncoupling (*3–5*). These compounds are all weak acids, and their uncoupling is thought to be attributable to their protonophoric actions, though the mechanism of uncoupling by aromatic amines such as the local anesthetics bupivacaine and dibucaine is controversial (*6,7*). The chemical structures of representative weakly acidic uncouplers are shown in Figure 3. The concentrations shown beside their structures are the approximate minimum concentrations for induction of full uncoupling activity determined by stimulation of state 4 respiration in isolated rat liver mitochondria.

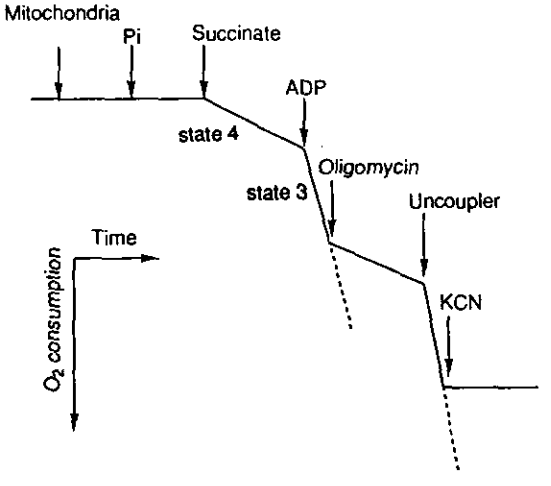

FIGURE 2. Effects of various inhibitors on the respiration of rat-liver mitochondria.

The most potent of these compounds are SF 6847 (*8*) and S-13 (*9*), exhibiting uncoupling activity at concentrations in the 10 nM range. Most powerful uncouplers induce uncoupling at concentrations of less than 1 μM. Complete uncoupling can be induced at about 0.05 mole of SF 6847 (*10*), and less than 0.2 mole of S-13 (*11*) per respiratory chain or per H^+–ATPase. These data indicate that uncouplers act as catalysts and not as specific inhibitors that bind firmly to some site on a component of the mitochondrial membrane. The common chemical features of uncouplers that are present in SF 6847 and S-13 consist of: an acidic dissociable group, an electron-withdrawing moiety, and a bulky hydrophobic group(s). In SF 6847, a phenolic OH-group surrounded by bulky di-*tert*-butyl groups is located at a certain spatial distance from the strongly electron-withdrawing malononitrile group. The geometric arrangement of these three groups is considered to be important for induction of strong uncoupling activity (*5*).

The replacement of the acid-dissociable group of a weakly acidic uncoupler by a nonacid dissociable moiety results in complete loss of uncoupling activity (*12*). Moreover, the finding that the resultant compound, devoid of an acid dissociable group, can cause uncoupling is concluded to be because of its contamination with the parent compound by an acid dissociable group (*12*) or the molecular conversion of the compound to the acidic compound catalyzed by dimethylsulfoxide that is used as solvent in the stock solution (*13*).

Studies on the quantitative structure-uncoupling activity relationship indicate that uncoupling activity (BR) in mitochondria is, in most cases, depicted as a linear function of the hydrophobicity determined as the partition coefficient in the octanol and water system (P_{oct}) and the electron withdrawing power represented by the acid dissociation constant pK_a (*5*). As an example, the case for salicylanilides in rat liver mitochondria is shown in Eq. (1) (*11*):

$$\begin{aligned}\text{Log BR} &= 2.886 + 1.044 \log P_{oct} + 0.272\ pK_a \\ &\quad (\pm 0.372)\ (\pm 0.249) \qquad (-0.144) \\ &\quad n = 25,\ r = 0.903,\ s = 0.370 \end{aligned} \tag{1}$$

where values in parentheses are 95% confidence intervals, n is the number of compounds tested, r is the correlation coefficient, and s is the standard deviation. Thus, high hydrophobicity and strong electron-withdrawing properties are of primary importance for induction of uncoupling. However, the degree of contri-

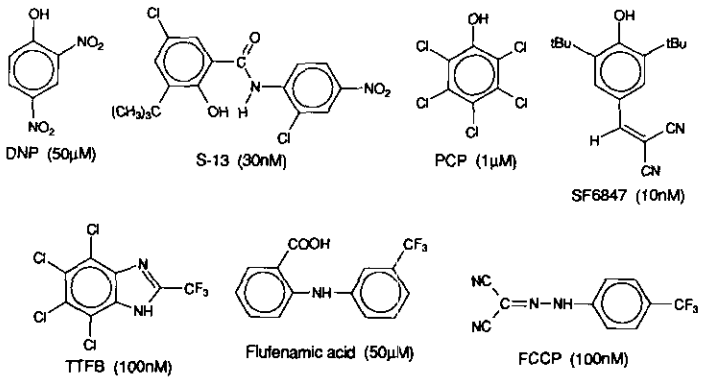

FIGURE 3. Structure and uncoupling activities (maximal stimulation of stage 4 respiration of isolated rat liver mitochondria) of representative weakly acidic uncouplers (*5,25*). The acidic proton and pK_a value, respectively, are as follows: DNP, phenolic OH, 4.1; S-13, phenolic OH, 6.57; PCP, phenolic OH, 4.80; SF 6847, phenolic OH, 6.83; TTFB, imidazole NH, 5.5 in 50% ethanol; flufenamic acid, COOH, 3.85; FCCP, secondary amine, 6.2 in 10% ethanol (*5,25*).

bution of these properties to uncoupling depends on the uncoupler. In the case of S-13, the most important factor is hydrophobicity, and the electron-withdrawing power is auxiliary (*11*).

As with salicylanilides, both hydrophobicity and electron-withdrawing ability are major factors for the activities of most weakly acidic uncouplers such as phenols, *N*-arylanthranilic acids, phenylhydrazones, and aryl indanediones (*14*). However, the determination of these two parameters simply and correctly is very difficult because these compounds are always only slightly soluble in water. Furthermore, determination of log P_{oct} values of more than 3 is very difficult under usual experimental conditions. A simple method is needed for determining these two parameters. The retention behaviors on HPLC under suitable conditions are very useful for this purpose. Log P_{oct} values of more than 6 (Fig. 4) and pK_a values can be determined easily by this method (*15*). The HPLC method is also shown to be very efficient for prediction of uncoupling activities (*11*). Furthermore, it is noteworthy that calculation of log P_{oct} from chemical structures is a valuable tool for estimating uncoupling activity.

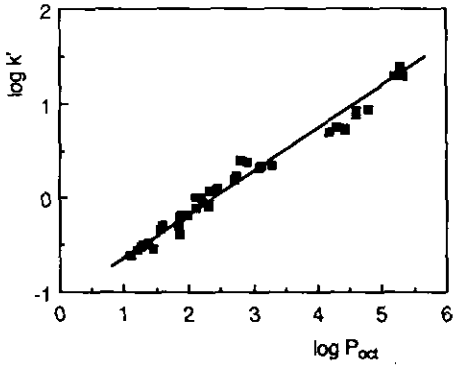

FIGURE 4. Linear correlation of the capacity factor k' in high performance liquid chromatography and the partition coefficient between octanol and water, P_{oct}. Adapted from Terada (*15*). Glycerol-coated controlled pore glass was used as a stationary phase in the chromatography.

Protonophoric Activity of Weakly Acidic Uncouplers

The simplest mechanism of the protonophoric action of a weakly acidic uncoupler is illustrated in Figure 5. At the membrane-water interface, the anionic form of the uncoupler, U^-, traps H^+ and becomes the neutral form UH. UH traverses the membrane to the opposite side where it releases H^+. U^- then returns to the original interface where it again traps H^+. By this uncoupler cycle, H^+ is transported into the inner side of mitochondria through the H^+ impermeable membrane, thus dissipating the H^+ gradient across the membrane, which results in uncoupling (*5,15*).

Since this cycle is governed by Brownian motion, the maximum number of cycles should be about 1000/sec. Cycling rates for potent uncouplers are consistent with this theoretical maximum value. SF 6847 was found to cycle about 800/sec when functioning at maximal efficiency (*10*), while S-13 cycles about 400/sec under usual experimental conditions (*11*). Because these compounds are the most potent uncouplers known at the present time, their cycling rates provide a measure for the limit of protonophoric activity. If an uncoupler that cycles more than 1000/sec is discovered, another mechanism needs to be considered.

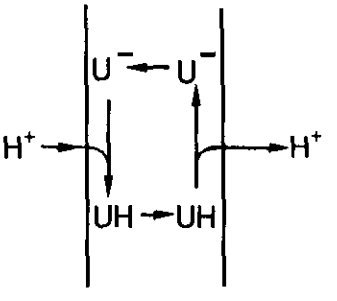

FIGURE 5. Protonophoric action of weakly acidic uncoupler.

Stability of Uncoupler Anions in the Membrane

The efficiency of uncoupling depends on the stability of uncoupler anions in the hydrophobic membrane (*5,16*). In general it is thought that ionic species of molecules cannot remain deep in a hydrophobic environment. However, according to the protonophoric mechanism in Figure 5, an uncoupler anion should remain stable in the membrane. Delocalization of charge in uncoupler anions can efficiently increase hydrophobicity (*5*). In the case of SF 6847, the electron-withdrawing ability of the malononitrile moiety should be highest when it is located in the same plane as that of the benzene ring ($\theta = 0$ in Fig. 6). However, such a flat structure can be shown by molecular orbital (MO) calculations to be most unstable in solution, although in the solid crystalline state SF 6847 takes a flat structure owing to forced packing. NMR and MO calculation studies in organic solution have indicated that the malononitrile moiety shows restricted intramolecular rotation in such a way that it oscillates 50° to either side from the position perpendicular to the benzene ring (Fig. 6). At 25 °C, the number of oscillations is about 10^6/sec for the neutral form of SF 6847, but it is only about 100/sec for the anionic form (SF^-). These results indicate that SF^- takes a more planar structure than SF 6847, thus making the electron-withdrawing power of the malononitrile group more efficient. Interestingly, in a solution containing the potassium ionophore valinomycin and K^+, the oscillatory motion of the malononitrile group of SF^- is greatly increased to about the same level as that of SF 6847. This increase in oscillatory motion localizes the negative charge at the phenoxide moiety, facilitating ion-pair formation of valinomycin-K^+ (*5*). These features indicate that SF 6847 is a well-designed molecular device in which the electronic structure is regulated according to the microenvironment.

When various alkyl chains were introduced onto the positions *ortho* to the phenolic OH in 4-hydroxybenzylidenemalononitrile (SF-2H), the activation energy (Ea) of the oscillation of the malononitrile moiety was found to increase with increasing alkyl chain length up to *tert*-butyl (SF 6847); i.e., the planarity of the molecule increased with increase in the length of alkyl chains. The value of E_a is the greatest for SF 6847 (64 KJ/mole), and lowest for SF-2H (42 KJ/mole) (*5,17*). The pK_a, which is a measure of the electron-withdrawing ability of the malononitrile group, is also related to this oscillation. Interestingly, the pK_a of SF 6847 (6.83) is smaller than that of SF-2H (7.25), even though SF 6847 contains a phenolic OH group that is sterically hindered by two bulky *tert*-butyl groups. Such a difference in pK_a values arises from the fact that the electron-withdrawing power of SF 6847 is greater than that of SF-2H due to the greater energy barrier for oscillations with the hindered phenol (*5,17*).

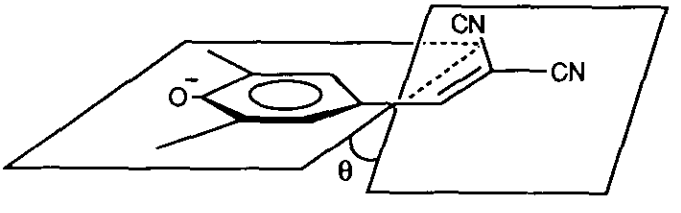

FIGURE 6. Restricted intramolecular rotation of the malononitrile moiety in SF 6847. Theta (θ) is the dihedral angle between the benzene ring and the malononitrile moiety; $\theta = 0$ when the two are coplanar.

According to the mechanism of protonophoric action in Figure 5, both hydrophobicity and a moderate pK_a should be of primary importance for uncoupling. SF 6847 is endowed with these two properties by its *tert*-butyl groups. In addition to contributing to the hydrophobicity of the compound, the *tert*-butyl groups exert two other effects: occlusion of the ionic charge of the phenoxide group from the environment, and enhancement of the planarity of the molecule, causing an increase of the acidity of the phenolic OH group and a delocalization of the polar ionic charge of the phenoxide group. As a result of these two effects, the stability of the SF 6847 anion in a hydrophobic environment is achieved along with a moderate pK_a value. In the series of SF 6847 derivatives, the activation energy E_a of the anionic form is well correlated with the uncoupling activity (*5*) (Fig. 7). A similar correlation is observed in the protonophoric activities of this series measured as the increase in the electronic conductivity in a planar phospholipid bilayer membrane. These results suggest that the oscillatory motion regulates the uncoupling activity based on the protonophoric action (*17*).

In the case of S-13, intramolecular hydrogen bond formation between an NH in the aniline moiety and phenolic OH in the salicylic acid moiety increases the hydrophobicity of both the neutral and anionic form of S-13 and stabilizes its anionic form, as depicted in Figure 8. The log P_{oct} value of the unsubstituted salicylanilide is estimated to be 1.95 without hydrogen bonding but is estimated to be 3.50 with hydrogen bonding. Thus, formation of a six-membered hydrogen bonded ring increases the hydrophobicity of the neutral form of S-13 about 35-fold. Furthermore, in the case of the S-13 anion, the negative charge is delocalized by the aromatic nature of the hydrogen bonded ring, which is

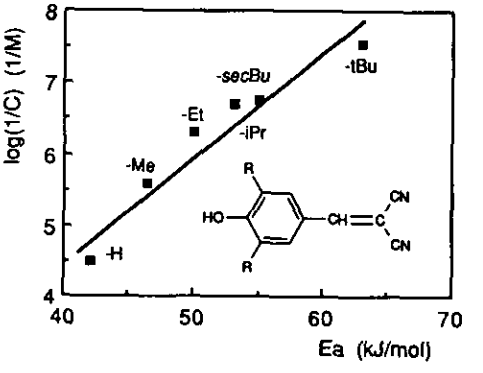

FIGURE 7. Dependence of uncoupling activity (log 1/C) on the activation energy of restricted rotation (E_a) of anionic forms of SF 6847 derivatives. Adapted from Terada et al. (*17*).

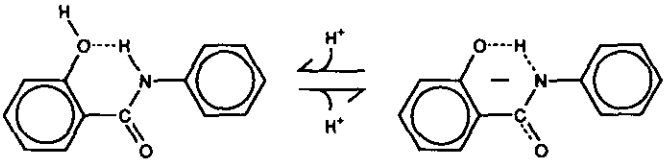

FIGURE 8. Formation of an intramolecular hydrophobic hydrogen bond in the salicylanilide molecule. Adapted from Terada et al. (*11*).

coplanar to the benzene ring of the salicylic acid moiety (*11,18*).

Uncouplers That Have No Protonophoric Activity

Some compounds that do not possess an acid dissociable group are also known to induce uncoupling. However, their activities are weaker than those of protonophoric uncouplers, and they show their actions at more than micromolar orders of concentration. It is generally observed that hydrophobic cations induce uncoupling in mitochondria but not in submitochondrial particles and chloroplasts where the orientation of membrane proteins and the sign of the membrane potential is the opposite to those of mitochondria (mitochondria are negative inside and chloroplasts are positive inside). On the other hand, hydrophobic anions such as picric acid and tetraphenyl borate induce uncoupling only in submitochondrial particles and chloroplasts (*19,20*). From these results, uncoupling is considered to be due to the dissipation of the membrane potential caused by the electrophoretic transfer of these hydrophobic ions to the inside space of membrane systems according to their membrane potential (*1,21*).

Uncoupling, however, cannot be based solely on this mechanism. Uncoupling by the cationic cyanine dyes tri-S-C_4(5) and tri-S-C_7(5) requires Pi; SH-reagents, such as *N*-ethylmaleimide, prevent uncoupling. These dyes stimulate state 4 respiration, but do not stimulate ATPase significantly (*22*). The uncoupling is proposed to be due to a modification of the state of the ADP/ATP transporter (*23*). The uncoupling mechanisms of SH-reactive compounds such as the Cu^{2+}-*o*-phenanthroline complex and aromatic isothiocyanates and Cd^{2+} and Ag^{+} are possibly similar to those caused by cyanine dyes, although their protein sites are unknown at present. Furthermore, the protonophoric action of picric acid is proposed to include uncoupling (*24*). Extensive studies are necessary to understand the mechanisms of uncoupling by these compounds.

Conclusion

Weakly Acidic Uncouplers Act As Nonsite-Specific Bioactive Compounds Acting on Biomembranes

The weakly acidic uncouplers are representative of protonophoric uncouplers and can be quite potent; however, there are other types of uncouplers with nonprotonophoric actions. Protonophoric uncouplers act on energy transducing biomembranes and induce uncoupling specifically and at very low concentrations. Uncoupling is caused primarily by interaction of the weakly acidic uncoupler with the phospholipid in the target membrane, making the membrane permeable to H^+, which results in uncoupling.

Now let us consider the mode of action of membrane targeting bioactive compounds that induce biological activity by modification of the state of a specific membrane protein. The mechanisms of induction of biological activity can be classified into two types (Fig. 9). In one case (Fig. 9*A*) compounds interact with a specific receptor site in the membrane. These bioactive compounds are classified as site-specific and their mechanism is summarized as follows:

Specific interaction with receptor → Specific biological activity

In the other case (Fig. 9*B*) compounds interact in a nonsite-specific manner. The mechanism of these compounds can be depicted as:

Nonspecific interaction with membranes → Specific biological response

Compounds of the first type can induce highly specific and potent biological activities because they have a specific binding receptor site in the membrane. Compounds of the second type generally do not express such specificity and potency because they do not have a specific protein binding site. In the latter case, a molecular device in the compound facilitates any observed specificity and potency. As an example, the action of weakly acidic uncouplers as nonsite-specific type compounds can show very high potency and specificity, as observed with SF 6847. Thus, nonsite-specific bioactive compounds can have very specific and potent actions, even though they do not have a specific receptor site in the membrane.

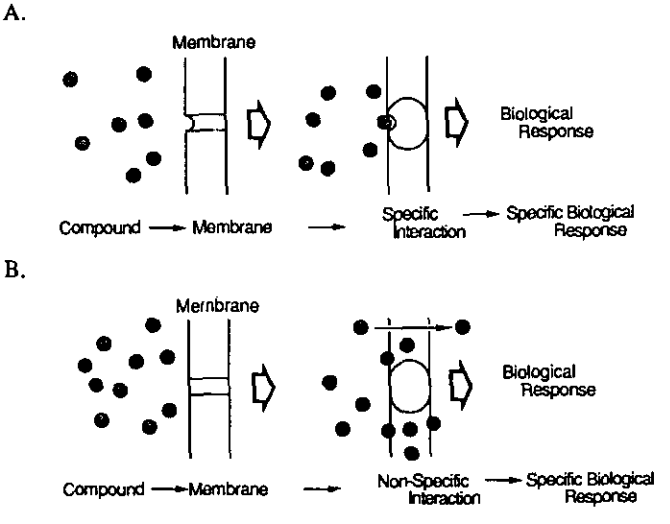

FIGURE 9. (*A*) Site-specific membrane-targeting bioactive compounds and (*B*) nonsite-specific membrane-targeting bioactive compounds.

These uncouplers possess a special feature in their molecule able to compensate for the lack of a receptor site. In the case of SF 6847, the restricted intramolecular oscillation of the malononitrile moiety regulated by the *ortho*-substituted *tert*-butyl groups is the molecular feature regulating the electronic structure of the compound. All potent nonsite-specific compounds would be expected to have a special feature in their molecules as well. Elucidation of these molecular features are crucial for understanding mechanisms of action and for the molecular design of new bioactive compounds.

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