Sakai, M., Fujii-Kuriyama, Y., Saito, T., & Muramatsu, M. (1981) *J. Biochem. (Tokyo) 89*, 1863–1868.
Staehelin, T., Erni, B., & Schreier, M. H. (1979) *Methods Enzymol. 60*, 136–165.
Thompson, R. C., & Stone, P. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 198–202.
Thompson, R. C., Dix, D. B., Gerson, R. B., & Karim, A. M. (1981) *J. Biol. Chem. 256*, 81–86.
Tsujimoto, Y., & Suzuki, Y. (1979) *Cell (Cambridge, Mass.) 18*, 591–600.
Woese, C. R. (1967) *Prog. Nucleic Acid Res. Mol. Biol. 7*, 107–172.
Yamane, Y., & Hopfield, J. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2246–2250.
Yarus, M. (1979) *Prog. Nucleic Acid Res. Mol. Biol. 23*, 195–225.

Dicyclohexylcarbodiimide Inhibits the Monoamine Carrier of Bovine Chromaffin Granule Membrane†

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ABSTRACT: The monoamine carrier of bovine chromaffin granule membrane catalyzes a H^+/neutral amine antiport. Dicyclohexylcarbodiimide (DCCD) inhibits this carrier in a time- and concentration-dependent manner as shown by the following evidence: (i) it inhibits the carrier-mediated pH gradient driven monoamine uptake without collapsing the pH gradient; (ii) it affects the binding of the specific inhibitors [2-^{3}H]dihydrotetrabenazine and [^{3}H]reserpine. The DCCD inhibition of the carrier occurs in the same concentration range as that of the ATP-dependent H^+ translocase. Saturation isotherms of [2-^{3}H]dihydrotetrabenazine binding indicate that DCCD decreases the number of binding sites without any change of the equilibrium dissociation constant. Kinetic studies of DCCD inactivation indicate that the modification of only one amino acid residue is responsible for the inhibition. Preincubation of the membranes with tetrabenazine protects the carrier against inactivation by DCCD: in this case, [2-^{3}H]dihydrotetrabenazine binding and pH gradient driven monoamine uptake are restored after washing out of DCCD and tetrabenazine. We suggest the existence in the monoamine carrier of a carboxylic acid involved in H^+ translocation, similar to those demonstrated not only in F_0–F_1 ATPases but also in cytochrome *c* oxidase, mitochondrial cytochrome b–c_1 complex, and nucleotide transhydrogenase. Protonation–deprotonation of this group would affect the binding of [2-^{3}H]dihydrotetrabenazine by the carrier.

In the adrenal medulla, the catecholamines adrenaline and noradrenaline are mainly located in specific organelles, the chromaffin granules. The large concentration gradient existing between these organelles and the cytosol is the result of an ATP-dependent active transport system located in the granule membrane (Kirshner, 1962; Carlsson et al., 1963). This system involves (i) an inwardly directed ATP-dependent H^+ translocase (H^+ pump), which generates an electrochemical proton gradient $\Delta\mu_{H^+}$ (inside acidic and positive) (Casey et al., 1977; Phillips & Allison, 1978; Johnson & Scarpa, 1979; Scherman & Henry, 1980a), and (ii) a specific monoamine carrier driven by the $\Delta\mu_{H^+}$, which catalyzes an electrodissipative H^+/neutral monoamine antiport (Johnson & Scarpa, 1979; Apps et al., 1980a; Scherman & Henry, 1980b; Kanner et al., 1980; Knoth et al., 1980). The monoamine carrier is inhibited by the drugs tetrabenazine (TBZ)[1] and reserpine (Pletscher, 1976; Scherman & Henry, 1980c).

In order to elucidate the mechanism of amine translocation, we have investigated the effect of chemical modifications of the catecholamine carrier. The activity of the carrier of chemically modified resealed chromaffin granule ghosts was estimated under conditions where the H^+ pump was bypassed: a pH gradient (ΔpH, inside acidic) obtained by a pH jump in the absence of ATP was imposed on ghosts, and the carrier-mediated (tetrabenazine sensitive) amine accumulation inside of the vesicles was followed (Schuldiner et al., 1978; Phillips, 1978). By this technique, we have already described an inhibition of the monoamine carrier by the histidine-specific reagent diethyl pyrocarbonate (Isambert & Henry, 1981). In the present paper, we describe an effect of dicyclohexylcarbodiimide (DCCD), a reagent rather selective for carboxylic acid. This reagent is known to inhibit H^+ translocases of various origins, including that of chromaffin granule membranes (Bashford et al., 1976; Giraudat et al., 1980; Apps et al., 1980b, 1983; Cidon & Nelson, 1983). We now provide evidence that it inhibits also the monoamine carrier in the same concentration range.

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[1] Abbreviations: DCCD, *N*,*N*′-dicyclohexylcarbodiimide; TBZ, tetrabenazine (2-oxo-3-isobutyl-9,10-dimethoxy-1,3,4,6,7,11b-hexahydro-2*H*-benzo[*a*]quinolizine); [^{3}H]TBZOH, 2-[^{3}H]hydroxy-3-isobutyl-9,10-dimethoxy-1,3,4,6,7,11b-hexahydro-2*H*-benzo[*a*]quinolizine; 5-HT, serotonin (5-hydroxytryptamine); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; ATPase, adenosinetriphosphatase.

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Experimental Procedures

Chemicals. 5-Hydroxy[G-^{3}H]tryptamine (12.3 Ci/mmol) was obtained through the Radiochemical Centre (Amersham, U.K.) and was used at a specific activity of 0.3 Ci/mmol. [2-^{14}C]Tyramine (50.4 mCi/mmol) was from CEA (France). [*benzoyl*-^{3}H]Reserpine (24.4 Ci/mmol) was from New England Nuclear Corp. (Boston, MA). Because of rapid radiolysis the product was periodically repurified by HPLC (Scherman & Henry, 1984). [^{3}H]TBZOH (10.4 Ci/mmol) was prepared as described (Scherman et al., 1983). TBZ and reserpine were from Fluka. DCCD was purchased from Merck. Stock solutions were prepared in absolute ethanol and were used immediately.

Chromaffin Granule Membrane Preparation. Bovine chromaffin granule membranes were prepared by osmotic lysis of granules isolated by centrifugation on a 1.6 M sucrose layer. Unless indicated, membranes were frozen in liquid nitrogen and were stored at −80 °C (Smith & Winkler, 1967; Giraudat et al., 1980).

DCCD Treatment of Membranes. Membranes were thawed, centrifuged at 100000*g* for 15 min, and resuspended at 2.5 mg of protein/mL in 3 mM Hepes buffer (pH 7.5) containing 0.3 M sucrose. DCCD in ethanol or ethanol alone (final concentration ≤1%) was added under stirring, and the mixture was incubated at room temperature for 20 min, unless otherwise indicated. Membranes were washed, generally by a 30-fold dilution in 20 mM Hepes buffer (pH 7.5) or, alternatively, in 20 mM Mes buffer (pH 6.0) when they were assayed for ΔpH-driven amine uptake. They were centrifuged for 20 min at 40000*g* and resuspended in 0.3 M sucrose buffered at the pH of the wash medium.

ΔpH-Driven Amine Uptake. The incubation mixtures contained 0.3 M sucrose buffered with 50 mM Hepes (pH 8.5) or 20 mM Mes (pH 6.0), 2.5 μM TBZ where indicated, and 2.5 μM [^{3}H]5-HT. When TBZ was added to the medium, membranes were pretreated for 5 min at 20 °C with the same drug concentration. The incubation was carried out at 37 °C and was initiated by addition of the membranes in 20 mM Mes buffer (pH 6.0) containing 0.3 M sucrose. For [^{3}H]5-HT uptake, aliquots (40 μL) were withdrawn at intervals, diluted in ice-cold incubation buffer (2 mL), and rapidly filtered through 0.45-μm Millipore filter (HAWP) previously washed with the same medium. The filters were washed twice with the wash buffer (2 mL), and their radioactivity was measured by liquid scintillation in Aqualuma (Lumac, Schaesberg, Holland). For [^{14}C]tyramine uptake the following modification was adopted to prevent leakage of the amine: incubation mixtures (1 mL) incubated for various periods of time were rapidly filtered without dilution, and the Millipore filters were washed once with 1 mL of wash buffer.

[^{3}H]Reserpine and [^{3}H]TBZOH Binding. Membranes (150 μg of protein/mL for reserpine binding or 20–50 μg of protein/mL for TBZOH binding) were incubated in a medium containing [^{3}H]reserpine or [^{3}H]TBZOH, drugs where indicated, 40 mM Hepes (pH 7.5), and 0.3 M sucrose. Incubations were performed at 25 °C for 20 h for reserpine binding (Scherman & Henry, 1984) and for 3–20 h for [^{3}H]TBZOH binding. Bound radioactivity was determined by filtration on GF/C filters (Scherman & Henry, 1984).

ATPase Activity. ATP hydrolysis was assayed at 37 °C as described (Giraudat et al., 1980). Initial rates were determined from plots of the time course of the reaction.

Analytical Techniques. Proteins were estimated by the Lowry procedure or by the Bio-Rad assay (Bradford, 1976) with bovine serum albumin as a standard. Phospholipid estimation was done by assaying P_i liberated by perchloric acid hydrolysis.

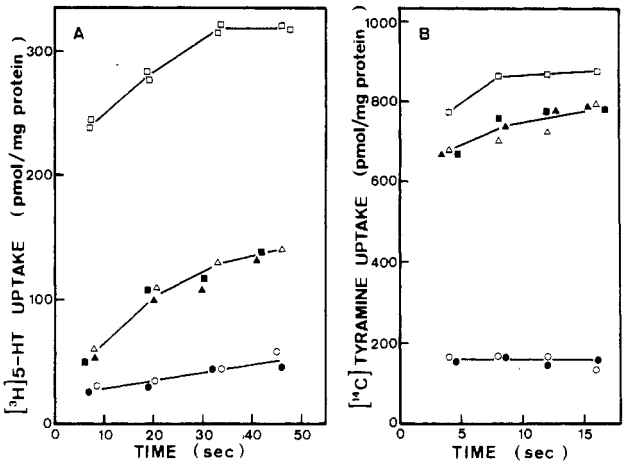

FIGURE 1: ΔpH-driven uptake of amine by DCCD-treated membranes. Membranes (2.5 mg of protein/mL) were treated by 120 μM DCCD (■, ▲, ●) or by 0.9% ethanol (□, △, ○) as described under Experimental Procedures. (A) [^{3}H]5-HT uptake. Membranes (3.4 mg of protein/mL) at pH 6.0 were diluted 5-fold in a medium at pH 8.5 with (△, ▲) or without (□, ■) 2.5 μM TBZ and containing 2.5 μM [^{3}H]5-HT. Controls were also performed by dilution of the membrane in a medium at pH 6.0 (○, ●). (B) [^{14}C]Tyramine uptake. The same membrane preparation was diluted 65-fold as in (A) in different media containing 1.5 μM [^{14}C]tyramine.

Results

Monoamine Carrier Activity of DCCD-Treated Membranes. When membranes were treated with 120 μM DCCD for 20 min, their monoamine carrier activity, determined as the TBZ-sensitive fraction of pH gradient driven [^{3}H]5-HT uptake, was inhibited (Figure 1A). This result has been observed in more than 10 experiments on frozen and on freshly prepared membrane preparations. Under the same conditions, the TBZ-resistant fraction of [^{3}H]5-HT uptake was not affected by DCCD (Figure 1A), and [^{14}C]tyramine uptake was only slightly inhibited (Figure 1B). These two transports are not carrier mediated and are only dependent upon the pH gradient. DCCD thus does not inhibit carrier-mediated [^{3}H]5-HT uptake by collapsing the pH gradient. Moreover, this inhibition does not originate in an effect of DCCD on H^+ translocation by the ATPase since this reaction is not involved in the experiments of Figure 1. The data thus suggest an inhibition of the monoamine carrier by DCCD.

[^{3}H]TBZOH and [^{3}H]Reserpine Binding by DCCD-Treated Membranes. (1) [^{3}H]TBZOH Binding. We have shown the presence of high-affinity binding sites for the TBZ derivative [^{3}H]TBZOH on the monoamine carrier (Scherman et al., 1983). Treatment of the membranes with 130 μM DCCD for 20 min at 25 °C results in an 85% decrease of [^{3}H]TBZOH binding (Table I). This result has been observed in more than 15 experiments; it has also been observed with freshly prepared membranes (data not shown).

To characterize the modification of [^{3}H]TBZOH binding sites, we analyzed the saturation isotherms for [^{3}H]TBZOH of membranes treated with a low concentration of DCCD (Figure 2). Control membranes had [^{3}H]TBZOH binding sites with a density (B_{max}) of 38 pmol/mg of protein and a K_D of 2.2 nM. In DCCD-treated membranes, B_{max} decreased without any concomitant change of K_D.

Table I: [^{3}H]TBZOH and [^{3}H]Reserpine Binding by DCCD-Treated Membranes[a]

	[^{3}H]TBZOH bound[c] (pmol/mg of protein)		[^{3}H]reserpine bound[c] (pmol/mg of protein)		
	control	+2 μM TBZ	control	+0.5 μM TBZ	+10 μM reserpine
control membranes[b]	10.2 ± 0.9	0.7 ± 0.2	2.3 ± 0.1	1.7 ± 0.1	0.2 ± 0.05
DCCD-treated membranes[b]	2.3 ± 0.3	0.6 ± 0.1	0.40 ± 0.01	0.34 ± 0.02	0.25 ± 0.03

[a] Results are mean ± SD (n = 3). [b] Membranes (1.85 mg of protein/mL) were treated by 130 μM DCCD. [c] [^{3}H]TBZOH and [^{3}H]reserpine concentration in the incubations was 1 nM.

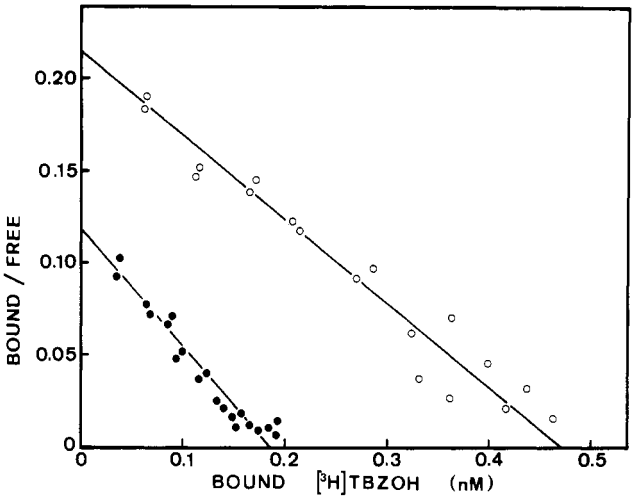

FIGURE 2: Effect of DCCD on [^{3}H]TBZOH binding. Membranes (2.5 mg of protein/mL) were treated by 45 μM DCCD (●) or 1% ethanol (○) and assayed for [^{3}H]TBZOH binding as described under Experimental Procedures. In untreated membranes the K_D was 2.2 nM, and the number of binding sites (B_{max}) was 38 pmol/mg of protein. In DCCD-treated membranes K_D was 1.6 nM while B_{max} was decreased to 15 pmol/mg of protein.

(2) [^{3}H]Reserpine Binding. [^{3}H]Reserpine binding to chromaffin granule membranes is described by curvilinear Scatchard plots (Scherman & Henry, 1984). This has been interpreted as indicating the existence of two classes of sites: (i) TBZ-resistant R_1 sites with a high affinity (K_D = 0.7 nM) and a low density (B_{max} = 7 pmol/mg of protein) and (ii) TBZ-sensitive R_2 sites with a low affinity (K_D = 25 nM) and a high B_{max} (60 pmol/mg of protein). The latter have been identified as [^{3}H]TBZOH binding sites. The two types of reserpine binding sites are borne by the monoamine carrier. It has also been shown that the kinetics of [^{3}H]reserpine binding is accelerated when the ATPase generates a $\Delta\mu_{H^+}$ (Weaver & Deupree, 1982; Scherman & Henry, 1984). In the absence of ATP, a 24-h incubation is required to reach equilibrium, whereas in the presence of ATP, complete binding is obtained after 3 h. We have investigated TBZ-resistant and TBZ-sensitive [^{3}H]reserpine binding sites of DCCD-treated membranes. Since DCCD affects $\Delta\mu_{H^+}$ generation, [^{3}H]-reserpine binding was measured on nonenergized membranes after a 20-h incubation at 25 °C. Under these conditions of assay, treatment of the membranes by 130 μM DCCD for 20 min induced a 90% inhibition of [^{3}H]reserpine binding to R_1 and R_2 sites (Table I).

The fact that DCCD treatment inhibits the binding of two specific ligands, TBZOH and reserpine, gives support to the contention that this treatment affects the monoamine carrier. The modification induced by DCCD is likely to be covalent since treated membranes were washed before being assayed. Moreover, the modified amino acid is in an hydrophobic environment since treatment with the water-soluble carbodiimide 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide did not inhibit (at concentrations up to 2 mM) [^{3}H]TBZOH binding (data not shown).

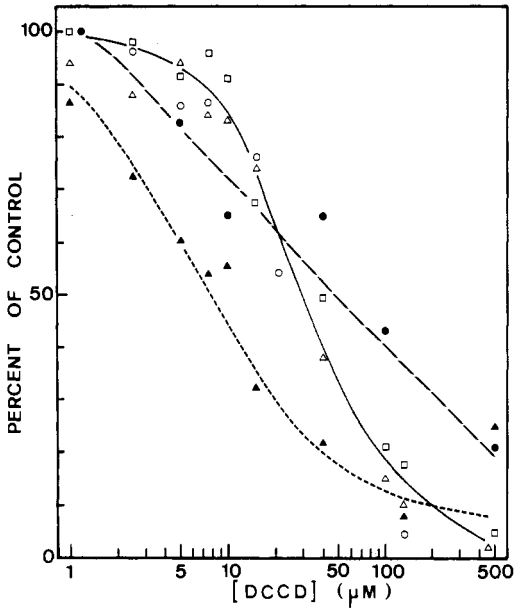

FIGURE 3: Effects of DCCD concentration on chromaffin granule membranes. Membranes (2.5 mg of protein/mL) were treated by DCCD at the indicated concentration or by 0.5% ethanol (control experiment). After washing, assays of [^{3}H]TBZOH specific binding (□), [^{3}H]reserpine specific binding in the absence [(△) R_2 sites] or presence [(▲) R_1 sites] of 500 nM TBZ, ATPase activity (●), and ΔpH-driven [^{3}H]5-HT uptake (○) were performed on the same samples of membranes. The concentrations of [^{3}H]TBZOH and [^{3}H]reserpine used for the assays were respectively 1.7 and 15.5 nM. Control activities, per milligram of protein, were 4.7 pmol for [^{3}H]TBZOH binding, 1.1 pmol for R_1 sites, 2.2 pmol for R_2 sites, 107 nmol/min for ATPase activity, and 800 pmol/min for [^{3}H]5-HT uptake.

Concentration and Time Dependency of DCCD Effects. (1) Concentration Dependency. Since we have noted various effects of DCCD treatment on the carrier, it was of interest to study the dose dependency of these effects to examine any causal relationship. In addition, it was interesting to compare the sensitivity of the ATPase and of the monoamine carrier to DCCD. For this type of experiment, either DCCD concentrations or DCCD/protein ratios have been considered in the literature [see, for instance, Cidon & Nelson (1983) and Apps et al. (1980b)]. Since the various assays of chromaffin granule membrane activities (ligand binding, [^{3}H]5-HT uptake, and ATPase) were performed at different protein concentrations, membranes at a constant concentration were treated by various DCCD concentrations; they were then washed and diluted in the various assay mixtures at optimal concentrations. A typical experiment is shown in Figure 3. The results can be summarized as follows: (i) The [^{3}H]-TBZOH binding sites and the carrier-mediated [^{3}H]5-HT uptake have a sensitivity to DCCD similar to that of the ATPase. (ii) The TBZ-resistant reserpine binding sites (R_1) are slightly more sensitive to DCCD than the TBZ-sensitive ones (R_2). For instance, at 5 μM DCCD, about 40% of R_1 sites are modified whereas only 5% of R_2 sites are affected. This difference of sensitivity has been observed in more than

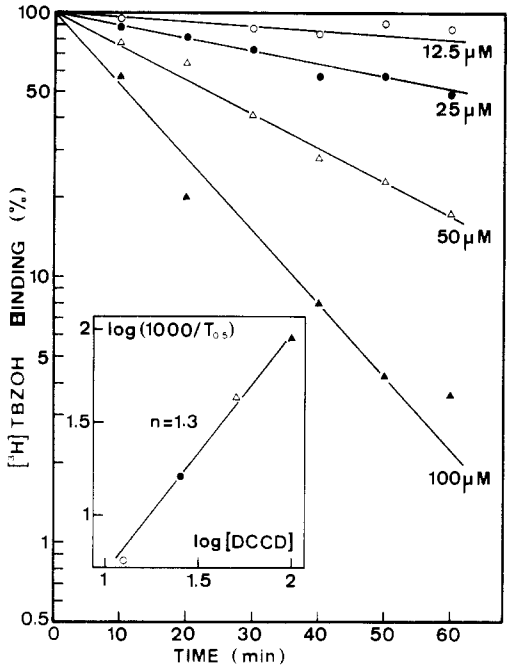

FIGURE 4: Kinetics of inactivation of [³H]TBZOH binding sites by DCCD. Membranes (2.5 mg of protein/mL) were treated for different time periods by 12.5 (○), 25 (●), 50 (△), or 100 μM (▲) DCCD or by 0.5% ethanol (control experiment). After the membranes were washed, they were assayed for [³H]TBZOH binding as described under Experimental Procedures with 3.4 nM [³H]TBZOH. Results are expressed as percent of binding to untreated membranes. Inset: The log of the reciprocal of the half-time of inactivation is plotted vs. the log of DCCD concentration. The straight line obtained has a slope of 1.3.

five experiments. (iii) The inhibition of R_2 sites and that of [³H]TBZOH binding sites occur simultaneously, thus confirming the identity of these sites.

(2) Kinetics of Inactivation. In order to study the kinetics of inactivation by DCCD, we examined [³H]TBZOH binding to membrane treated for different periods of time at several DCCD concentrations. Inhibition of [³H]TBZOH binding followed pseudo-first-order kinetics (Figure 4). A plot of the log of the reciprocal of the half-time of inactivation vs. the log of DCCD concentration gave a straight line with a slope of 1.3 (Figure 4, inset). This result indicates that inhibition of [³H]TBZOH binding originates in the interaction of approximately one molecule of DCCD per [³H]TBZOH binding site unit.

Effect of Preincubation of the Membranes with TBZ Derivatives on DCCD-Induced Inhibitions. Since TBZ derivatives are ligands of high affinity for the monoamine carrier, their protective effect against DCCD-induced modifications was tested. These experiments required as a preliminary step that membranes incubated with saturating concentrations of TBZ derivatives could be washed to assay the carrier. Washing was achieved by dilution and by centrifugation and was more efficient when performed in the presence of liposomes (Kanner et al., 1979). By simple dilution, more than 60% of [³H]TBZOH binding (Table II, line 2) and 30% of [³H]5-HT uptake were recovered. The inhibition observed after washing was attributed to residual TBZ. A more efficient washing was obtained with a liposome suspension (Figure 5). In the case of 5-HT uptake, the TBZ content of the washed membrane preparation had to be more carefully controlled since membranes were less diluted in the assay mixture than in the case of [³H]TBZOH binding, and in addition, 5-HT had an affinity for the carrier much lower than that of TBZ

Table II: Effect of Preincubation of the Membranes with TBZ on DCCD-Induced Inhibitions[a]

treatment of the membranes before washing[b]	[³H]TBZOH-specific binding (pmol/mg of protein)	ATPase activity [nmol of P_i min^{-1} (mg of protein)$^{-1}$]
control	11.7 ± 0.5	66 ± 0.7
TBZ[c]	7.4 ± 1.2	73 ± 1.2
DCCD	0.6 ± 0.1	43 ± 1.6
TBZ and DCCD[c]	7.5 ± 0.5	43 ± 0.7

[a] Results are mean ± SD (n = 3). [b] Membranes (2.5 mg of protein/mL) were treated for 15 min with 0.75% ethanol (control) or 175 μM DCCD. [c] Membranes were preincubated for 20 min at room temperature with 0.5 μM TBZ before ethanol or DCCD addition.

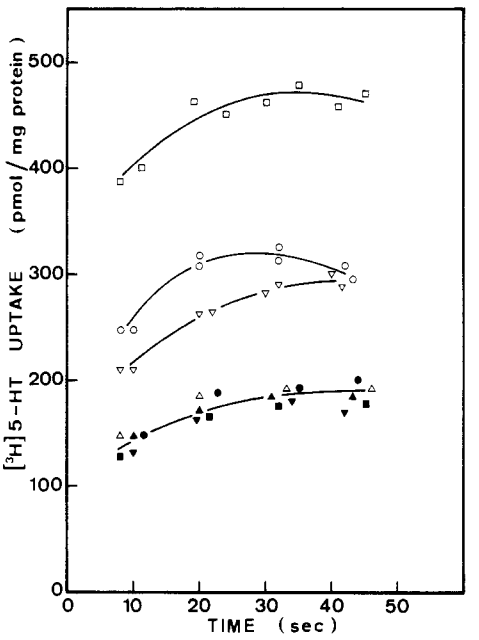

FIGURE 5: TBZ protection vs. inactivation by DCCD of the ΔpH-driven [³H]5-HT uptake. Membranes preincubated in the absence (□, ■, △, ▲) or in the presence (○, ●, ▽, ▼) of 0.5 μM TBZ were treated for 15 min with 175 μM DCCD (△, ▲, ▽, ▼) or with 0.4% ethanol (□, ■, ○, ●). They were washed by dilution in 2.5 mM Hepes buffer (pH 7.5) and by centrifugation for 20 min at 40000g. The pellets were washed by a liposome suspension (3 mM phospholipid) buffered at pH 7.5 and finally by a 20 mM Mes buffer (pH 6.0) solution. The membranes resuspended in 0.3 M sucrose buffered at pH 6.0 were assayed for [³H]5-HT uptake in the absence (open symbols) or in the presence (closed symbols) of 2 μM TBZ. Liposomes were prepared by sonication of asolectin suspended in 40 mM Hepes (pH 7.5)–4 mM EDTA. The suspension was centrifuged at 40000g for 20 min before it was to be used.

derivatives (Scherman et al., 1983).

Preincubation of the membranes with 500 nM TBZ prior to DCCD addition resulted in a full protection of [³H]TBZOH binding sites (Table II, line 4). Similar results have been obtained in eight experiments with TBZ or TBZOH as the protective agent. TBZ is more efficient than TBZOH (data not shown), a result that may reflect the difference of affinity of these two drugs for [³H]TBZOH binding sites (Scherman et al., 1983). Preincubation with TBZ also protected the carrier-mediated pH gradient driven [³H]5-HT uptake (Figure 5). This result has been obtained in five different experiments. Preincubation with TBZ partially protected total [³H]reserpine binding, and an analysis of [³H]reserpine binding in the presence of 0.5 μM TBZ showed that the TBZ-resistant R_1 sites were not protected, thus suggesting a protection of R_2 sites (data not shown). Under the same experimental conditions the ATPase activity was not protected (Table II), showing that the observed protection of the carrier was not

an artifact such as the inactivation of DCCD (180 μM) by TBZ (0.5 μM).

Discussion

DCCD inactivates the monoamine carrier of chromaffin granules on the basis of the following evidence: (i) DCCD inhibits the carrier-mediated pH gradient driven monoamine uptake without collapsing the pH gradient; (ii) DCCD treatment affects TBZOH and R_2 reserpine binding sites, which are localized on the carrier; (iii) TBZ derivatives protect the carrier against DCCD modification. To our knowledge, this is the first demonstration of a direct effect of DCCD on the monoamine carrier. An inhibition of the pH gradient driven monoamine uptake by this reagent has been reported (Schuldiner et al., 1978; Apps et al., 1980b). No interpretation was given in the first publication. In the second, this inhibition was attributed to an increase of monoamine efflux. Such an explanation is unlikely since DCCD treatment also inhibits the initial rate of uptake, at a time where amine efflux is limited.

DCCD is very reactive, but under the conditions used (100 μM, corresponding to 40 nmol/mg of protein, for 20 min at 20 °C), the observed modifications were quite specific, since kinetic studies of DCCD inactivation showed that the inhibition of [^{3}H]TBZOH binding was due to the modification of only one amino acid of the carrier. The specificity of this modification is also supported by the protection afforded by TBZ derivatives. In addition, the ATPase of chromaffin granules and the monoamine carrier were affected in the same concentration range (Figure 3), thus supporting the contention of a high reactivity of the carrier. This similar reactivity of the carrier and of the ATPase suggests that the inhibition of ATP-dependent monoamine uptake is not entirely due to the inhibition of the H^+ pump, as generally accepted.

The monoamine carrier is modified by diethyl pyrocarbonate (Isambert & Henry, 1981) and by DCCD, but these two reagents have different effects. Diethyl pyrocarbonate, an histidine modifier, inhibited the pH gradient driven amine uptake without affecting [^{3}H]TBZOH binding. DCCD affected both, indicating different targets for the two reagents. We have shown that DCCD cannot react with a carrier to which TBZ is bound (protective effect of TBZ). We have also shown that [^{3}H]TBZOH cannot bind to DCCD-modified molecules, since, in the case of partial inhibition by DCCD, residual binding occurs on intact molecules (K_D is not affected; Figure 2). TBZ binding and DCCD modification are thus mutually exclusive processes. The simplest interpretation of these results is to assume that a highly DCCD-sensitive residue, probably a carboxylate, is located at the vicinity of the TBZ binding site: TBZ protection would thus originate in steric effects.

An alternative interpretation of the data is to assume two conformations of the carrier: the first one, DCCD sensitive, does not bind the amines, whereas in the other one, the amine site exists, but the residue reacting with DCCD is inactivated or masked. This two-conformation hypothesis is interesting for the following reasons: (i) It does not imply the presence of a carboxylate at the amine binding site; since the carrier binds the neutral form of amines (Scherman & Henry, 1981; Scherman & Henry, 1983; Ramu et al., 1983; Johnson et al., 1984), it is more satisfactory to postulate a neutral binding site. (ii) If, in the amine binding conformation, protection against DCCD results from chemical inactivation, it could be by protonation of a carboxylate, since a protonated carboxylic residue is insensitive to DCCD (Kurzer & Douraghi-Zadeh, 1967). This hypothesis might be interesting to consider in the interpretation of the coupling between H^+ and amine translocation. It has to be noted that DCCD, which has been known for a long time as a blocker of H^+ movements in F_0–F_1 proton pumps (Beechey et al., 1967) has now been reported to inhibit H^+ translocation in other membrane systems such as cytochrome *c* oxidase (Casey et al., 1979), mitochondrial cytochrome b–c_1 complex (Degli Esposti et al., 1983), and nucleotide transhydrogenase (Pennington & Fisher, 1981). It is thus tempting to speculate that a carboxylic acid with a similar reactivity is present in H^+-driven carriers such as the monoamine carrier. This hypothesis is substantiated by the fact that tributyltin, another inhibitor of H^+ pumps, also blocks [^{3}H]TBZOH binding (data not shown).

The reported experiments give also some information on reserpine binding. The existence of two classes of reserpine binding sites and the identity of R_2 and [^{3}H]TBZOH binding sites are confirmed by the dose dependency of inhibition curves (Figure 3). It is also confirmed by the protection experiments since TBZ seems to protect R_2 but not R_1 sites. Contrasting with our previous findings (Scherman & Henry, 1984) which involved R_1 high-affinity sites in the uptake process, in the present experiments R_1 sites are blocked without inhibition of uptake. This conclusion is supported not only by the experiments of Figure 3 but also by the protection experiments since TBZ protects monoamine uptake but not R_1 sites. It has to be noted that in the present work monoamine uptake is driven by an imposed pH gradient whereas in the previous one it was driven by the $\Delta\mu_{H^+}$ generated by the H^+ pump which generates a ΔpH and a $\Delta\psi$. A possible explanation to the discrepancy between the two sets of experiments might then be a difference in the mechanism of the carrier under the different conditions. Sites R_1 would operate only in the presence of a transmembrane potential $\Delta\psi$. We (Scherman & Henry, 1980b) and others (Johnson & Scarpa, 1979; Njus & Radda, 1978) have postulated ΔpH- and $\Delta\mu_{H^+}$-dependent steps in the mechanism of amine translocation. The two conformation model would thus provide insights only on the ΔpH-dependent step.

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Registry No. DCCD, 538-75-0; TBZOH, 3466-75-9; serotonin, 50-67-9; tyramine, 51-67-2.

References

Apps, D. K., Pryde, J. G., & Phillips, J. H. (1980a) *FEBS Lett. 111*, 386.

Apps, D. K., Pryde, J. G., Sutton, R., & Phillips, J. H. (1980b) *Biochem. J. 190*, 273.

Apps, D. K., Pryde, J. G., & Sutton, R. (1983) *Neuroscience 9*, 687.

Bashford, C. L., Casey, R. P., Radda, G. K., & Ritchie, G. A. (1976) *Neuroscience 1*, 399.

Beechey, R. B., Robertson, A. M., Holloway, C. T., & Knight, J. G. (1967) *Biochemistry 6*, 3867.

Bradford, M. (1976) *Anal. Biochem. 72*, 248.

Carlsson, A., Hillarp, N. A., & Waldeck, B. (1963) *Acta Physiol Scand., Suppl. No. 215*, 1.

Casey, R. P., Njus, D., Radda, G. K., & Sehr, P. A. (1977) *Biochemistry 16*, 972.

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

Cidon, S., & Nelson, N. (1983) *J. Biol. Chem. 258*, 2892.

Degli Esposti, M., Meier, E. M. M., Timoneda, J., & Lenaz, G. (1983) *Biochim. Biophys. Acta 725*, 349.

Giraudat, J., Roisin, M. P., & Henry, J. P. (1980) *Biochemistry 19*, 4499.
Isambert, M. F., & Henry, J. P. (1981) *FEBS Lett. 136*, 13.
Johnson, R. G., & Scarpa, A. (1979) *J. Biol. Chem. 254*, 3750.
Johnson, R. G., Carty, S., & Scarpa, A. (1984) *Abstracts of the International Symposium on Molecular Biology of Peripheral Catecholamine Storing Tissues*, p 51, Colmar, France.
Kanner, B. I., Fishkes, H., Maron, R., Sharon, I., & Schuldiner, S. (1979) *FEBS Lett. 100*, 175.
Kanner, B. I., Sharon, I., Maron, R., & Schuldiner, S. (1980) *FEBS Lett. 111*, 83.
Kirschner, N. (1962) *J. Biol. Chem. 237*, 2311.
Knoth, J., Handloser, K., & Njus, D. (1980) *Biochemistry 19*, 2938.
Kurzer, F., & Douraghi-Zadeh, K. (1967) *Chem. Rev. 67*, 107.
Njus, D., Radda, G. K. (1978) *Biochim. Biophys. Acta 463*, 219.
Pennington, R. H., & Fisher, R. R. (1981) *J. Biol. Chem. 256*, 8963.
Phillips, J. H. (1978) *Biochem. J. 170*, 673.
Phillips, J. H., & Allison, Y. P. (1978) *Biochem. J. 170*, 661.
Pletscher, A. (1976) *Bull. Schweiz. Akad. Med. Wiss. 32*, 181.
Ramu, A., Levine, M., & Pollard, H. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2107.
Scherman, D., & Henry, J. P. (1980a) *Biochim. Biophys. Acta 599*, 150.
Scherman, D., & Henry, J. P. (1980b) *Biochim. Biophys. Acta 601*, 664.
Scherman, D., & Henry, J. P. (1980c) *Biochem. Pharmacol. 29*, 1883.
Scherman, D., & Henry, J. P. (1981) *Eur. J. Biochem. 116*, 535.
Scherman, D., & Henry, J. P. (1983) *Mol. Pharmacol. 23*, 431.
Scherman, D., & Henry, J. P. (1984) *Mol. Pharmacol. 25*, 113.
Scherman, D., Jaudon, P., & Henry, J. P. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 584.
Schuldiner, S., Fishkes, H., & Kanner, B. I. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3713.
Smith, A. D., & Winkler, H. (1967) *Biochem. J. 103*, 480.
Weaver, J. H., & Deupree, J. D. (1982) *Eur. J. Pharmacol. 80*, 437.

Role of Phospholipid and Protein–Protein Associations in Activation and Stabilization of Soluble Ca^{2+}-ATPase of Sarcoplasmic Reticulum

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ABSTRACT: The effect of increasing concentrations of the nonionic detergent Triton X-100 on catalytic activity, stability, phospholipid content, and aggregational state of solubilized Ca^{2+} ion activated adenosinetriphosphatase (Ca^{2+}-ATPase) of sarcoplasmic reticulum has been investigated. Increasing concentrations of Triton X-100 in the range 0.2–0.6% (w/v) inhibited ATP hydrolysis and *p*-nitrophenyl phosphate hydrolysis in parallel to the extent of 50% and 95%, respectively. Inactivation of *p*-nitrophenyl phosphate hydrolysis by preincubation in excess ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) at 25 °C was monophasic and first order at all concentrations of Triton X-100. The rate constant for inactivation increased sharply in the range 0.1–0.6% Triton X-100. At higher concentrations, the increase was less marked. Protein–protein associations of the solubilized ATPase were assessed by glutaraldehyde cross-linking and by ultracentrifugation in sucrose gradients. Both methods indicated a decrease in these associations in the 0.1–0.5% range. Cross-linking studies established that above 0.5% Triton X-100 the enzyme is >90% monomeric. The amount of phospholipid associated with the ATPase, recovered from sucrose gradients, decreased from about 50 mol of phospholipid/mol of ATPase at 0.1% Triton X-100 to about 3 mol of phospholipid/mol of ATPase at 0.5% and higher concentrations. Monomeric ATPase and aggregated ATPase isolated from equilibrium mixtures of these components had similar phospholipid/protein ratios. The results indicated that with increasing Triton X-100 concentrations, inhibition of catalysis, destabilization, loss of protein–protein associations, and loss of phospholipid occur concurrently. However, it was possible to maintain the monomeric state, reverse the inhibition of ATP and *p*-nitrophenyl phosphate hydrolysis, and restore stability by adding soybean phospholipid to the monomeric enzyme in 2% Triton X-100. This suggests that associated phospholipid, and not protein–protein association, is the principle determinant of the activity and stability of Ca^{2+}-ATPase in Triton X-100 solutions. We propose that mixtures of micelles containing one, two, or more ATPases are in slow equilibrium and each ATPase is equally unstable in excess EGTA. It is evident that while Triton X-100 can substitute for phospholipids in supporting catalytic activity, although at a slower rate especially with *p*-nitrophenyl phosphate as substrate, it cannot substitute for phospholipids in maintaining a stable native enzyme structure, and this suggests a specific phospholipid–ATPase interaction.

Intrinsic membrane proteins differ from soluble proteins in that the surface of at least part of the molecule is hydrophobic and associated with phospholipid. It has become clear that in many cases the catalytic activity of membrane proteins and the maintenance of their stable native structure depend, among other things, on the phospholipid–protein interaction. The

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