Journal of Bioenergetics and Biomembranes, Vol. 22, No. 5, 1990

Dicyclohexylcarbodiimide as Inducer of Mitochondrial Ca^{2+} Release

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Received October 16, 1989; revised March 26, 1990

Abstract

The effect of the alkylating reagent dicyclohexylcarbodiimide (DCCD) on mitochondrial Ca^{2+} content was studied. The results obtained indicate that DCCD at a concentration of 100 μM induces mitochondrial Ca^{2+} efflux. This reaction is accompanied by an increasing energy drain on the system, stimulation of oxygen consumption, and mitochondrial swelling. These DCCD effects can be partially suppressed by supplementing the incubation medium with 1 mM phosphate. By electrophoretic analysis on polyacrylamide-sodium dodecyl sulfate, it was found that DCCD binds to a membrane component with an M_r of 20 to 29 kDa.

Key Words: Dicylcohexylcarbodiimide: DCCD: Ca^{2+} release; Kidney mitochondria.

Introduction

Cellular Ca^{2+} homeostasis is believed to be controlled in part by its uptake and release from mitochondria (Carafoli, 1987). The route by which Ca^{2+} release is performed is largely unknown. A variety of different compounds and agents cause Ca^{2+} release from Ca^{2+}-loaded mitochondria. These include, among others, the inhibitor of the calcium uniporter ruthenium red (Compton *et al.*, 1978), sulfhydryl reagents (Beatrice *et al.*, 1980; Palmer and Pfeiffer, 1981; Kapoor and van Rossum, 1984; Chavéz *et al.*, 1987; Chávez and Holguín, 1988; Chávez *et al.*, 1989), inorganic phosphate (Rossi and Lehninger, 1964; Coelho and Vercesi, 1980), phosphoenolpyruvate (Chudapongse and Haugaard, 1973; Peng *et al.*, 1974) atractylate (Asimakis and Sordahl, 1977; Chávez and Osornio, 1988), and palmitoyl CoA (Sul *et al.*, 1976; Wolkowicz and McMillin-Wood, 1980. A question that is still

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0145-479X/90/1000-0679$06.00/0

unanswered is whether the increased permeability to Ca^{2+} efflux represents the opening of a specific pore or nonspecific changes in the permeability properties of the lipid bilayer (Ackerman and Nicholls, 1983).

During the course of experiments, by studying the mercury-induced loss of mitochondrial Ca^{2+} (Chávez and Holguín, 1988), we found this reaction to be insensitive to ruthenium red. This would indicate that matrix Ca^{2+} is released by a pathway different from that of ruthenium red-sensitive uniport, probably through a H^+/Ca^{2+} exchange reaction. To explore this possibility we used dicyclohexylcarbodiimide (DCCD), a reagent that blocks membrane H^+ channels (Beechley *et al.*, 1966; Beattie and Villalobo, 1982) and the activity of the mitochondrial K^+/H^+ antiport (Martin *et al.*, 1984, 1986). However, unexpectedly we found that DCCD, at a concentration of 100 μM, induced a fast and extensive mitochondrial Ca^{2+} release. Further investigation showed that this reaction was accompanied by an increasing energy drain on the system, stimulation of oxygen consumption, and mitochondrial swelling. Addition of 1 mM phosphate to the incubation medium resulted in a partial protection against the deleterious effect of DCCD. Studies of [^{14}C]-DCCD binding to the membrane protein revealed that radioactivity was incorporated to membrane components with apparent molecular weight ranging from 20 to 29 kDa.

To explain the Ca^{2+} releasing effect of DCCD, it must be considered that in addition to its reaction with carboxylic groups, the alkylating reagent reacts with membrane thiol groups (Beavis and Garlid, 1988) and inhibits the energy-linked transhydrogenase (Phelps and Hatefi, 1981). These factors have been implicated in the regulation of intramitochondrial Ca^{2+} content.

Material and Methods

Rat kidney mitochondria were obtained according to the procedure described (Chávez *et al.*, 1985). Spectrophotometric determination of Ca^{2+} movements were performed at 685–675 nm, using Arsenazo III as the indicating dye (Kendrick, 1976). Alternatively cation transport was estimated by the filtration technique with $^{45}CaCl_2$ (specific activity 1000 cpm/nmol). Radioactivity retained by the filters was determined by drying the filters and counting ^{45}Ca in a toluene-based scintillation medium. Oxygen consumption was analyzed polarographically with a Clark type electrode. Membrane energization was monitored by dual wavelength spectroscopy at 533–511 nm with safrine, as reported by Akerman and Wikström (1976). Mitochondrial swelling was followed by recording the changes in optical density at 546 nm. The oxidation–reduction state of mitochondrial pyridine nucleotides was monitored spectrophotometrically by dual wavelength at 370–340 nm.

Binding of [^{14}C]-DCCD to mitochondrial membrane was analyzed also by the filtration technique (specific activity 6500 cpm/nmol). Polyacrylamide gel electrophoresis of mitochondrial proteins labeled with [^{14}C]-DCCD (specific activity 6500 cpm/nmol) was performed in the presence of 2% sodium dodecyl sulfate and 9% polyacrylamide in cylindrical gels. Radioactivity distribution in 2-mm slices of the gels was determined in a scintillation counter. Protein was determined by the method of Lowry *et al.* (1951).

Results

Figure 1 shows Ca^{2+} movements in control and DCCD-treated mitochondria suspended in the standard assay media in the presence and absence of 1 mM phosphate. In both conditions, increasing concentrations of DCCD induced mitochondrial Ca^{2+} release; however, the extent of the efflux reaction

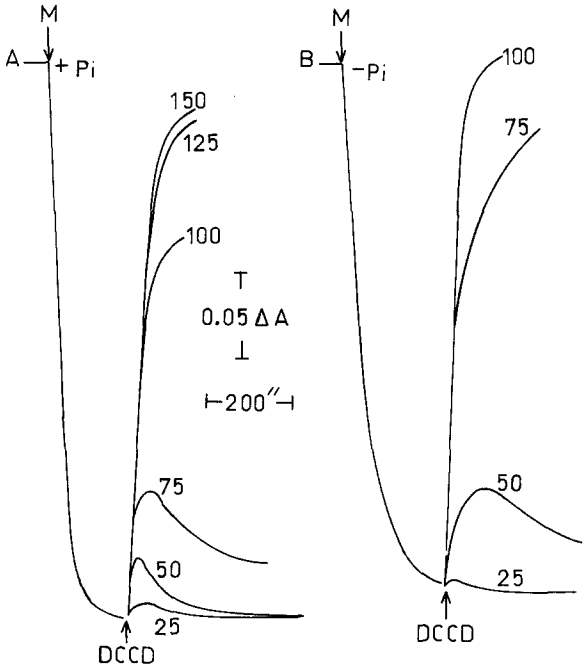

Fig. 1. Induction of mitochondrial Ca^{2+} release by increasing concentrations of DCCD. Mitochondrial protein (M, 2 mg) was added to incubation media containing 250 mM sucrose, 10 mM succinate, 10 mM HEPES, 10 mM acetate, 50 μM $CaCl_2$, 200 μM ADP, 10 μg rotenone, 5 μg oligomycin, 50 μM Arsenazo III, and, where indicated, 1 mM phosphate (Pi). The media were adjusted to pH 7.3 with KOH. The numbers alongside the traces indicate the final concentration of DCCD added. Final volume 3 ml, temperature 25° C.

was different when the medium was supplemented with phosphate. Trace A shows that in the presence of 1 mM phosphate, the addition of 75 μM DCCD induced the release of approximately 25% of the accumulated cation, whereas with no phosphate added (trace B), the addition of 75 μM DCCD released nearly 80% of the intramitochondrial Ca^{2+} content. The depletion of matrix Ca^{2+} brought about by DCCD proved to be insensitive to ruthenium red (not shown). This finding would indicate that apparently DCCD promotes a nonspecific increase in membrane permeability. The protective role of phosphate resembles the data reported by Palmer and Pfeiffer (1981), which indicate that N-ethylmaleimide and diamide are unable to promote a prompt Ca^{2+} release in the presence of phosphate.

The protective effect of phosphate raises the question of whether it depends on its specific interaction with Ca^{2+} within the inner mitochondrial compartment, or to the movement of phosphate carrier across the inner membrane. To answer such a question, mitochondria were allowed to accumulate Ca^{2+} and phosphate (Fig. 2A); after this period, 20 μM mersalyl was added, and then 75 μM DCCD. After inhibition of phosphate transport by mersalyl (Fonyo and Bessman, 1968), the addition of 75 μM DCCD

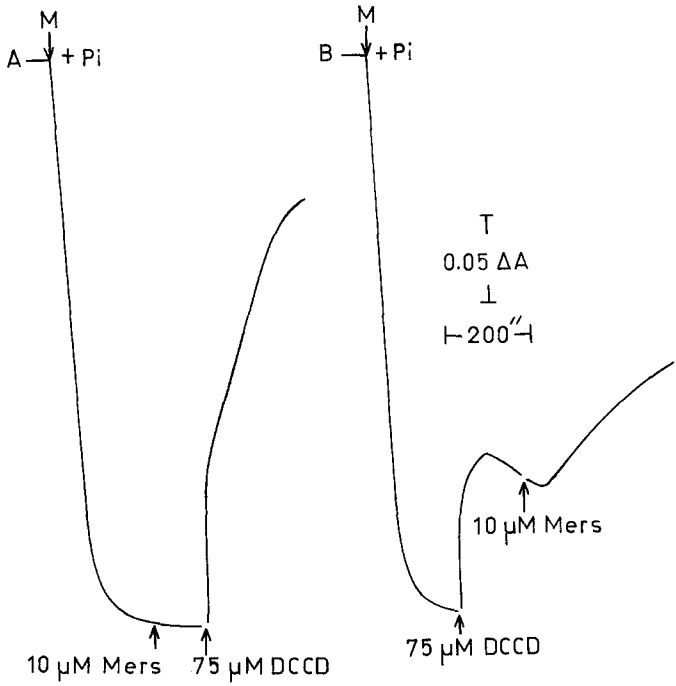

Fig. 2. Effect of mersalyl on DCCD-induced mitochondrial Ca^{2+} release. Experimental conditions are as described in the legend of Fig. 1. The indicated concentrations of mersalyl and DCCD were added.

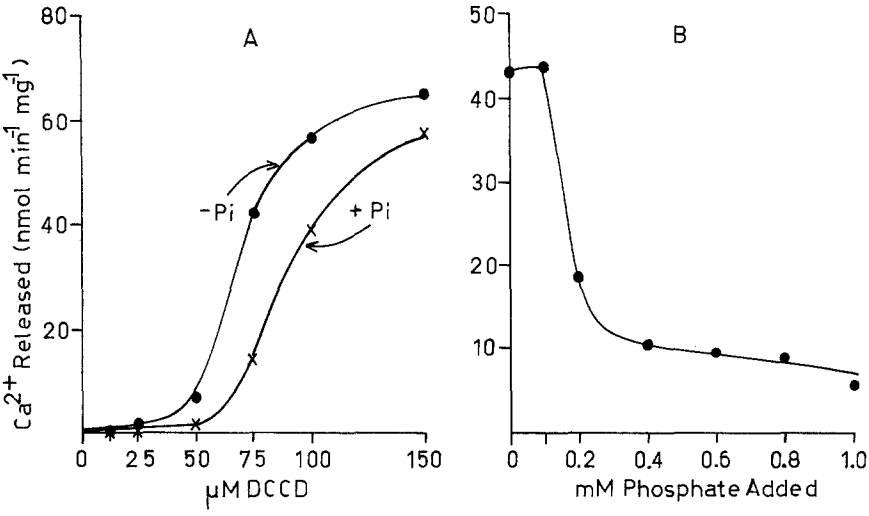

Fig. 3. Effect of phosphate on DCCD-induced mitochondrial Ca^{2+} release. Mitochondrial protein (2 mg) was added to a basic medium similar to that described in the legend of Fig. 1, except that $^{45}CaCl_2$ (50 μM) was used and no Aresenazo III was added. After 3 min of incubation time, the indicated concentrations of DCCD were added. After 1 min, aliquots of 0.2 ml were withdrawn and filtered through a Millipore filter, 0.45 μM pore size, to estimate radioactivity. Temperature 25° C.

produced a fast calcium efflux, similar to that observed in the absence of phosphate (see Fig. 1B). It is worthwhile mentioning that when mersalyl was added after DCCD (Fig. 2B), the failure of this reagent to induce a large Ca^{2+} release persisted.

Titration of Ca^{2+} efflux with DCCD, in the absence and in the presence of phosphate, resulted in curves consisting of an initial lag phase followed by a stimulation of Ca^{2+} release (Fig. 3A). Half-maximal rate of Ca^{2+} efflux was attained with 94.1 μM DCCD in the presence of phosphate; in contrast, only 72.4 μM DCCD was required in the absence of the anion. Figure 3B shows the inhibitory effect of increasing concentrations of phosphate on the DCCD-induced Ca^{2+} release. When Ca^{2+} release was stimulated by 100 μM DCCD, the $K_{0.5}$ required to inhibit the reaction was attained with 0.2 mM phosphate.

The effect of DCCD on membrane energization and the effect of phosphate on this reaction are shown in Fig. 4. Trace A indicates that with phosphate absent form the incubation medium, 50 μM DCCD induced a transient membrane depolarization; this result was not obtained when mitochondria were incubated in the presence of 1 mM phosphate (trace B). Nevertheless, by increasing the concentration of DCCD to 100 μM, mitochondria became de-energized in both conditions, with or without phosphate. Trace D indicates that phosphate effectively protects against the effect of

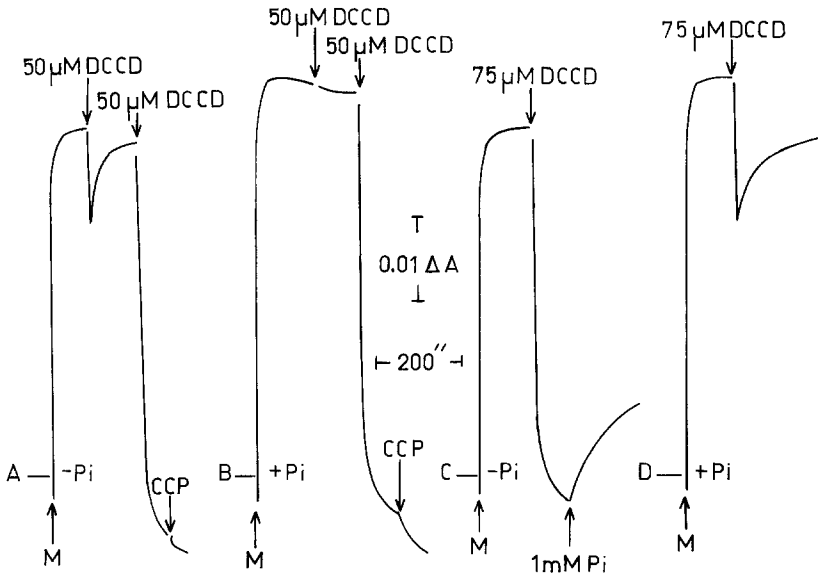

Fig. 4. Collapse of the transmembrane potential induced by DCCD mitochondria, Protein (M, 2 mg) was suspended in the standard medium described in Fig. 1, except that 10 μM safranine replaced Arsenazo III. Also Ca^{2+} was absent from the medium. Where indicated, 1 mM phosphate (Pi) was added. Other additions are as indicated in the figure. Final volume 3 ml, temperature 25° C.

75 μM DCCD on membrane depolarization. By comparing these results with those of Fig. 1, a close relationship between the effect of DCCD (± phosphate) on membrane $\Delta\Psi$ and Ca^{2+} release can be seen.

The relation between mitochondrial Ca^{2+} loss and oxidation of pyridine nucleotides has been well documented (Lehninger *et al.*, 1978; Palmer and Pfeiffer, 1981; Bellomo *et al.*, 1982; Beatrice *et al.*, 1984; Chávez and Jay, 1987). Thus, to determine if DCCD also induces a diminution in the NAD(P)H/NAD(P) ratio, the experiment depicted in Fig. 5 was performed. The results obtained show that DCCD induce a fast oxidation of pyridine nucleotides. The extent of this reaction was also dependent on the addition of phosphate to the incubation medium. As observed (trace A), with phosphate added, 75 μM DCCD promoted oxidation of 8 nmol NAD(P)H mg^{-1}. In contrast, without phosphate, DCCD (75 μM) induced oxidation of 12 nmol NAD(P)H mg^{-1}.

DCCD brought about a release of the resting state respiration (Fig. 6); interestingly, the effect of DCCD followed the established pattern with respect to the presence of phosphate, i.e., DCCD stimulated State 4 at a faster rate in the absence than in the presence of phosphate. Without phosphate 75 μM DCCD stimulated oxygen consumption at a rate of 86 natoms

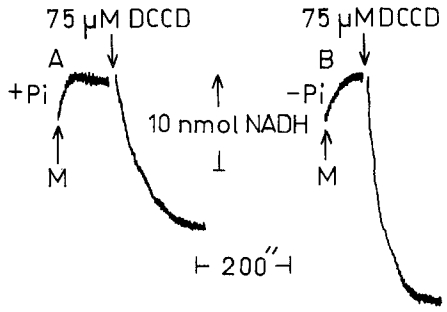

Fig. 5. Effect of DCCD on the redox state of pyridine nucleotides. Mitochondrial protein (M, 2 mg) was added to a basic medium as described in Fig. 1, except that Ca^{2+} was not added. When indicated, 1 mM phosphate (Pi) was added. Other additions are as indicated. Final volume 3 ml, temperature 25° C.

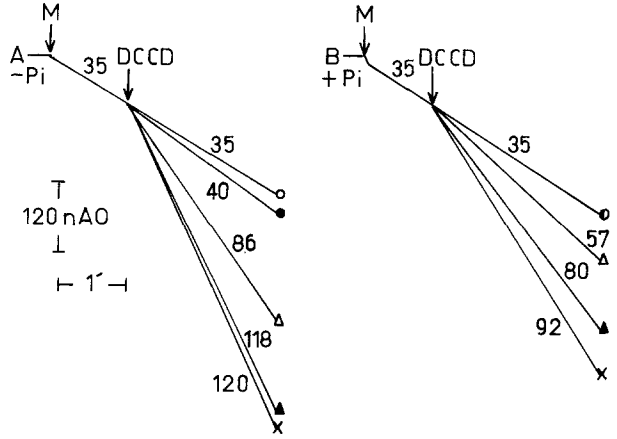

Fig. 6. Effect of DCCD on mitochondrial oxygen consumption in the absence or presence of phosphate. Mitochondria (M, 2 mg protein) added to a basic medium as described in Fig. 5. Where indicated, 1 mM phosphate was added. DCCD additions were as follows: ○, 25 μM; ●, 50 μM; △, 75 μM; ▲, 100 μM; ×, 125 μM. Final volume 3 ml temperature 25° C.

O/min/mg, whereas with phosphate added, the rate was diminished to 57 natoms O/min/mg. This stimulatory effect of DCCD on oxygen consumption is in close agreement with the collapse of membrane potential induced by the alkylating reagent.

Compounds which induce Ca^{2+} efflux from mitochondria work through a mechanism that leads to mitochondrial swelling (Broekemeier *et al.*, 1989). Accordingly, it was observed that DCCD induced mitochondrial volume changes (Fig. 7). Traces A and B show that increasing concentrations of DCCD induced a large-amplitude swelling, which was more apparent in the absence of phosphate. These results would agree with a membrane damage induced by the binding of DCCD.

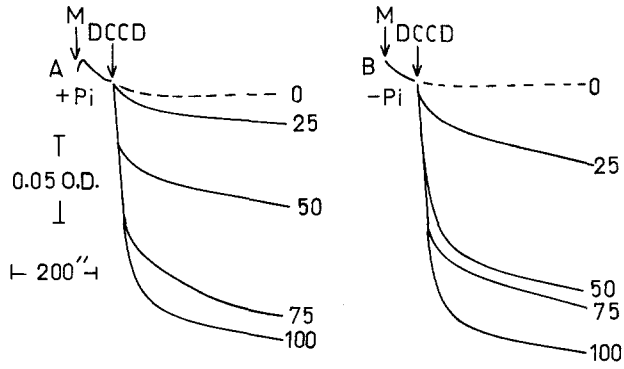

Fig. 7. Effect of DCCD on mitochondrial volume in the presence or absence of phosphate. Experimental conditions are identical to those described in the legend of Fig. 6.

At this stage of the experimental work, it can be concluded that the magnitude of the effects produced by DCCD is related to the presence of phosphate in the incubation medium. Therefore, it was considered of interest to explore if phosphate modifies the binding of DCCD to the inner membrane. We found that DCCD bound to a similar extent regardless of the presence or absence of phosphate, i.e., 42 and 39 nmol/mg, respectively.

Previous reports have shown that dicyclohexylcarbodiimide reacts quite specifically with a low-molecular-weight membrane polypeptide (see Beechey *et al.*, 1966 for example). Taking this into account, we decided to investigate the membrane proteins bound to [^{14}C]-DCCD in our experimental conditions. Figure 8 shows that radioactivity was mainly associated with a polypeptide band in the region of M_r 20–29 kDa.

Discussion

The results presented demonstrate that Ca^{2+} release from kidney mitochondria, induced by DCCD, occurs in parallel with NAD(P)H oxidation, membrane de-energization, and mitochondrial swelling. These findings are analogous to those reported previously with diverse Ca^{2+} releasing agents (for example, see Beatrice *et al.*, 1980; Palmer and Pfeiffer, 1981; Coelho and Vercesi, 1980; Chávez *et al.*, 1989). Hence it appears that modification of carboxylic groups, similarly to –SH groups, promotes a nonspecific increase in inner membrane permeability. This latter supposition is supported by the fact that DCCD-induced Ca^{2+} loss is not inhibited by ruthenium red.

Certainly, the concentrations of DCCD used to block carboxylic groups, and in consequence to inhibit oxidative phosphorylation (Beechey *et al.*, 1966), proton translocation of cytochrome oxidase (Casey *et al.*, 1979), or K^+/H^+

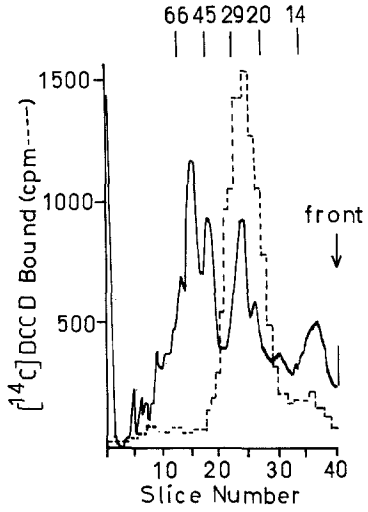

Fig. 8. Radioactive profile of mitochondrial proteins labeled with [^{14}C]-DCCD. Mitochondrial protein (2 mg) was incubated under similar conditions as those described in Fig. 5, except that 100 μM [^{14}C]-DCCD was added. After 3 min of incubation time, the medium was layered in 35 ml of 330 mM sucrose and spun down 10 min at 34,000 $\times$ g. The pellets were treated with 2% sodium dodecyl sulfate and 2-mercaptoethanol; 200 μg of protein was submitted to electrophoresis. The gels were stained with Coomasie blue and scanned at 540 nm (solid line). Dashed line indicates the radioactive profile.

exchange (Martin *et al.*, 1984, 1986), are quite lower than those used in our experiments to induce mitochondrial Ca^{2+} efflux. However, it is well known that the use of low concentrations of DCCD requires the preincubation of the reagent for long periods of time with mitochondria. In contrast, the observations described here resulted from the interaction of DCCD with the inner membrane during short periods of time. This indicates that the extent of DCCD reaction with the mitochondrial membrane depends on the concentration of the reagent as well as on the time of reaction (Beavis and Garlid, 1988).

A notable feature of Ca^{2+} release induced by increasing concentrations of DCCD is the cooperative pattern followed, in the absence or in the presence of phosphate (n_H values of 5.88 and 5.84, respectively). Therefore it appears that the opening of a passage for Ca^{2+} loss involves a conformational transition which can be induced by the cooperative binding of DCCD to the membrane.

With respect to the protective effect of phosphate against the deleterious effects of DCCD, it is conceivable that the movement of phosphate carrier across the inner membrane induces conformational transitions that result in control of mitochondrial Ca^{2+} content. This suggestion would ascribe to the phosphate carrier a similar role that the adenine nucleotide carrier

plays in mitochondrial Ca^{2+} homeostasis (Chudapongse and Haugaard, 1973).

With respect to the binding of DCCD to the inner membrane, it should be noted that DCCD is a highly hydrophobic reagent. Thus, it probably reacts at a site buried in the hydrophobic milieu of the membrane. The above can be sustained by the fact that water-soluble carbodiimides, such as 1-ethyl-3-(dimethylaminopropyl)carbodiimide, even at a concentration of 500 μM failed to induce Ca^{2+} release (not shown). Based on the results obtained from the electrophoretic analysis of [^{14}C]-DCCD-labeled mitochondria, it may be reasonably assumed that the binding site is localized to a monomeric subunit of the energy-linked transhydrogenase. Indeed, the experiments of Phelps and Hatefi (1981) demonstrated that DCCD inhibits such enzyme. By the same token, it should be pointed out that the energy-linked transhydrogenase plays a key role in the redox state of $NADP^+$ which is related to Ca^{2+} retention and efflux (Vercesi, 1987). Finally, we cannot rule out the possibility of DCCD exhibiting an uncoupler-like action. In this regard, Katre and Wilson (1978) have demonstrated that the uncoupler 2-nitro-4-azidocarbonyl-cyanide phenylhydrazone binds to a membrane proteolipid of 12–15 kDa, and they have suggested that this site may involve the DCCD-binding site. In our conditions a dimeric form of such proteolipid would be the binding site of DCCD.

References

Akerman, K. E. O., and Wikström, M. F. K. (1976). *FEBS Lett.* **68**, 191–197.

Akerman, K. E. O., and Nicholls, D. G. (1983). *Rev. Physiol. Biochem. Pharmacol.* **95**, 149–201.

Asimakis, G. K., and Sordahl, L. A. (1977). *Arch. Biochem. Biophys.* **179**, 200–210.

Beatrice, M. C., Palmer, J. W., and Pfeiffer, D. R. (1980). *J. Biol. Chem.* **255**, 8663–8671.

Beatrice, M. C., Stiers, D. L., and Pfeiffer, D. R. (1984). *J. Biol. Chem.* **259**, 1279–1287.

Beattie, D. S., and Villalobo, A. (1982). *J. Biol. Chem.* **257**, 14745–14752.

Beavis, A. D., and Garlid, K. D. (1988). *J. Biol. Chem.* **263**, 7574–7580.

Beechey, R. B., Holloway, C. T., Knight, I. G., and Roberton, A. M. (1966). *Biochem. Biophys. Res. Commun.* **23**, 75–80.

Bellomo, G., Jewell, S. A., Thor, H., and Orrenius, S. (1982). *Proc. Natl. Acad. Sci. USA* **79**, 6842–6846.

Broekemeir, K. M., Dempsey, M. E., and Pfeiffer, D. R. (1989). *J. Biol. Chem.* **264**, 7825–7830.

Carafoli, E. (1987). *Annu. Rev. Biochem.* **56**, 395–433.

Casey, R. P., Thelen, M., and Azzi, A. (1979). *Biochem. Biophys. Res. Commun.* **87**, 1044–1051.

Chávez, E., and Jay, D. (1987). *J. Bioenerg. Biomembr.* **19**, 571–580.

Chávez, E., and Holguín, J. A. (1988). *J. Biol. Chem.* **263**, 3582–3587.

Chávez, E., and Osornio, A. (1988). *Int. J. Biochem.* **20**, 731–736.

Chávez, E., Briones, R., Michel, B., Bravo, C., and Jay, D. (1985). *Arch. Biochem. Biophys.* **242**, 493–497.

Chávez, E., Jay, D., and Bravo, C. (1987). *J. Bioenerg. Biomembr.* **19**, 285–295.

Chávez, E., Zazueta, C., Díaz, E., and Holguín, J. A. (1989). *Biochim. Biophys. Acta* **986**, 27–32.

Chudapongse, P., and Haugaard, N. (1973). *Biochim. Biophys. Acta* **307**, 599–603.
Coelho, J. L. and Vercesi, A. E. (1980). *Arch. Biochem. Biophys.* **204**, 141–147.
Compton, M., Moser, R., Lüdi, H., and Carafoldi, E. (1978). *Eur. J. Biochem.* **82**, 25–31.
Fonyo, A., and Bessman, S. P. (1968). *Biochem. Med.* **2**, 145–163.
Kapoor, S. C., and van Rossum, G. D. V. (1984). *Biochem. Pharmacol.* **33**, 1771–1778.
Katre, N. V., and Wilson, D. F. (1978). *Arch. Biochem. Biophys.* **191**, 647–656.
Kendrich, N. C. (1976). *Ann. Biochem.* **76**, 487–501.
Lehninger, A. L., Vercesi, A., and Babanunmi, E. (1978). *Proc. Natl. Acad. Sci. USA* **75**, 1690–1694.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). *J. Biol. Chem.* **193**, 265–275.
Martin, W. H., Beavis, A. D., and Garlid, K. D. (1984). *J. Biol. Chem.* **258**, 2062–2065.
Martin, W. H., DiResta, D. J., and Garlid, K. D. (1986). *J. Biol. Chem.* **261**, 12300–12305.
Palmer, J. W., and Pfeiffer, D. R. (1981). *J. Biol. Chem.* **256**, 6742–6750.
Peng, C. F., Price, D. W., Bhuvaneswaran, C., and Wadkins, C. L. (1974). *Biochem. Biophys. Res. Commun.* **56**, 134–141.
Phelps, D. C., and Hatefi, Y. (1981). *J. Biol. Chem.* **256**, 8217–8221.
Rossi, C. S., and Lehninger, A. L. (1964). *J. Biol. Chem.* **239**, 3971–3980.
Sul, H. S., Shrago, E., and Shug, A. L. (1976). *Arch. Biochem. Biophys.* **172**, 230–237.
Vercesi, A. E. (1987). *Arch. Biochem. Biophys.* **252**, 171–178.
Wolkowicz, P. E., and McMillin-Wood, J. (1980). *Biochem. J.* **186**, 257–266.