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Effects of Inhibitors of Ion-Motive ATPases on the Plasma Membrane Potential of Murine Erythroleukemia Cells

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Summary. The membrane electric effects of N,N′-dicyclohexylcarbodiimide (DCCD) and vanadate were studied in murine erythroleukemia cells (MELC), comparing the patch-clamp technique and the accumulation ratio (AR_{exp}) of [^{3}H]-tetraphenylphosphonium (TPP^+). Electrophysiological measurements showed that both these inhibitors produce, at micromolar concentrations, a 20–30 mV hyperpolarization of resting potential ($\Delta\psi_p$) of MELC, which is abolished when the electrochemical equilibrium potential of K^+ (E_K) is brought close to zero.

DCCD and vanadate turned out to have distinct targets on the plasma membrane of MELC (an H^+ pump and the Na^+,K^+-ATPase, respectively).

Measurements of AR_{exp} showed that: (i) patch-clamp measurements of $\Delta\psi_p$ were equivalent to those based on AR_{exp} of antimycin-pretreated cells (AR_{ANT}); (ii) DCCD produced a strong increase in AR_{ANT}, that was antagonized by carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone (FCCP) and diethylstilbestrol (DES); (iii) vanadate determined a marked increase in AR_{ANT} that was insensitive to FCCP, but antagonized by ouabain; (iv) incubation in high K^+ medium (*H*K) brought AR_{ANT} to 1.0 in the controls, but did not lower this ratio below 3.0 in the presence of DCCD or vanadate; (v) the total amount of TPP^+ taken up by the cells was in any case water extractable by a freezing and thawing procedure.

On the whole, our data indicate that DCCD and vanadate hyperpolarize the MELC by increasing the K^+ conductance and, at the same time, enhance the TPP^+ binding, probably by changing the electrostatic potential profile of the plasma membrane. These effects seem to involve functional modifications of the target pumps, apparently related to the ion-occluding state of these enzymes.

Key Words ion-motive ATPase inhibitors · leukemia cells · resting potential · potassium conductance · TPP^+ binding · electrostatic potential of the plasma membrane

Introduction

Ion-motive ATPases (Pedersen & Carafoli, 1987*a*,*b*) mediate crucial cellular processes such as ATP synthesis or ion translocation against energy barriers. In general, they are membrane-intrinsic proteins, composed of two functionally distinct moieties: a portion interacting with ATP in the cellular aqueous compartment (the catalytic portion) and the ion-translocating portion across the membrane (Amzel & Pedersen, 1983; Al-Awqati, 1986; Skou, 1988; Futai, Noumi & Maeda, 1989).

Various inhibitors greatly contributed to characterize diverse types of ion motive ATPases and to elucidate their functional properties (Glynn & Karlish, 1975; Penefsky, 1979; Goffeau & Slayman, 1981; Pedersen & Carafoli, 1987*a*). In particular, DCCD specifically blocks the proton translocation step through the transmembrane moiety of H^+-ATPases (Hoppe & Sebald, 1984), while vanadate predominantly interferes with the ion-translocating activity of Na^+,K^+-ATPases (Cantley, Resh & Guidotti, 1978; Glynn & Richards, 1982; Beaugé, 1988).

Interestingly, both DCCD (De La Peña et al., 1982; Baroni et al., 1988) and vanadate (Lichtshtein, Mullikin-Kilpatrick & Blume, 1982) have been reported to hyperpolarize the plasma membrane of many different types of cells, including yeasts and neuroblastoma cells. These studies have been performed essentially by measuring the intracellular accumulation of the lipophylic cation TPP^+, but the hyperpolarizing properties of vanadate were confirmed by direct electrophysiological measurements. Although the hyperpolarization induced by DCCD in yeasts was traced to a K^+ efflux through K^+ channels (De La Peña et al., 1982; Baroni et al., 1988), while that sustained by vanadate was accompanied by an increase in the membrane conductance (Lichtshtein et al., 1982), the mechanism underlying these processes remains obscure so far.

We considered it worthwhile to explore this

topic by integrating the TPP^+ method and the patch-clamp technique, both of which have been employed widely on MELC (Arcangeli & Olivotto, 1986; Arcangeli et al., 1987*a,b*, 1989*a,b*). It was thought that this integration should provide information about the membrane energy profile, which might unravel the electric events, including the surface-charge effects, that occur in the membrane itself, when the ion-translocating moiety of a pump is blocked by specific inhibitors. In fact, the above-mentioned profile depends not only on the resting potential ($\Delta\psi_p$), but also on the membrane dipole potential and on the diffuse double-layer surface potential (McLaughlin, 1977, 1989). While $\Delta\psi_p$ can be exactly measured by the patch-clamp technique, accumulation of TPP^+ in cells and organelles is a function of both the resting potential and a complex of TPP^+ interactions with membranes, collectively referred to as "binding."

According to a widely accepted model, worked out on neutral phospholipid bilayers, binding consists of a confined adsorption of lipophilic ions to potential wells, formed near the membrane-water interfaces by the combination of repulsive electrostatic and attractive hydrophobic energy terms (Neumcke & Läuger, 1969; Ketterer, Neumcke & Läuger, 1971; Flewelling & Hubbell, 1986*a,b*). This model implies that lipid bilayers possess a dipole potential of several hundred millivolts positive in the interior, a feature of great importance to structural and functional properties of membrane proteins, including ion channels (Jordan, 1983). Variations of this potential, modifying the energy barrier in the middle of the membrane, may change the binding wells at the water interfaces. In natural membranes, containing negatively charged lipids, the latter generate a negative surface potential that represents another electrostatic parameter influenced (and mirrored) by the binding of lipophilic ions (Andersen et al., 1978; Bakker, 1982).

In this paper, we present patch-clamp and TPP^+ measurements aimed at clarifying the effects of DCCD and vanadate on both $\Delta\psi_p$ and the membrane electrostatic potential profile in MELC. Electrophysiological data provided evidence that these inhibitors hyperpolarize the plasma membrane by increasing the K^+ conductance, while TPP^+ experiments indicated that this hyperpolarization is a direct consequence of the inhibitor interaction with the target pumps. Integration of data obtained with the two techniques allowed the conclusion that DCCD and vanadate cause a marked increase of TPP^+ binding, suggesting that changes of the electrostatic potential profile underlie the membrane electric effects of these inhibitors.

Materials and Methods

Cell Culture

MELC (strain 745A, originally obtained from Dr. Friend and kindly provided by Dr. G.B. Rossi, Istituto Superiore di Sanità, Rome) were routinely cultured in RPMI 1640 medium, supplemented with 5% foetal calf serum (FCS) ("complete standard medium"), as previously described (Arcangeli & Olivotto, 1986). Cells were harvested from preparatory cultures at 1.8–2.0 $\times$ 10^6 cells/ml, centrifuged at 250 $\times$ *g* for 10 min, and, unless otherwise indicated, resuspended in freshly prepared complete medium at 9 $\times$ 10^5 cells/ml. Usually 8 ml of this suspension were transferred into tissue culture flasks with a culture surface of 25 cm^2 and incubated at 37°C in a humidified atmosphere (5% CO_2 in air).

Electrophysiological Techniques

$\Delta\psi_p$ was measured using an amplifier Axopatch 1-D (Axon Instruments), under the current-clamp configuration of the whole-cell patch-clamp technique (Hamill et al., 1981) on cells with an average diameter of about 8–9 μm. All experiments were done at room temperature (20–22°C). In about 10% of the experiments a remarkable swelling of the cells occurred: these experiments were discarded on the assumption that the cells were not in good physiological condition. Glass pipettes (borosilicate, Hilgenberg, Germany), with a resistance of 5–10 MΩ, were filled with an internal solution adjusted at pH 7.3, containing (in mM): NaCl 14, KCl 120, $MgCl_2$ 2, 4-(2-hydroxtyethyl)-1-piperazineethanesulfonic acid (HEPES) 10 and Na_2ATP 3; the final Ca^{2+} concentration was buffered with 10 mM ethylenebis (oxyethylenenitrilo)-tetra acetic acid (EGTA) at 10^{-7} M (*p*Ca 7) or about 10^{-9} M (*p*Ca 9) by adding 4 mM or no $CaCl_2$.

When needed, DCCD or Na-orthovanadate were added to these solutions, at the final concentrations of 5 and 25 μM, respectively. External solutions were delivered with hypodermic needles inserted into a capillary with a small hole (inner diameter 0.4 mm) positioned in the vicinity of the cell under study. Unless otherwise indicated, the external solution was adjusted at pH 7.4 and contained (in mM): NaCl 140, KCl 3, $MgCl_2$ 2, $CaCl_2$ 2, glucose 5 and HEPES 10. Modifications of the electrochemical equilibrium potential of K^+ (E_K) were obtained by changing the concentrations of KCl, either in the external or in the internal solution, and adjusting accordingly the NaCl concentration in order to keep constant the osmolarity. All seals were over 10 GΩ. The plasma membrane resistance was measured by injecting a series of 5-pA impulses (10 Hz) and recording the subsequent shifts of $\Delta\psi_p$ (Fig. 1*D*). Analog signals, filtered at 1 kHz, were recorded on a video tape, through a PCM processor (Sony, model 701ES) and analyzed off-line using the pCLAMP hardware-software system (Axon Instruments).

Measurement of Acid Extrusion

Acid efflux from the cells was detected by continuously monitoring the extracellular pH (pH_e) with a conventional combination pH electrode, in a water-jacketed chamber at 37°C, containing 1.5 ml of an unbuffered saline of the following composition (in mM): NaCl 143, KCl 3, $CaCl_2$ 2 and $MgCl_2$ 2. Acid loading of cells was performed essentially according to Swallow, Grinstein & Rotstein, (1988). Cells (4–6 $\times$ 10^6) were incubated in 2 ml of

RPMI 1640 medium, containing 10 mM HEPES and 40 mM NH_4Cl for 15 min at 37°C. After this incubation, cells were centrifuged for 5 min at 200 × *g*, gently resuspended in the unbuffered saline and immediately transferred into the chamber. Cells were magnetically stirred and the pH_e monitored for about 10 min. Initial pH_e at the moment of cell resuspension in the saline varied between 7.50 and 7.70.

Determination of Intracellular ATP

Intracellular ATP was determined by high pressure liquid chromatography (HPLC), according to Pogolotti and Santi (1982). Preparation of cell extracts was as follows: cells were seeded in complete medium at 9×10^5/ml in 75-cm^2 tissue culture flasks and incubated for 1 hr at 37°C. At the end of this incubation cells were harvested, centrifuged at 250 × *g* at 4°C for 5 min and the pellet extracted with 1 M perchloric acid at 2°C. Acid extract was frozen and thawed twice in liquid nitrogen, maintained for 30 min in ice and then centrifuged at 13,000 rpm for 20 min at 4°C in a Heraeus centrifuge (Biofuge 17 RS). The supernatant was collected, buffered with Tris HCl 1 M + KOH 2 N, and then centrifuged at 3,000 rpm for 5 min in a Heraeus Biofuge A centrifuge. The supernatant was filtered and used for ATP determination by means of a HPLC apparatus (Waters).

Determination of Intracellular Water and Intra- and Extracellular Ion Concentrations

Intracellular water (H_2O_i), Na^+ and K^+ concentrations in intra- ($[Na^+]_i$, $[K^+]_i$), and extracellular ($[Na^+]_e$, $[K^+]_e$) media were determined as previously described (Arcangeli & Olivotto, 1986; Arcangeli et al., 1987*a*).

Determination of TPP^+ Accumulation Ratio (AR_{exp})

This ratio was determined as previously described (Arcangeli & Olivotto, 1986).

The Estimate of Water-Extractable TPP^+

Water-extractable TPP^+ was determined by labeling MELC with ^{3}H-TPP^+ as previously described (Arcangeli & Olivotto, 1986). At the end of labeling, cells were centrifuged at 250 × *g* for 10 min and the pellet was resuspended in 250 μl of distilled water. Cells were immediately frozen and thawed twice, then centrifuged in a Beckman Microfuge at 12,000 × *g* for 5 min. Radioactivity in the supernatant was measured in a Packard Tri Carb 460 D scintillation counter. The percentage of water-extractable TPP^+ (TPP^+_{we}) was calculated by dividing the dpm contained in the supernatant, obtained as above, by the total dpm extracted from the cells as previously described (Arcangeli & Olivotto, 1986).

The Rationale of TPP^+ Measurements

Following previous contributions to this topic (Ketterer et al., 1971; McLaughlin, 1977; Rottenberg, 1979; Felber & Brand, 1982; Azzone, Pietrobon & Zoratti, 1984; Ritchie, 1984; Flewelling & Hubbell, 1986*a*,*b*), we based the use of TPP^+ on the following rationale.

In view of the scanty endoplasmic reticulum of MELC (Arcangeli & Olivotto, 1986), we have assumed that mitochondria are the only cellular organelles whose membrane potential could affect the amount of TPP^+ taken up by the cells. Hence

$$AR_{exp} = \frac{TPP_i^+}{H_2O_i} \cdot \frac{1}{[TPP^+]_e} = 1 + C\Delta\psi_p + C\Delta\psi_m + C_B \qquad (1a)$$

where AR_{exp} represents the TPP^+ accumulation ratio between the intracellular and the extracellular compartments; TPP_i^+ is the total TPP^+ taken up by the cells; H_2O_i is the intracellular water, $[TPP^+]_e$ is the TPP^+ concentration in the bulk extracellular water; $C\Delta\psi_p$ is the *AR* value accounted for by the $\Delta\psi_p$ contribution to AR_{exp}; $C\Delta\psi_m$ is the *AR* value accounted for by the contribution to AR_{exp} of the mitochondrial resting potential ($\Delta\psi_m$); C_B is the *AR* value accounted for by the contribution to AR_{exp} of the binding (*see* Introduction) and the term 1 is the value assumed by AR_{exp} when all the above contributions are null, so that at the equilibrium $\frac{TPP_i^+}{H_2O_i} = [TPP^-]_i = [TPP^+]_e$ where $[TPP^+]_i$ is the TPP^+ concentration in the total cellular water.

At the equilibrium partitioning, the TPP^+ accumulation ratio between the extracellular and the extramitochondrial cell compartments is governed by $\Delta\psi_p$ according to the Nernst equation, as follows

$$\Delta\psi_p TPP^+ = -61.5 \log\left(\frac{[TPP^+]_{em}}{[TPP^+]_e} = AR_p\right) \qquad (2a)$$

where $[TPP^+]_{em}$ is the TPP^- concentration in the extramitochondrial compartment and $-61.5 = 2.3 \cdot RT/F$.

By analogy, the accumulation ratio between the cell intra- and extramitochondrial compartments is governed by the mitochondrial potential ($\Delta\psi_m$) as follows

$$\Delta\psi_m TPP^- = -61.5 \log\left(\frac{[TPP^+]_{im}}{[TPP^+]_{em}} = AR_m\right) \qquad (3)$$

where $[TPP^-]_{im}$ is the TPP^+ concentration in the intramitochondrial compartment.

According to Eq. (2a), when $\Delta\psi_p$ is zero, $AR_p = 1$. Under these conditions $C\Delta\psi_p$ of Eq. (1a) is zero; on the other hand, when $\Delta\psi_p$ is different from zero, its contribution to AR_{exp} will depend both on AR_p and on the relative volume of the extramitochondrial compartment as compared to the cell total volume. Thus, on the whole

$$C\Delta\psi_p = (AR_p - 1) \cdot (S_t - S_m)/S_t \qquad (4a)$$

where S_t and S_m are the cell total volume and the volume of the total mitochondrial compartment.

By analogy

$$C\Delta\psi_m = (AR_m - 1) \cdot S_m/S_t. \qquad (5a)$$

Recalling Eqs. (2a) and (3), one can express AR_m as a function of $[TPP^+]_e$, so that Eq. (5a) becomes

$$C_{\Delta\psi m} = \frac{[TPP^+]_{im}}{AR_p [TPP^+]_e} - 1 \cdot S_m/S_t \quad (5b)$$

which expresses how in Eq. (1a) AR_{exp} depends on $\Delta\psi_m$.

In MELC, $S_m = 0.039\, S_t$, or $(S_t - S_m)/S_t = 0.96$ (Arcangeli & Olivotto, 1986). Hence Eq. (4a) becomes

$$C_{\Delta\psi p} \simeq AR_p - 1. \quad (4b)$$

Hence, whenever $C_B = 0$, and provided that $\Delta\psi_m$ is abolished by the mitochondrial inhibitor antimycin A (*ANT*) (Nicholls, 1982), it follows that

$$AR_{ANT} = 1 + C\Delta\psi_p \approx AR_p \quad (1b)$$

and, substituting in Eq. (2a)

$$\Delta\psi_p TPP^+ = -61.5 \log AR_{ANT}. \quad (2b)$$

On the other hand, whenever C_B is not negligible

$$AR_{ANT} = 1 + C\Delta\psi_p + C_B. \quad (1c)$$

In cells, where, like in MELC, $\Delta\psi_p$ is mainly governed by the K^+ conductance (Arcangeli et al., 1987*a,b*, 1989*b*), $C\Delta\psi_p$ can be brought close to zero when E_K is approximated to zero by incubating the cells in high K^+ external medium (*HK*). Then, in this medium and in ANT, one has

$$AR_{HK+ANT} = 1 + C_B HK \quad (1d)$$

which gives an estimate of the binding in *HK*.

Materials

Tissue culture flasks were purchased from Sterilin; the petri dishes used in patch-clamp experiments were from Falcon. RPMI 1640 medium was obtained from GIBCO Laboratories; FCS was from Flow Laboratories. DCCD, Na-orthovanadate, DES and FCCP were purchased from Aldrich; antimycin A and ouabain were from Sigma. Oligomycin and ATP were obtained from Boehringer Mannheim. Amiloride was a kind gift of Merk Sharp and Dohme. ^{3}H-TPP^+ (bromide salt, sp act 24 Ci/mmol) was obtained from Amersham Radiochemical Centre and diluted with cold TPP^+ provided by ICN, K and K Laboratories. All other reagents were of analytical grade.

TPP^+, DCCD, DES, FCCP, antimycin A and oligomycin were dissolved in ethanol and diluted in the selected media immediately before use. At the corresponding dilutions, ethanol was always ineffective on the parameters under study.

Results and Discussion

The Patch-Clamp Demonstration of the $\Delta\psi_p$ Hyperpolarizing Effects of DCCD and Vanadate

The effects of ATPase inhibitors on $\Delta\psi_p$ were first analyzed by a series of patch-clamp experiments, whereby the inhibitors were added to the cells from the inside. This allowed the use of very low inhibitor concentrations and ruled out possible hyperpolarizing effects caused by vanadate anion diffusion from the outside into the cells (Lichtshtein et al., 1982).

Figure 1 shows typical whole-cell recordings of $\Delta\psi_p$, either in the absence or in the presence of the ATPase inhibitors. Using the control pipette solution, $\Delta\psi_p$ did not undergo any significant variation during the measuring time (Fig. 1*A*). When the pipette solution contained 5 μM DCCD (Fig. 1*B*) or 25 μM vanadate (Fig. 1*C*), a substantial hyperpolarization of $\Delta\psi_p$ occurred constantly, although at variable times after the beginning of the measurement. Table 1,*A* shows that, at the steady state, both DCCD and vanadate produce, in physiological media, an average significant hyperpolarization (20 mV), with respect to the controls. When E_K was lowered from -98 to -10 mV by increasing $[K^+]_e$ in the perfusion solutions, $\Delta\psi_p$ followed E_K either in the absence or in the presence of the inhibitors (Table 1,*A*). The same occurred when E_K was changed to -18 mV, essentially by lowering the K^+ concentration in the internal solution (Table 1,*B*). The fact that in the controls $\Delta\psi_p$ depends on E_K indicates that a marginal potassium conductance is present under these conditions. Thus, under the simplifying assumption of ohmic relations, the resting equilibrium condition gives $g_K(\Delta\psi_p - E_K) = -g_L(\Delta\psi_p - E_L)$, where g_K, E_K, g_L and E_L are the conductances and Nernst potentials for potassium and the leakage pathways, respectively. The ratio $g_K/g_L = -(\Delta\psi_p - E_L)/(\Delta\psi_p - E_K)$ is reported in Table 1,*A* either for starting (g_{Ki}/g_L) or steady-state (g_{Kf}/g_L) conditions, assuming $E_K = -98$ and $E_L = 10$ (Arcangeli et al., 1989*b*). Notice that all the g_{Ki}/g_L values were not significantly different from the g_{Kf}/g_L value of the control. On the contrary, g_{Kf}/g_L increased in the presence of inhibitors. A comparative index of this effect is reported in Table 1,*A* as g_{Kf}/g_{Ki}, which shows that the K^+ conductance is increased more than twofold by both inhibitors.

On the whole, data in Table 1 demonstrate that the hyperpolarization induced by DCCD and vanadate in physiological media depends on the increase in K^+ conductance, in keeping with the indirect indications previously reported in yeasts (De la Peña et al., 1982; Baroni et al., 1988).

This increase most likely accounts for the report that, when added to the external medium, vanadate produces a $\Delta\psi_p$ hyperpolarization associated to a diminution of the electrical resistance of the plasma membrane (Lichtshtein et al., 1982). We confirmed this finding. In fact 5 mM extracellular vanadate determined a 30–40 mV hyperpolarization of $\Delta\psi_p$ (Fig. 1*D*), associated to an increase in the membrane conductance, as revealed by the response to current

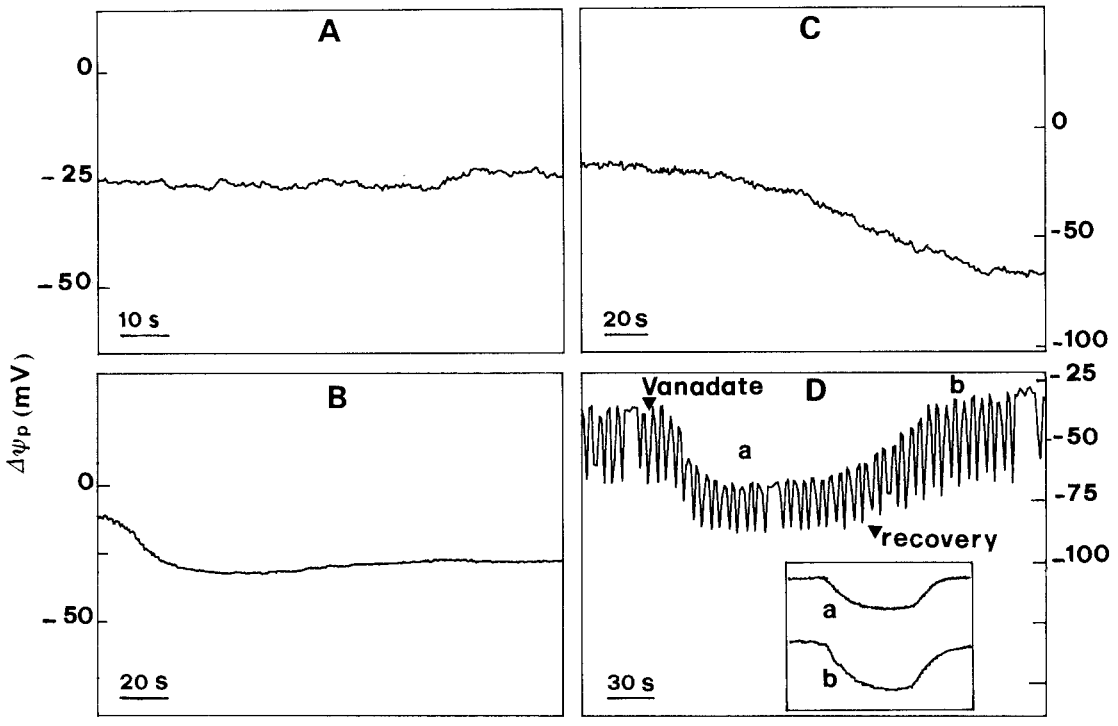

Fig. 1. Patch-clamp recordings of DCCD and vanadate effects on $\Delta\psi_p$ and on the plasma membrane conductance. (*A–D*) = $\Delta\psi_p$ records obtained in the whole-cell configuration as indicated in Materials and Methods. (*A*) control. $\Delta\psi_p$ does not vary significantly during the recording time. (*B*) The pipette solution contained 5 μM DCCD. The $\Delta\psi_p$ hyperpolarizes within the first minute and reaches a steady-state value more negative than the initial one. (*C*) The pipette solution contained 25 μM vanadate. $\Delta\psi_p$ shows a progressive hyperpolarization followed by a steady-state value. (*D*) Measurement of the plasma membrane conductance in the absence and in the presence of vanadate. The pipette contained the standard control saline, while 5 mM vanadate was added to the external solution at the time indicated by the first arrow. The membrane conductance was measured by injecting low frequency (10 Hz) 5-pA current pulses as reported in Materials and Methods. On the average, the membrane conductance was 6.67 GΩ (±0.441 SD; n = 15), corresponding to 0.15 nS, in the absence, and 3.85 GΩ (±0.181 SD; n = 10), corresponding to 0.26 nS, in the presence of vanadate. After recovery, both steady state $\Delta\psi_p$ and conductance return to the initial values. Under the conditions used in all these experiments, electrochemical equilibria were as follows (in mV): $E_K = -104$; $E_{Na} = +56$; $E_{Cl} = 0$. *Inset:* The hyperpolarizing response to one current pulse during (trace *a*) and after (trace *b*) vanadate perfusion.

pulses injected into the cell (*see* inset in Fig. 1*D*). The simple application of the procedure used for Table 1,*A* gave $g_{Kf}/g_{Ki} = 3.53$. With the help of the voltage response shown in Fig. 1*D*, we have calculated $g_{Ki} + g_L = 0.15$ nS and $g_{Kf} + g_L = 0.26$ nS. This gave us, in this particular example, a direct quantification of $g_{Ki} = 0.061$ nS and $g_{Kf} = 0.185$ nS, or $g_{Kf}/g_{Ki} = 3.04$. This value is not far from the previous 3.53, supporting the validity of the method worked out to measure the relative g_K increase in Table 1,*A*.

Since, in MELC, $\Delta\psi_p$ is mainly regulated by Ca^{2+}-dependent K^+ channels (K_{Ca}) (Arcangeli et al., 1987*a*,*b*, 1989*b*), the hyperpolarization produced by ATPase inhibitors could be reasonably traced back to a $[Ca^{2+}]_i$ increase, consequent to the block of Ca^{2+}-ATPases (Pedersen & Carafoli, 1987*a*). However, this explanation does not easily apply to our data, since the hyperpolarizing activity of DCCD and vanadate was maintained when Ca^{2+} in the pipette was lowered below 10^{-9} M, a concentration usually incompatible with the activation of these channels (Hamill, 1983). The involvement of other types of K^+ channels, such as those recently identified as being inhibited by ATP (Ashcroft, Harrison & Ashcroft, 1984), is equally difficult to conceive, since 3 mM ATP was present in the pipette solution used in these experiments.

Cell Parameters Affected by the ATPase Inhibitors and Identification of the Target Pumps

A deeper insight into the mechanism of the hyperpolarization sustained by DCCD and vanadate was sought by analyzing the alteration induced in intact cells and, hence, trying to identify the ion-motive ATPases principally affected.

Table 1. Effects of DCCD and vanadate on $\Delta\psi_p$ measured by the patch-clamp technique at the beginning or at the steady state of measurement

	Start		Steady state			
	$\Delta\psi_p$ (mV)	g_{Ki}/g_L	$\Delta\psi_p$ (mV)	LK ($E_K = -98$) g_{Kf}/g_L	g_{Kf}/g_{Ki}	HK ($E_K = -10$) $\Delta\psi_p$ (mV)
A						
Control	−24.0 ±3.5 (7)	0.48 ±0.07 (7)	−17.4 ±2.8 (7)	0.35 ±0.04 (7)	0.75 ±0.05 (7)	−3.7 ±2.9 (4)
DCCD 5 μM	−21.0 ±5.5 (3)	0.42 ±0.1 (3)	−38.6* ±1.2 (5)	0.82 ±0.04 (5)	2.18 ±0.37 (3)	−6.0 ±3.6 (4)
Vanadate 25 μM	−18.7 ±3.7 (6)	0.38 ±0.07 (6)	−37.5** ±6.1 (6)	1.08 ±0.29 (6)	2.66 ±0.86 (6)	−8.0 ±5.2 (4)
B			($E_K = -18$)			
Control	−23.5 ±7.17 (12)		−9.7 ±3.87 (12)			
DCCD 5 μM	−20 ±5.62 (6)		−10.2 ±5.46 (6)			
Vanadate 25 μM	−19.8 ±7.14 (8)		−13.3 ±5.25 (8)			

*$P < 0.001$; **$P < 0.01$ (Student's t test for independent samples).

Experimental conditions were as reported in the legend to Fig. 1 *A–C*. *Start* refers to $\Delta\psi_p$ values recorded soon after the whole-cell configuration was attained, *steady state* to the plateau subsequently attained and maintained for at least 2 min. g_{Ki} and g_{Kf} refers to g_K calculated at the start or at the steady state, respectively. Values are means ± SEM of the number of experiments listed in parentheses.

A: E_K was varied from −98 to −10 mV, passing from a perfusing solution with 3 mM K^+ (low K^+ = LK) to another with 80 mM K^+ (high K^+ = HK). Osmolarity was compensated by changing $[Na^+]_e$. $[K^+]_i$ = 120 mM. For calculation of g_K/g_L and $1 + g_{Ki}/g_K$, *see* text.

B: $[K^+]_i$ was 20 mM and $[K^+]_e$ 10 mM. By these means, E_K was maintained constantly at −18 mV.

As shown in Table 2, the rate of H^+ extrusion from acid-loaded MELC is strongly reduced by DCCD, as well as by another classical H^+-ATPase inhibitor, DES. Although these data support the existence of a plasma membrane H^+ pump, the activity of the latter is not the only mechanism of proton extrusion from MELC, since a marked inhibition of this parameter is produced also by a specific inhibitor of the Na^+/H^+ antiport, such as amiloride (Benos, 1982). This antiport could explain the finding, shown in Table 3, that DCCD, although ineffective on $[K^+]_i$ and (H_2O_i), increases $[Na^+]_i$. The lack of effect on $[K^+]_i$ clearly indicates that no inhibition of the Na^+,K^+-ATPase is brought about by DCCD up to 200 μM. On the other hand, the $[Na^+]_i$ increase probably reflects an activation of the Na^+/H^+ antiport following the diminution of the proton pump activity.

Contrary to DCCD, vanadate is a potent inhibitor of the MELC Na^+,K^+-ATPase, causing a dose-dependent decrease in $[K^+]_i$ and an increase in $[Na^+]_i$ (Table 3). At the concentration of 5 mM, vanadate completely mimics the effects of ouabain, namely the most specific inhibitor of this pump (Wallick & Shwartz, 1988).

On the whole, we conclude that DCCD and vanadate differ substantially as to their preferential target ATPase on the MELC plasma membrane, which are a H^+ pump and the Na^+,K^+-ATPase, respectively. On the contrary, as shown in Table 4, both 50 μM DCCD and 1 mM vanadate produce only a modest decrease in intracellular ATP content, similar to that produced by oligomycin at 4 $\mu g/10^7$ cells. Since, as indicated by the drastic effect of antimycin, the mitochondrial contribution to this parameter is decisive, the data in Table 4 suggest that, at the tested concentrations, DCCD and vanadate behave similarly to oligomycin at nonsaturating doses, with respect to their inhibitory activity on the mitochondrial F_0-F_1-ATPase.

Table 2. Effect of various inhibitors on H^- efflux rate in acid-loaded MELC

	H^+ extrusion rate (pmol/min/10^6 cells)
Control	2.71 ± 0.16 (5)
DCCD 50 μM	1.30 ± 0.07 (2)*
DCCD 200 μM	1.40 ± 0.09 (7)*
Amiloride 200 μM	1.39 ± 0.09 (2)*
DES 50 μM	1.27 ± 0.18 (3)*

* $P < 0.001$ (Student's t test for independent samples).
Cells were resuspended at 2.5×10^6/ml in 2 ml of a modified RPMI medium, buffered with 10 mM HEPES and containing 40 mM NH_4Cl. When needed, DCCD was added to the cell suspension at the concentrations indicated. After 15 min of incubation at 37°C, cells were centrifuged and gently resuspended in 1.5 ml of the unbuffered saline (*see* Materials and Methods) and transferred into the jacketed chamber at 37°C. pH_e was continuously recorded and the initial rate of acid extrusion measured as described in Materials and Methods. Amiloride and DES were added into the chamber after the beginning of pH_e recording. Values are means ± SEM of the number of experiments listed in parentheses.

The Equivalence of TPP^+ and Patch-Clamp Measurements of $\Delta\psi_p$ under the Standard Conditions

In Table 5, the electrophysiological measurements of $\Delta\psi_p$ obtained with the whole-cell configuration in the control cells are compared to values of $\Delta\psi_p TPP^+$ calculated in antimycin-pretreated cells, according to Eq. (2b). As shown, the two types of measurements agree very well over the range of $\Delta\psi_p$ variations induced in MELC by changing the cell inoculum (Arcangeli & Olivotto, 1986; Arcangeli et al., 1989*a*). Furthermore, whatever the method of measurement used, $\Delta\psi_p$ of leukemia cells follows the variations of E_K produced by the increase of $[K^+]_e$, in keeping with the prominent role of the K_{Ca} channels in the regulation of this potential. While confirming the validity of Eq. (2b) under the standard conditions of culture, these results indicate that all the parameters, except $C\Delta\psi_p$, identified in Eq. (1a) as potentially affecting AR_{exp}, are negligible under these conditions. This means that: (i) the contribution of $\Delta\psi_m$ to the cell uptake of TPP^+ is abolished by antimycin; and (ii) the TPP^+ binding is negligible, confirming our previous findings (Arcangeli & Olivotto, 1986). In turn, the equivalence of the patch-clamp measurements with those obtained by means of the TPP^+ technique, which leaves the internal cellular milieu unaltered, indicates that our electrophysiological records are not biased by the unavoidable alterations of this milieu in the whole-cell configuration.

The Effects of ATPase Inhibitors on AR_{exp}

Basing on the above, we performed the following analysis of the effects of ATPase inhibitors on AR_{exp} (Table 6).

The Effects of Oligomycin

AR_{exp} is not significantly affected in the controls either by inhibitors of the respiratory chain (antimycin) or by protonophores (FCCP). This behavior indicates that the contribution of $\Delta\psi_m$ to AR_{exp} ($C\Delta\psi_m$) is negligible, without necessarily implying that $\Delta\psi_m$ is null. In fact, taking into account the small percentage of cell total volume occupied by mitochondria (3.9%), it can be calculated that in MELC, up to $\Delta\psi_m$ values of -60 mV, $C\Delta\psi_m$ remains within the SE limits of AR_{exp}. However, these low values of $\Delta\psi_m$ contrast with the marked mitochondrial contribution to cellular ATP, witnessed by the fall of this parameter produced by antimycin treat-

Table 3. Effects of various ATPase inhibitors on $[Na^+]_i$, $[K^+]_i$ and H_2O_i

	$[K^+]_i$ (mM)	$[Na^+]_i$ (mM)	H_2O_i (nl/10^6 cells)
Control	152.3 ± 9.9 (8)	14.1 ± 2.0 (8)	343.6 ± 26.9 (8)
DCCD 50 μM	151.5 ± 12.3 (5)	35.9 ± 6.6 (5)	364.6 ± 39.9 (5)
DCCD 200 μM	159.5 ± 13.5 (2)	51.8 ± 8.8 (2)	346.5 ± 68.5 (2)
Vanadate 1 mM	81.0 ± 12.0 (2)	63.0 ± 12.0 (2)	294.5 ± 14.8 (2)
Vanadate 5 mM	20.0 ± 3.0 (2)	135.5 ± 18.5 (2)	346.5 ± 27.5 (2)
Ouabain 6 mM	32.4 ± 3.0 (7)	138.3 ± 14.0 (7)	398.0 ± 46.0 (8)

Cells were seeded at 9×10^5/ml in complete standard medium for 90 min. Inhibitors, at the concentrations indicated, were added 1 hr after cell seeding, except ouabain, that was added 30 min after seeding. At the end of incubation, cells were processed as described in Materials and Methods. Values are means ± SEM of the number of experiments listed in parentheses.

Table 4. Effects of various inhibitors on intracellular ATP content

	ATP (nmol/10^6 cells)
Control	3.40 ± 0.35 (12)
Antimycin A	1.17 ± 0.24 (8)
Oligomycin (4 μg)	2.54 ± 0.34 (6)
Oligomycin (40 μg)	1.74 ± 0.28 (7)
DCCD 50 μM	2.78 ± 0.43 (6)
Vanadate 1 mM	2.86 ± 0.75 (3)
Vanadate 5 mM	1.26 ± 0.75 (2)

Cells were seeded into 75-cm^2 tissue culture flasks and incubated in complete standard medium for 90 min. Antimycin A (20 μM) was added 30 min after seeding; oligomycin (4 or 40 μg/10^7 cells), DCCD (50 μM) and vanadate (1 and 5 mM) were added 1 hr after cell seeding. At the end of incubation, cells were processed for ATP determination as indicated in Materials and Methods. Values are means ± SEM of the number of experiments listed in parentheses.

ment (Table 4). This seems to rule out the possibility that the low $\Delta\psi_m$ of leukemic cells reflects a de-energized state of mitochondria, a conclusion confirmed by the effects of a specific inhibitor of F_0-F_1-ATPase, such as oligomycin. In fact, as shown in Table 6 and Fig. 2, this inhibitor caused a strong increase of AR_{exp} that was abolished by cell pretreatment with either antimycin or FCCP. These data are consistent with the predictions of the chemiosmotic theory (Mitchell, 1961) for tightly coupled mitochondria, whereby the block of F_0,F_1-ATPase interrupts the drop of potential required to sustain the H^+ current across this enzyme; this produces the maximal $\Delta\psi_m$ hyperpolarization, provided the respiratory chain is not impaired and the inner membrane remains impermeable to H^+ (Nicholls, 1982).

While raising interesting questions on the functionality of MELC mitochondria, the above experiments demonstrate that cell pretreatment with antimycin totally abolishes the contribution of $\Delta\psi_m$ to AR_{exp}, even when this contribution tends to become very high (as it is in oligomycin-treated cells). In fact, AR_{ANT} and the corresponding $\Delta\psi_p$ TPP^+ values calculated by Eq. (2b), in no case resulted in significantly different values in oligomycin-treated cells as compared to the controls (about −20 mV).

The Effects of DCCD

Up to 50 μM, DCCD produced a highly variable, but always considerable, increase of AR_{exp}. Furthermore, when the cells were pretreated with antimycin, AR_{ANT} was enhanced by DCCD at rather constant levels (≃10), which were significantly higher than those displayed by the cells treated with antimycin alone or with antimycin plus oligomycin. On the contrary, the effects of DCCD were totally abolished by cell pretreatment with FCCP, showing a relevant difference in the sensitivity of these effects to protonophores or respiratory chain inhibitors.

The dose response of AR_{exp} to DCCD in cells pretreated, or not, with antimycin is given in Fig. 2. In the absence of antimycin, the function has bell-

Table 5. Comparison between TPP^+ and patch-clamp measurements of $\Delta\psi p$ under the standard conditions

Inoculum	$\Delta\psi_p$ (mV)				
	10^5 *L*K	(E_K) (mV)	9×10^5 *L*K	9×10^5 *H*K	(E_K) (mV)
$\Delta\psi_p$ (whole-cell)	−53.7 ±9.1 (9)	−98	−20.2 ±1.6 (15)	−6.8	−10
$\Delta\psi_p$ TPP^+ (−61.5 log AR_{ANT})	−58.2 ±5.0 (3)	−87	−18.2 ±3.7 (8)	−1.6	−3.8

Cells were seeded at 10^5 or 9×10^5/ml in complete standard medium (low K^+ = *L*K) or in the high K^+ medium (high K^- = *H*K) prepared by substituting all NaCl of the standard medium with 108 mM KCl plus 24 mM $KHCO_3$ (final $[K^+]_e$ = 132 mM). Antimycin A (20 μM) was added 30 min after cell seeding. After 60 min of incubation, cells were used either for patch-clamp experiments (whole-cell configuration) or processed for determination of AR_{exp}. Values of $\Delta\psi_p$ TPP^+ were calculated from this ratio by means of Eq. (2b) (*see* Materials and Methods) and are means ± SEM of the number of experiments listed in parentheses. Whole-cell measurements refer to values recorded at the steady state of the traces and are means ± SEM of the number of determinations listed in parentheses. E_K values derive from the following parameters: $\Delta\psi_p$ (whole cell): $[K^+]_i$ = 120 mM; $[K^+]_e$ = 3 mM (*L*K) or 80 mM (*H*K). $\Delta\psi_p$ TPP^+: $[K^+]_i$ = 152 mM (*see* Table 3); $[K^+]_e$ = 6 mM (*L*K) or 132 mM (*H*K) (Arcangeli et al., 1987*a*).

Table 6. Effects of ATPase inhibitors on AR_{exp} and their response to various cell pretreatments

ATPase inhibitor		Oligomycin ($\mu g/10^7$ cells)		DCCD (μM)		DES (μM) 50	Vanadate (mM)		Ouabain (mM) 6
		4	40	50	200		1	5	
Pretreatment									
—	2.66 ±0.21 (21)	18.49 ±2.9 (10)	41.34 ±7.5 (7)	45.90 ±10.9 (10)	17.83 ±1.8 (6)	2.14 ±0.34 (4)	58.61 ±10.2 (5)	62.99 ±11.9 (9)	2.68 ±0.24 (11)
Antimycin	2.13 ±0.35 (8)	2.54 ±0.7 (5)	2.84 ±0.6 (3)	10.39 ±1.9 (6)	18.79 ±3.2 (4)	1.52 ±0.19 (5)	17.03 ±3.3 (5)	34.82 ±3.8 (7)	—
FCCP	2.29 ±0.03 (2)	2.73 ±0.01 (2)	—	3.13 ±0.55 (5)	—	—	—	20.03 ±3.0 (6)	—
DES	—	—	—	5.58 ±0.04 (2)	—	—	—	—	—
OUAB + FCCP	—	—	—	2.91 ±0.90 (3)	—	—	—	6.03 ±0.70 (3)	—

Cells were incubated in complete standard medium at 9×10^5/ml. ATPase inhibitors were added after 1 hr of incubation at the final concentrations indicated. Two min after this addition, cells were labeled with ^{3}H-TPP$^+$ and AR_{exp} was determined as described in Materials and Methods. Pretreatments were performed by adding the various substances at the following times before the addition of ATPase inhibitors: antimycin (20 μM), ouabain (OUAB) (6 mM), 30 min; FCCP (1 μM), DES (50 μM), 5 min. Values are means ± SEM of the number of experiments listed in parentheses.

shaped kinetics, with a peak at about 75 μM, followed by a rapid fall. On the contrary, in antimycin-pretreated cells, AR_{exp} shows a parabolic kinetics and an asymptotic approach to about 18 between 100 and 200 μM. On the whole, we conclude that DCCD causes two distinct effects on AR_{exp} of MELC: and (i) an antimycin- and FCCP-sensitive stimulation, which reflects the $\Delta\psi_m$ enhancement produced by the block of F_0-F_1-ATPase and is abolished by the dose excess; (ii) an antimycin-insensitive but FCCP-sensitive stimulation, which accounts for the whole TPP$^+$ accumulation beyond 150 μM DCCD. Since we know from oligomycin experiments that the $\Delta\psi_m$ hyperpolarization produced by the block of the F_0-F_1-ATPase is entirely prevented by the antimycin pretreatment, these data suggest that DCCD causes an intracellular TPP$^+$ accumulation that cannot be attributed to mitochondrial hyperpolarization. On the other hand, being prevented by FCCP, this accumulation seems sustained by a proton disequilibrium across, or within, cellular membranes.

The Effects of DES

While the effects of DCCD on AR_{exp} are shared by another widely used inhibitor of H$^+$-ATPase, such as N-ethylmaleimide (*not shown*), a totally different behavior is displayed by DES. In fact, as shown in Table 6, up to concentrations of 50 μM, that block the H$^+$ extrusion rate to the same extent as DCCD (*see* Table 2), DES has no significant effect on AR_{exp}, either in the absence or in the presence of antimycin.

The DES-Antagonization of DCCD Effects

Pretreatment with DES antagonized the DCCD-dependent increase of AR_{exp}, so that $AR_{DES+DCCD}$ was much lower than AR_{DCCD}, and even significantly lower than $AR_{ANT+DCCD}$. In other words, DES did not only prevent the $\Delta\psi_m$ hyperpolarization, but it also substantially antagonized the effects of DCCD on the extramitochondrial parameters affecting AR_{exp}.

These data deserve special attention in view of the peculiarity of DES interaction with the F_0-F_1-ATPase (McEnery & Pedersen, 1986; McEnery, Hullihen & Pedersen, 1989). Like oligomycin and DCCD, DES blocks the proton-translocating channel of this ATPase, but, unlike the other two inhibitors, it opens an alternative proton pathway at the F_0-lipid interface. This bypasses the effects of the block of F_0, operated either by DES itself or by other F_0 inhibitors, such as DCCD. Under these conditions, DES should fail to induce any $\Delta\psi_m$ hyperpolarization but should also antagonize the hyperpolarizing activity of DCCD. By analogy, assuming that the block of the proton channel of the plasma membrane H$^+$-ATPase is implicated in the $\Delta\psi_p$ hyperpolarization sustained by DCCD (recall Fig. 1 and Table 1), the bypass of this block via the alternative pathway created by DES would explain both the lack of effects of the latter on AR_{exp} and its antagonization of the extramitochondrial activity of DCCD. This interpretation would imply that the hyperpolarizing effects of DCCD involve functional changes of the target pump antagonized by a H$^+$

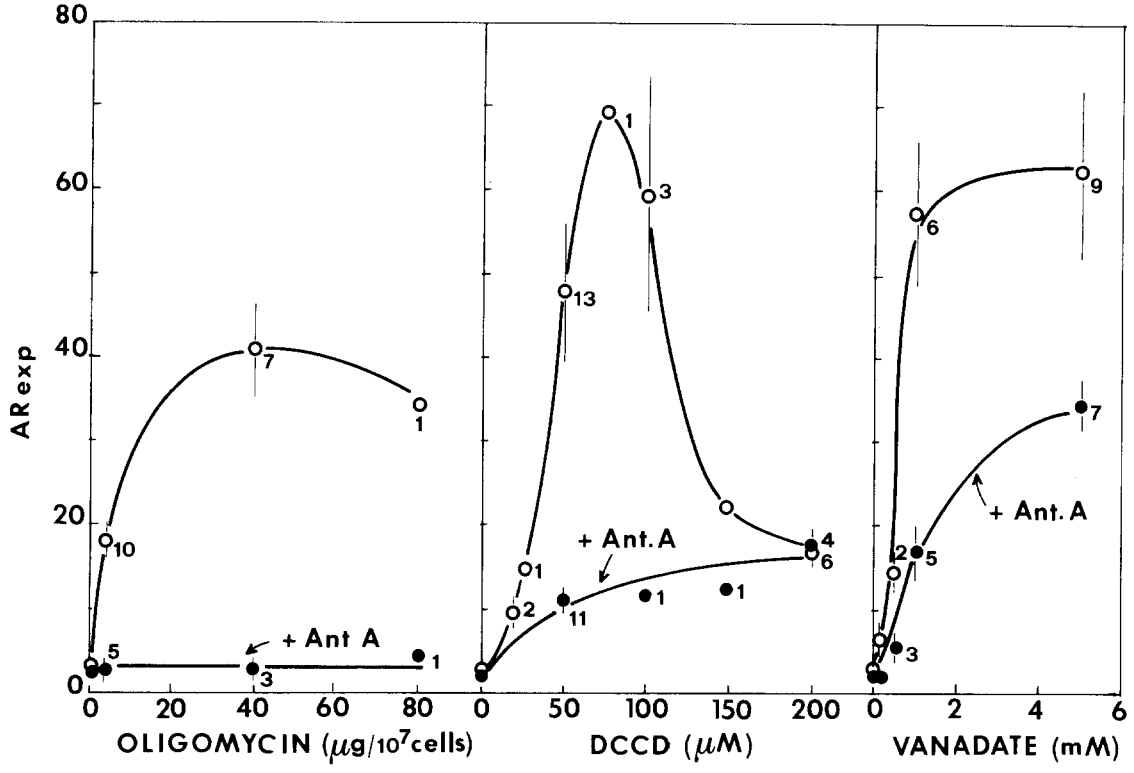

Fig. 2. Dose response of AR_{exp} to ATPase inhibitors in control and antimycin-pretreated cells. Experimental conditions were the same as reported in the legend to Table 6. Values are means ± SEM of the number of experiments indicated by the numbers next to the symbols.

bypass at the boundaries of the pump itself. In this light, the reversal of DCCD effects by FCCP could mimic the DES dissipation of the proton gradient within, or in the vicinity of, the H^+ pump.

The Effects of Vanadate and Their Antagonization by Ouabain

As illustrated in Table 6 and Fig. 2, vanadate (1–5 mM) enhanced AR_{exp} of the controls up to about 60 and AR_{ANT} up to asymptotic values as high as 34. This leads to the conclusion that, like DCCD, this inhibitor caused a marked $\Delta\psi_m$ hyperpolarization, displaying an oligomycin-similar activity on mitochondria. This similarity is also suggested by the vanadate effects on cellular ATP (*see* Table 4). On the other hand, the bulk of the AR_{exp} response to vanadate, unlike that to DCCD, is equally insensitive to FCCP as to antimycin, reflecting the difference in the target affected by the two ATPase inhibitors. However, the following effects of ouabain indicate that, also in the case of vanadate, functional changes of the target pump are implicated in this response. In fact, 6 mM ouabain, that blocked the Na^+,K^+-ATPase to the same extent as 5 mM vanadate (*see* Table 3), had no effect on AR_{exp} but drastically prevented the FCCP-insensitive effects of vanadate. It is worth noting here that vanadate and ouabain block the Na^+,K^+-ATPase with different mechanisms (Beaugè, 1988; Hootman & Ernst, 1988), which, paradoxically, involve antagonistic effects on the so-called "occluded state" of the pump. The latter is an intermediate state characterized by the sequestration of K^+ within the pump molecule during transport (Post, Hegyvary & Kume, 1972; Glynn & Richards, 1982; Forbush, 1987). In fact vanadate stabilizes this state, while ouabain prevents it (Glynn & Richards, 1982). Thus, it is tempting to speculate that the stabilization of the occluded state is necessary, if not sufficient, to confer on the pump the properties that induce the increase in extramitochondrial uptake of TPP^+.

To summarize, data presented in Table 6 lead to the conclusion that both DCCD and vanadate determine a marked increase of AR_{exp} that includes two contributions: first, an antimycin- and FCCP-sensitive component due to the increase in $\Delta\psi_m$; and second, the bulk of which is antimycin and FCCP insensitive is apparently sustained by modifications

Table 7. Effects of HK medium on AR_{exp} and estimate of TPP^+ binding either in the absence or in the presence of DCCD and vanadate

	AR_{HK+ANT}	$HK\text{-}TPP^+_{we}$ (%)	$AR_{HK+FCCP}$	AR_{ANT}[a]	TPP^+_{we} (%)	$C_B HK$ $(AR_{HK+ANT}{}^{-1})$[b]	$C\Delta\psi_p^{*c}$	C_B[d]	$C_B/C\Delta\psi_p^*$
ATPase inhibitor									
—	1.24 ±0.4 (4)	118 ±40	—	2.13	112 ±12	0.24	1.13	0	—
DCCD 50 μM	3.09 ±0.25 (6)	106 ±9	1.77 ±0.29 (2)	10.39	99.1 ±16	2.09	3.24	6.15	1.9
Vanadate 5 mM	3.34 ±0.26 (3)	97.1 ±13	—	34.8	112 ±4.3	2.34	10.4	23.49	2.2

[a] Taken from Table 6.
[b] Calculated from Eq. (1d).
[c] Calculated from the $\Delta\psi_p$ values, measured in patch clamp (Table 1 and Fig. 1), according to the formula: $\Delta\psi_p = -61.5 \log(1 + C\Delta\psi_p^*)$, or $C\Delta\psi_p^* = 10^{\Delta\psi_p/-61.5} - 1$.
[d] Calculated according to Eq. (1c) by the formula: $C_B = AR_{ANT} - (1 + C\Delta\psi_p)$.
Cells were incubated at 9×10^5/ml either in complete standard or in HK medium containing 132 mM K^+ ($E_K = -3.9$ mV). ATPase inhibitors were added after 1 hr of incubation at the final concentrations indicated. Two min after this addition, cells were labeled with ^{3}H-TPP^+ and AR_{exp} was determined as described in Materials and Methods. Pretreatments with antimycin A (20 μM) or FCCP (1 μM) were performed by adding these substances 30 or 5 min, respectively, before the addition of the ATPase inhibitors. Water extraction of TPP^+ taken up by the cells was carried out as described in Materials and Methods; values are reported as percentages of water-extractable TPP^+ on the total TPP^+ taken up either in HK or in standard medium (HK-TPP^+_{we} and TPP^+_{we}, respectively). Values are means ± SEM of the number of experiments listed in parentheses. For explanation *see* text.

of the target pumps that are conferred by some inhibitors and antagonized by others.

The Effects of High $[K^+]_e$ on AR_{exp} in the Absence or in the Presence of ATPase Inhibitors and the Estimate of TPP^+ Binding

Patch-clamp experiments demonstrated that the $\Delta\psi_p$ hyperpolarization produced by DCCD and vanadate, being abolished in HK media, is sustained by the increase in K^+ conductance. Incubation in these media was then used to discriminate the extramitochondrial effects of these inhibitors due to variations of either the K^+ conductance, or TPP^+ binding, calculated according to Eq. (1d). As shown in Table 7, incubation in HK medium brought AR_{ANT+HK} close to 1 ($\Delta\psi_p \simeq 0$), while it did not lower this parameter below 3.0 in DCCD- or vanadate-treated cells. When compared to AR_{ANT} values taken from Table 6, these data allow the following conclusions: (i) the bulk of the extramitochondrial effects of DCCD and vanadate (revealed by AR_{ANT}) are $[K^+]_e$ dependent, reflecting the increase in the K^+ conductance. In the light of information gathered in the preceding section, such increase should be regarded now as the consequence of the functional modifications of the target pumps induced by the ATPase inhibitors. (ii) Both DCCD and vanadate determine a substantial increase in TPP^+ binding in HK medium ($C_B HK$). In the case of DCCD, this effect is again abolished by FCCP (compare the effects of DCCD on AR_{HK+ANT} with those on $AR_{HK+FCCP}$; $P < 0.05$), suggesting that the K^+ conductance and the TPP^+ binding are increased through a similar mechanism reversed by the protonophore.

Electrophysiological measurements of $\Delta\psi_p$, performed in standard medium, were then utilized to calculate the parameter $C\Delta\psi_p^*$, i.e., the expected contribution of $\Delta\psi_p$ to AR_{exp} in this medium. This allowed us to estimate the expected contribution to AR_{exp} of TPP^+ binding in physiological media (C_B) in the absence or in the presence of DCCD and vanadate. As shown, this parameter, that is null in the controls, is increased by the inhibitors to values that are proportional to $C\Delta\psi_p^*$, so that $C_B/C\Delta\psi_p^*$ is roughly constant and equal to $C_B HK$ ($\simeq 2$). In other words, DCCD and vanadate apparently modify to the same extent the processes underlying the TPP^+ binding either in the standard or in the HK medium, while the absolute amount of bound TPP^+ increases proportionally to the $[TPP^+]_i$ attained within the cell as a function of the resting potential. This trend agrees with that described for the bound-surface density of TPP^+ in artificial phospholipid vesicles, which increases proportionally to the probe concentration up to an electrostatic saturation at 600 μM (Flewelling & Hubbell, 1986*a*).

Finally, Table 7 shows that the amount of TPP^+ recoverable from the cells by means of a freezing-thawing extraction in water (TPP^+_{we}) represents the totality of TPP^+ taken up by the cells in all the conditions tested, either in the absence or in the presence of DCCD and vanadate. This finding rules

out any hydrophobic sequestration of the probe within cellular components and fits in with the TPP^+ adsorption at the boundaries of lipid bilayers as a function of their electrostatic potential profile. In this light one should conclude that DCCD and vanadate change this profile in MELC plasma membrane.

Concluding Remarks

Patch-clamp and TPP^+-data presented in this paper converge to show that DCCD and vanadate, although acting on different targets, increase the K^+ conductance across the plasma membrane of leukemia cells. This increase requires the direct and specific interaction of these inhibitors with the target ATPases, while it is antagonized by inhibitors that block the same pumps with different mechanisms (DES or ouabain). A totally speculative explanation of this finding could be that, upon interaction with the effective inhibitor, the ion-translocating moiety of the pump is transformed into a K^+-permeable channel. In this context it should be noted that a mutant in the plasma membrane H^+-ATPase gene of *Saccharomyces cerevisiae*, with a reduced H^+-ATPase activity, when examined at the single-channel level, exhibited the activation of a K^+ channel that was hardly distinguishable from the H^+-ATPase itself (Ramirez et al., 1989).

The other important information to emerge from this work derived from the integration of patch-clamp and TPP^+ data, showing that both DCCD and vanadate alter the complex set of membrane electrostatic parameters reflected by the TPP^+ binding (*see* Introduction and comments on Table 7). It is worth recalling here that the internal energy profile of lipid bilayers can be changed by molecules modifying their dipole potential (Hladky, 1979; Kleijn et al., 1983; Flewelling & Hubbell, 1986*b*). We propose here that DCCD and vanadate may alter substantially the membrane energy profile by varying the internal dipole potential and/or the double-layer surface potential. This effect, undetectable by means of electrophysiological measurements, could: (i) alter the TPP^+ adsorption at the membrane water interfaces, accounting for the increase of the binding demonstrated in Table 7 and, hence, of the alterations of AR_{exp} reported in Table 6; and (ii) be monitored by potential-sensitive proteins spanning the membrane. One of these molecules could be a voltage-sensitive K^+ channel either pre-existing in a silent configuration or newly formed across the pump upon interaction with specific inhibitors. Alternatively, changes of the electrostatic potential profile sustained by the ATPase inhibitors could drastically enhance the Ca^{2+} concentration at the boundaries of the plasma membrane, thus activating calcium-sensitive K^+ conductance. This can occur even at prohibitive concentrations of this ion in the bulk cellular water. The crucial role of the electrostatic surface potential in determining the Ca^{2+} concentration necessary at the binding sites for triggering exocytosis has been clearly established by McLaughlin and Whitaker (1988) in sea urchin eggs.

However, the fact that, in the case of the Na^+,K^+-ATPase, the increase in K^+ conductance is activated by vanadate and prevented by ouabain suggests that putative changes of the electrostatic potential profile of the membrane are somehow related to the creation of the occluded state of this pump. This would favor the hypothesis that occlusion of cations within the ion-translocating moiety of the pumps could locally modify the membrane internal energy profile by interacting, for instance, with the membrane dipole potential (Flewelling & Hubbell, 1986*a*). This interpretation could also apply to the effects of DCCD on the H^+-ATPase of the plasma membrane, if DCCD creates the occlusion of H^+ within or in the vicinity of the H^+ pump. The bypass of this occlusion by FCCP and DES would thus explain the reversal of DCCD effects operated by these substances on either the TPP^+ binding or the K^+ conductance.

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