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REVIEW

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## MEMBRANE ENERGIZATION BY PROTON PUMPS IS IMPORTANT FOR COMPARTMENTALIZATION OF DRUGS AND TOXINS: A NEW TYPE OF ACTIVE TRANSPORT

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### Summary

Many organelles are energized by proton pumps: mitochondria form an inside-negative membrane potential by means of the respiratory chain and endomembrane structures, such as lysosomes and synaptic vesicles, establish an internal acidic pH by means of a vacuolar-type H<sup>+</sup>-ATPase (V-ATPase). Various amphipathic drugs such as local anesthetics and neuron blockers are accumulated in acidic organelles upon energization by proton pumps. However, this process does not require any transporters specific for the drugs: these drugs penetrate through the lipid bilayer against a concentration gradient so as to accumulate inside the energized organelles. Essentially the

same transport process takes place in liposomes that have been reconstituted with purified V- or F-ATPase. Various hydrophobic cations are also accumulated in mitochondria by a similar mechanism. The energy-dependent but transporter-independent accumulation does not belong to the known transport categories and seems to represent a new type of transport which may be important for understanding the mode of action of drugs and toxins.

Key words: proton pump, compartmentalization, drug, toxin, V-ATPase, F-ATPase.

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### Introduction

It is well established that V-ATPases acidify endomembrane systems such as endosomes and lysosomes. The resultant acidic pH is important for a variety of biological reactions, including proteolysis and the invasion of foreign substances (e.g. toxins and viruses) into cells (Nelson, 1989; Forgac, 1989). V-ATPases in plasma membranes extrude protons and acidify the restricted extracellular environment, which is essential for bone resorption (Vaananen *et al.* 1990; Chatterjee *et al.* 1992) and the metabolism of electrolytes in the kidney and bladder (Gluck and Nelson, 1992). They also energize the membrane across which nutrient absorption occurs in the insect midgut (Wieczorek, 1992). Another important role of an electrochemical proton gradient (proton-motive force; pmf) is the concentration of various substances. The pmf (in mV) has two components: a membrane potential ( $\Delta\Psi$ ) and a transmembrane pH gradient ( $\Delta\text{pH}$ ). The pmf is defined by the following equation:

$$\text{pmf} = \Delta\Psi - 2.3RT\Delta\text{pH}/F, \quad (1)$$

where  $R$  is the gas constant,  $T$  is the temperature in K and  $F$  is the Faraday constant. Using pmf as a driving force, transporters accumulate substrates against a concentration gradient (Table 1). Several substrates/proton antiporters and uniporters

have been identified in endomembrane systems (Njus *et al.* 1986; Kanner and Schuldiner, 1987; Johnson, 1988; Anraku, 1987; Schuldiner *et al.* 1995). In brain synaptic vesicles, at least four kinds of transport systems for neurotransmitters have been identified: the uptake of acetylcholine, monoamines or GABA/glycine may be catalyzed by substrate/proton antiporters (Njus *et al.* 1986; Kanner and Schuldiner, 1987; Marshall and Parsons, 1987; Haigh *et al.* 1994; Johnson, 1988; Sudhof and Jahn, 1991; Schuldiner *et al.* 1995), whereas the vesicular glutamate transporter seems to be a uniporter that transports glutamate anions electrophoretically (Maycox *et al.* 1988; Cidon and Shira, 1989; Shioi and Ueda, 1990; Moriyama *et al.* 1990). All these neurotransmitter transporters have been identified in microvesicles, the counterparts of brain synaptic vesicles in various endocrine cells (Annaert *et al.* 1993; Thomas-Reets *et al.* 1993; Erickson *et al.* 1994; Moriyama *et al.* 1992, 1995, 1996; Moriyama and Yamamoto, 1995a,b). These transport systems appear to use a chemiosmotic energy-coupling mechanism: a primary transporter (pump) energizes the membranes and a secondary transporter then drives the

uphill transport of the substrates (Table 1) (see also Harvey and Slayman, 1994).

However, some systems do not belong to the transport categories mentioned above. For instance, exogenous phenazinemethosulfate incorporated into thylakoid membranes accelerates the rate of electron flow more than tenfold by catalyzing an oxidation and reduction cycle of endogenous electron donors and acceptors (Nelson *et al.* 1972; Trebst,

1974). Quinone derivatives in liposomal membranes transfer protons from extravesicular ascorbate into internal ferricyanate, resulting in the formation of a proton gradient (inside acidic). In these cases, the oxidation/reduction potential converts an electrochemical proton gradient without any membrane enzymes: that is, accumulation against a concentration gradient can occur without secondary or primary transporters. This type of transport has received little attention so far. However, I observed that a similar type of transport often occurs when the compartmentalization of various drugs and toxins is measured. In this article, I briefly discuss the significance of energy-dependent but transporter-independent drug transport for understanding the mode of action of drugs and toxins.

Table 1. *Types of transport systems in biological membranes*

System	Transporter	Energy coupling
Passive transport or diffusion		
Simple diffusion	No	No
Facilitated diffusion (uniport)	Yes	No
Primary active transport	Yes	Yes
Secondary active transport		
Symport	Yes	Yes
Antiport	Yes	Yes
Uniport	Yes	Yes
Transporter-independent transport (simple concentration)	No	Yes
Group translocation	Yes	Yes

### Energy-dependent but transporter-independent accumulation of neuron blockers and local anesthetics in secretory vesicles

Most neuron blockers and local anesthetics are amphipathic amines. Since methylamine and aminoacridine derivatives, such as Acridine Orange, penetrate through membranes in a neutral form and are accumulated as a protonated form inside acidic compartments (Schuldiner *et al.* 1972; Dean *et al.* 1984)

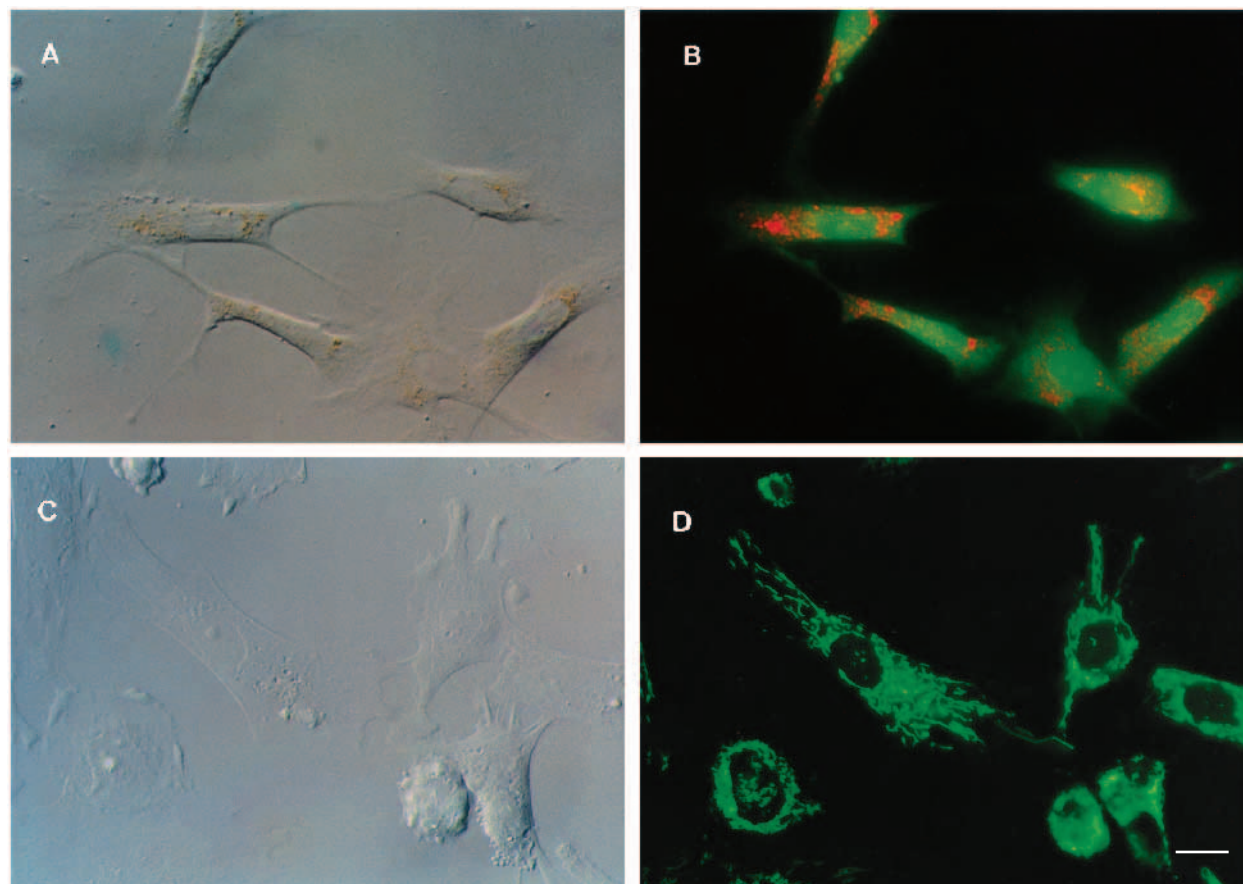


Fig. 1. Vital staining of acidic organelles and mitochondria with Acridine Orange and Rhodamine 123. Pinealocytes from rat pineal glands were cultured and stained with Acridine Orange at  $5 \mu\text{mol l}^{-1}$  (A,B) or Rhodamine 123 at  $5 \mu\text{mol l}^{-1}$  (C,D). Nomarski images (A,C) and fluorescence micrographs (B,D) are shown. Scale bar,  $20 \mu\text{m}$ .

(Fig. 1A,B), similar accumulation could be expected for these psychomimetic drugs (Moriyama *et al.* 1991a, 1993b). Upon the addition of ATP, chromaffin granule membrane vesicles took up radiolabeled chlorpromazine, haloperidol and propranolol (Moriyama *et al.* 1993b). This uptake was sensitive to proton conductors such as 3,5-di-*t*-butyl-4-hydroxybenzylidene malonitrile (SF6847) and to V-ATPase inhibitors such as bafilomycin A1, indicating that an electrochemical proton gradient established by a V-ATPase drove the uptake. Ammonium ions and nigericin plus K<sup>+</sup>, which dissipate  $\Delta\text{pH}$ , abolished the uptake, whereas valinomycin plus K<sup>+</sup>, a dissipator of  $\Delta\Psi$ , had no effect or was slightly stimulatory, indicating that  $\Delta\text{pH}$ , but not  $\Delta\Psi$ , is a driving force for the uptake. The ATP-dependent uptake was insensitive to reserpine, tetrabenazine, imipramine (inhibitors of vesicular or plasma membrane monoamine transporter) (Njus *et al.* 1986; Johnson, 1988) and to vanadate and verapamil (inhibitors of drug-transporting P-glycoprotein) (Gottesman and Pastan, 1988; Tsuruo, 1991), suggesting that the accumulation process is diffusion across the lipid bilayer and that transporters specific for these drugs are not involved in this concentration process. Similar ATP-dependent and proton-conductor-sensitive accumulation of these compounds was observed in synaptic vesicles and reconstituted proteoliposomes containing only purified V-ATPase as a protein component. Furthermore, proteoliposomes containing purified F-ATPase (bacterial F<sub>1</sub>F<sub>o</sub>-ATP synthase) accumulated these neuron blockers using only  $\Delta\text{pH}$  as a driving force (Fig. 2; Table 2). These results confirmed the hypothesis that the drugs are accumulated in an energy-dependent but transporter-independent manner (Moriyama *et al.* 1993b).

The energy-dependent but transporter-independent transport of drugs shows the following properties. (1) This process occurs against a concentration gradient and requires an energy supply

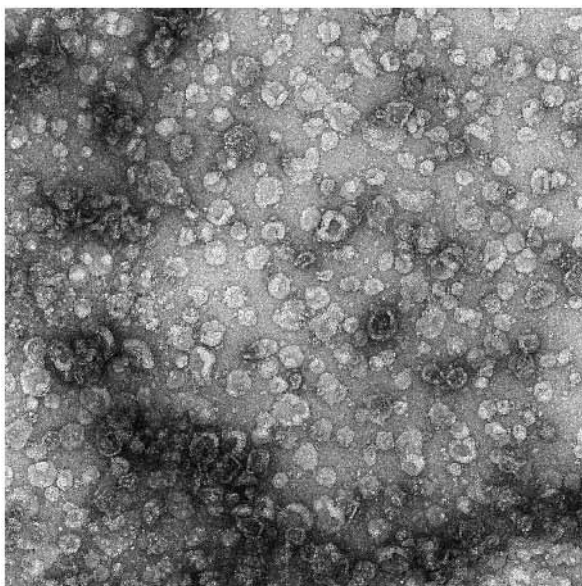


Fig. 2. An electron micrograph of proteoliposomes reconstituted with purified F-ATPase. Bacterial F-ATPase was purified and reconstituted into asolectin liposomes as described by Moriyama *et al.* (1991b).

through a primary proton pump. (2) This process is mediated by permeation through the lipid bilayer, and no specific transporters are involved. According to the classification of transport systems (Table 1) (Harvey and Slayman, 1994), this type of transport does not belong to any known transport category and represents a new type of 'active transport' (Table 1).

Accumulation of chlorpromazine in acidic compartments has been observed in cultured cells (M. Hayashi and Y. Moriyama, in preparation). Amphetamine derivatives (Sulzer and Rayport, 1990; Rudnick and Wall, 1992) or dopamine analogs (Johnson *et al.* 1982; Gasnier *et al.* 1986) are accumulated in acidic organelles in a similar manner. The energy-dependent but transporter-independent accumulation of amphipathic drugs into acidic organelles also occurs *in vivo*.

As discussed by Schuldiner and Avron (Schuldiner *et al.* 1972), amphipathic amines possessing a proton-accepting group with a pK much greater than physiological pH may accumulate in proportion to  $\Delta\text{pH}$  according to the following equation:

$$[\text{base}]_{\text{out}}/[\text{base}]_{\text{in}} = [\text{H}^+]_{\text{out}}/[\text{H}^+]_{\text{in}}. \quad (2)$$

Since more than 2 units of  $\Delta\text{pH}$  was formed at the steady state of energized chromaffin granules or synaptic vesicles, more than a 100-fold concentration gradient of neuron blockers and related drugs could be attained in cultured cells as well as in isolated membrane vesicles (Moriyama *et al.* 1993b). Thus, when administered at micromolar levels in the blood, these drugs could be concentrated more than 100-fold in synaptic vesicles. They could be secreted into the synaptic cleft through exocytosis upon nerve stimulation so as to cause effective nerve blocking due to binding to the receptors on postsynaptic membranes (Fig. 3). The concentrations of these drugs at presynaptic terminals may be important for effective nerve blocking. Therefore, synaptic vesicles may be one of the important target sites of psychomimetic drugs.

Table 2. Liposomes reconstituted with purified F-ATPase accumulate chlorpromazine using  $\Delta\text{pH}$  as a sole driving force

Addition	$\Delta\text{pH}$ (%)	$\Delta\Psi$ (%)	Chlorpromazine uptake (nmol mg <sup>-1</sup> protein)
None (control)	0	0	0.32
ATP	22	14	0.89
ATP + valinomycin	55	0	1.16
ATP + NH <sub>4</sub> Cl	0	41	0.18
ATP + valinomycin + NH <sub>4</sub> Cl	0	0	0.23

ATP-dependent formation of  $\Delta\text{pH}$  (inside acidic) or  $\Delta\Psi$  (inside positive) in reconstituted liposomes (see Fig. 2) (4  $\mu\text{g}$  of protein) was measured fluorometrically as described previously (Moriyama *et al.* 1991b) and expressed as percentage quenching (%). The uptake of radiolabeled chlorpromazine (1  $\mu\text{mol l}^{-1}$ ) by proteoliposomes under the same conditions is also shown (Moriyama *et al.* 1993b).

Additions: ATP (1 mmol l<sup>-1</sup>); valinomycin (0.1  $\mu\text{mol l}^{-1}$ ); NH<sub>4</sub>Cl (5 mmol l<sup>-1</sup>).

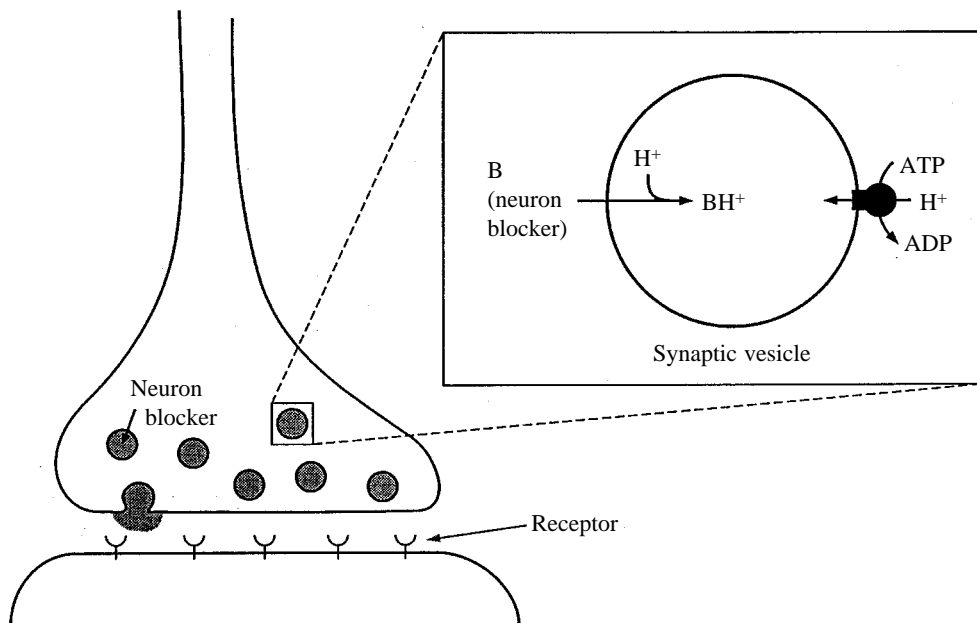


Fig. 3. Accumulation of neuron blockers and local anesthetics in synaptic vesicles and release at presynaptic nerve terminals.

#### Neuron-blocker-induced selective perturbation of transmitter uptake

When excess amounts of neuron blockers or local anesthetics are present, the entry of these psychomimetic drugs may lower  $\Delta\text{pH}$  and increase  $\Delta\Psi$  in synaptic vesicles, because  $\Delta\text{pH}$  is converted to  $\Delta\Psi$  upon the accumulation of the protonated forms of drugs under ideal conditions (Fig. 3). Chlorpromazine strongly dissipated  $\Delta\text{pH}$  but slightly increased  $\Delta\Psi$  without affecting V-ATPase activity (Fig. 4). Under these conditions, the uptake of neurotransmitters into synaptic vesicles must be selectively perturbed: the uptake of monoamines or GABA, which is driven by  $\Delta\text{pH}$ , was decreased, while the uptake of L-glutamate, an excitatory neurotransmitter taken up by the  $\Delta\Psi$ , was stimulated to some extent (Moriyama *et al.* 1993b). All amphipathic neuron blockers and local anesthetics tested so far exhibited essentially the same tendency. The pharmacological significance of these phenomena is not known at present. However, the administration of excess amounts of local anesthetics or neuron blockers always causes 'excitation' as a side effect (Tucker and Mather, 1980; Runciman, 1987), which is consistent with the possible increase in the amount of glutamate released from presynaptic terminals due to increased glutamate uptake into synaptic vesicles. Ammonia is known to be a neurotoxin which affects glutaminergic neurotransmission under hyperammonemic conditions such as hepatic encephalopathy (Szerb and Butterworth, 1992). Enhanced glutamate release into the synaptic cleft, which is at least partly due to selective perturbation of transmitter uptake (Albrecht *et al.* 1994), is one mechanism for ammonium neurotoxicity (Moroni *et al.* 1983; Butterworth *et al.* 1991).

#### Accumulation of anti-neoplastic agents in acidic organelles

It is well known that daunomycin and some anti-neoplastic

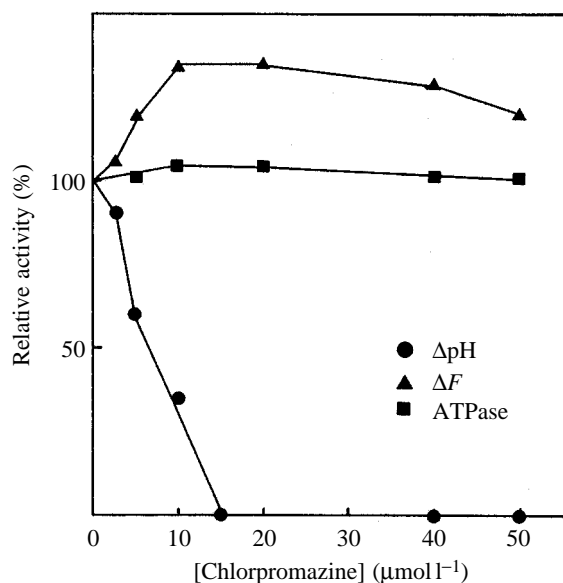


Fig. 4. A representative experiment showing that accumulation of chlorpromazine causes selective perturbation of the driving force in synaptic vesicles. Data from Moriyama *et al.* (1993b) with slight modifications. ATP-dependent formation of  $\Delta\text{pH}$  (circles), formation of  $\Delta\Psi$  (triangles) and ATPase activity (squares) in the absence or presence of chlorpromazine were assayed. Results are expressed as relative activities taking 70%  $\Delta F \text{ min}^{-1}$ , 26%  $\Delta F \text{ min}^{-1}$  and 2.6 units  $\text{mg}^{-1}$  protein, respectively, as 100%.  $\Delta F$  is the degree of fluorescent intensity.

agents such as doxorubicin and vinblastine are accumulated in acidic compartments *in vivo* (Willingham *et al.* 1986) (Fig. 5A), this being one of the important pathways for the extrusion of anti-neoplastic agents from drug-resistant cells (Beck, 1987; Sehested *et al.* 1988; Marquardt and Center, 1991). However, P-glycoprotein, an active drug extrusion pump, was not detected in acidic organelles (Willingham *et al.*

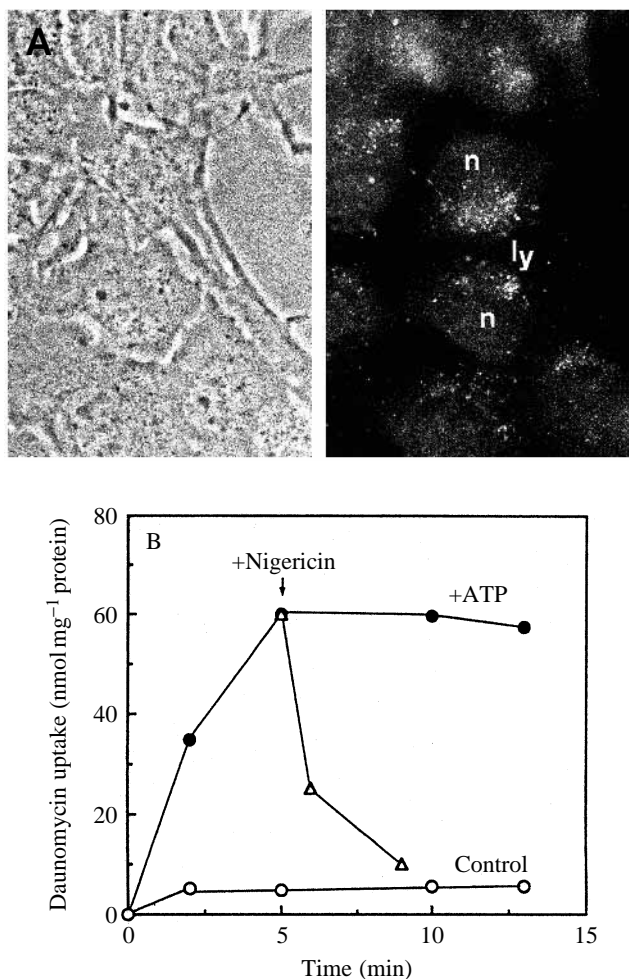


Fig. 5. Accumulation of daunomycin in acidic organelles. (A) Daunomycin ( $20\mu\text{mol l}^{-1}$ ) was incubated with cultured BNL CL.2 cells, normal mouse embryonic liver cells, for 5 min at  $37^\circ\text{C}$ . After washing the cells with Hanks buffer, daunomycin in the cells was observed by Nomarski (right) or fluorescence (left) microscopy. n, nucleus; ly, lysosome. (B) A representative experiment showing the time course of uptake of daunomycin by chromaffin vesicles measured in the absence (open circles) or presence (filled circles) of ATP. At the time indicated by an arrow, nigericin ( $2\mu\text{mol l}^{-1}$ ) was added to dissipate  $\Delta\text{pH}$ . For details, see Moriyama *et al.* (1994).

1987), so the mechanism by which daunomycin is accumulated in acidic compartments is unclear. We showed that the accumulation of daunomycin and related anti-neoplastic agents is also energy-dependent but transporter-independent (Moriyama *et al.* 1994). These compounds are also amphipathic amines and are accumulated in chromaffin vesicles upon the addition of ATP (Fig. 5B). The uptake was insensitive to various transporter inhibitors and to inhibitors of P-glycoprotein, but sensitive to bafilomycin A1, concanamycins and various proton conductors such as SF6847, indicating that an electrochemical proton gradient established by V-ATPase drove the uptake (Moriyama *et al.* 1994). Dissipation of  $\Delta\text{pH}$  by ammonium ions or nigericin plus  $\text{K}^+$  diminished the uptake, whereas treatment with valinomycin

plus  $\text{K}^+$  to cancel the  $\Delta\Psi$  slightly stimulated the uptake, indicating that  $\Delta\text{pH}$  was the sole driving force for the uptake. Similar ATP-dependent uptake can be reconstituted in proteoliposomes containing purified V- or F-ATPase. Daunomycin disappeared from acidic organelles *in vivo* when the internal pH of the acidic organelles became neutral after addition of bafilomycin A1, concanamycins or nigericin (Moriyama *et al.* 1994).

$\text{Ca}^{2+}$  channel blockers and calmodulin inhibitors are known to increase the toxicity of daunomycin (Tsuruo, 1991). This effect could be explained by inhibition of P-glycoprotein by these compounds so as to increase the free concentration of daunomycin in the cytoplasm (Tsuruo, 1991). However, these compounds are also amphipathic in nature and dissipate  $\Delta\text{pH}$  of acidic organelles so as to inhibit the accumulation of daunomycin in acidic organelles (Moriyama *et al.* 1994). The effect may also increase the free concentration of daunomycin in the cytoplasm, resulting in elevated cytotoxicity. Therefore, the amphipathic nature of these compounds may be one of the reasons why a variety of chemically unrelated compounds equally enhance the cytotoxicity of daunomycin.

#### Accumulation of hydrophobic cations in mitochondria

The concept of energy-dependent but transporter-independent accumulation of drugs can be expanded to other organelles such as mitochondria. It is well known that mitochondria accumulate hydrophobic cations, such as Rhodamine 123 or JC-1, as a consequence of electrophoretic movement of cations across the mitochondrial membrane (Fig. 1C,D) (Johnson *et al.* 1980; Smiley *et al.* 1991). Rhodamine 123 and related compounds are used as anti-neoplastic agents and the specific accumulation of these compounds in mitochondria is important for their selective cytotoxicity (Modica-Napolitano and Aprile, 1987). Methylphenyltetrahydropyridine (MPTP) is a neurotoxin causing artificial parkinsonism (Langston *et al.* 1983; Heikkila *et al.* 1984). MPTP is oxidized *in vivo* to the active form 1-methyl-4-phenylpyridinium ( $\text{MPP}^+$ ) (Markey *et al.* 1984).  $\text{MPP}^+$  accumulates in mitochondria in neuronal or other cells and inhibits complex I of the respiratory chain, causing ATP depletion and cell death (Mizuno *et al.* 1987). This accumulation has been shown to be energy-dependent (Ramsay and Singer, 1986), but so far no transporters responsible for the uptake have been identified. We suggest that, like Rhodamine 123,  $\text{MPP}^+$  is also taken up in a transporter-independent manner. First,  $\text{MPP}^+$  is a relatively hydrophobic and permeant cation, which is taken up by chromaffin cells in a transporter-independent manner (Reinhard *et al.* 1990). Second, the involvement of tetraphenylboron ( $\text{TPB}^-$ ) significantly increased the uptake of  $\text{MPP}^+$ , possibly due to increased hydrophobicity and reduction of the charge upon the formation of the  $\text{MPP}^+/\text{TPB}^-$  complex (Aiuchi *et al.* 1988), as in the case of the tetramethylphenylphosphonium ion ( $\text{TPMP}^+$ ), an indicator of the membrane potential (Szmelcman and Adler, 1976). Third,

the uptake of MPP<sup>+</sup> was observed in liposomes when only an inside-negative membrane potential was imposed with K<sup>+</sup>/valinomycin (Y. Moriyama, unpublished observation).

It is noteworthy that MPP<sup>+</sup> is recognized as a substrate by plasma membrane and vesicular monoamine transporters (Chiba *et al.* 1985; Darchen *et al.* 1988; Danielis and Reinhard, 1988; Reinhard *et al.* 1990; Moriyama *et al.* 1993a). This is the reason why MPP<sup>+</sup> is retained for a long time in dopaminergic neurons and adrenal glands (Johannessen *et al.* 1986). Thus, I suggest that transporter-dependent transport of MPP<sup>+</sup> is involved in the long-term and selective accumulation of MPP<sup>+</sup> in dopaminergic neurons and, in turn, that transporter-independent but energy-dependent accumulation occurs in neuronal mitochondria and causes an energy crisis, resulting in cell death.

### Conclusion

Some drugs or toxins are actively accumulated through lipid bilayer in organelles energized by proton pumps. This type of transport belongs neither to 'simple diffusion' nor to 'facilitated diffusion', because this process involves 'uphill' transport (Table 1). I would like to propose that this type of drug accumulation is termed 'simple concentration' (Table 1). Simple concentration is frequently seen upon administration of various kinds of drugs and toxins. After accumulation in the organelles or in restricted compartments, these drugs may exert their pharmacological effects and/or perturb the organellar functions, thereby exerting their toxicological effects. Therefore, 'simple concentration' is important for understanding modes of action of drugs and toxins. In this article, I have only discussed the accumulation of amphipathic drugs and hydrophobic cations. It is also possible that some acids, such as salicylic acid, may accumulate in alkaline environments (Oku, 1992) and that hydrophobic anions, such as suramin (an inhibitor of various enzymes, Moriyama and Nelson, 1988), are distributed among subcellular organelles by means of a similar mechanism.

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