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Contribution of a H^+ Pump in Determining the Resting Potential of Neuroblastoma Cells

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Abstract. The aim of this work was to examine the effects of changes in external K^+ concentration (K_o) around its physiological value, of various K^+ channels blockers, including internal Cs^+, of vacuolar H^+-ATPase inhibitors and of the protonophore CCCP on the resting potential and the voltage-dependent K^+ current of differentiated neuroblastoma x glioma hybrid NG108-15 cells using the whole-cell patch-clamp technique. The results are as follows: (i) under standard conditions (K_o = 5 mM) the membrane potential was -60 ± 1 mV. It was unchanged when K_o was decreased to 1 mM and was depolarized by 4 ± 1 mV when K_o was increased to 10 mM. (ii) Internal Cs^+ depolarized the membrane by 21 ± 3 mV. (iii) The internal application of the vacuolar H^+-ATPase inhibitors N-ethylmaleimide (NEM), NO_3^- and bafilomycin A1 (BFA) depolarized the membrane by 15 ± 2, 18 ± 2 and 16 ± 2 mV, respectively. (iv) When NEM or BFA were added to the internal medium containing Cs^+, the membrane was depolarized by 45 ± 1 and 42 ± 2 mV, respectively. (v) The external application of CCCP induced a transient depolarization followed by a prolonged hyperpolarization. This hyperpolarization was absent in BFA-treated cells. The voltage-dependent K^+ current was increased at negative voltages and decreased at positive voltages by NEM, BFA and CCCP. Taken together, these results suggest that under physiological conditions, the resting potential of NG108-15 neuroblastoma cells is maintained at negative values by both voltage-dependent K^+ channels and an electrogenic vacuolar type H^+-ATPase.

Key words: Neuroblastoma cells — K^+ channels — Vacuolar H^+-ATPase — Resting potential

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Introduction

The resting potential (V_R) of eukaryotic cells is generally assumed to be mainly controlled by a selective permeability of the membrane to K^+ ions. This concept is based on the fact that increasing the external K^+ concentration depolarizes the membrane which, at high external K^+ concentrations, behaves as a K^+ electrode. At lower external K^+ concentrations, resting membranes generally show less K^+ concentration dependence, which has been explained in terms of the Goldman-Hodgkin-Katz equation taking into account the contribution of permeabilities to other monovalent ions (Hodgkin & Katz, 1949; *see* Chang, 1983; Offner, 1991). Moreover, external or internal application of K^+ channel blockers generally induces a membrane depolarization assumed to result from a decrease in K^+ permeability. The resting potential of neuroblastoma cells, like that of other neuronal cells, has also been thought to be primarily determined by a relatively high permeability of the membrane to K^+ ions (*see* Spector, 1981). However, further examination of this problem has shown that the resting potential of neuroblastoma cells is almost insensitive to K^+ channel blockers and to alterations in the external K^+ concentration around its physiological value (Miyake & Kurihara, 1983*a*; Rouzaire-Dubois & Dubois, 1991). The conclusion from these works was that K^+ channels do not significantly contribute to determining the resting potential under physiological conditions. The question raised by this conclusion is: What is the mechanism which determines the resting potential? According to Miyake and Kurihara (1983*b*), the resting potential is mainly controlled by nonselective channels which are at the origin of a diffusion potential directly controlled by the surface potential. While attractive, this conclusion seems unlikely since the resting potential is insensitive not only to changes in external cation concentrations

(except at high K^+ concentrations which open K^+ channels) but also to changes in external or internal Cl^- concentrations (Miyake & Kurihara, 1983*a*; Rouzaire-Dubois & Dubois, 1991). Another hypothesis could be that the resting potential is generated by an electrogenic pump. In several cell types, the Na^+/K^+ pump significantly contributes to determining the resting potential. This does not seem to be the case in neuroblastoma cells since the Na^+/K^+ pump inhibitor, ouabain, does not alter the resting potential (Miyake & Kurihara, 1983*a*).

Recently, it has been shown that the insertion of a yeast H^+-ATPase in the plasma membrane of normal fibroblasts renders them tumorigenic (Perona & Serrano, 1988). Moreover, it has been suggested that, in contrast to most normal cells, tumorigenic cells may express a vacuolar-type H^+-ATPase in their plasma membrane (Martinez-Zaguilan, Martinez & Gillies, 1991). ATP-dependent proton extrusion is known to occur in certain epithelia (Torres-Zamorano et al., 1992), in osteoclasts (Wang et al., 1992) and in macrophages (Swallow, Grinstein & Rothstein, 1990; Tapper & Sundler, 1992). In these cells, proton pumping is thought to be mediated by an electrogenic vacuolar-type H^+-ATPase whose properties have been reviewed by Pedersen and Carafoli (1987) and Forgac (1989) (*see also* volume 172, 1992 of *J. Exp. Biol.*, dedicated to vacuolar ATPases). If such a proton pump exists in the membrane of neuroblastoma cells, it should contribute to the genesis of resting potential and may shunt the K^+ diffusion potential when the open probability of K^+ channels is low. The aim of the present work was to test this latter hypothesis by studying the effects of various K^+ channel blockers or H^+-ATPase inhibitors on the resting potential and membrane current of the mouse neuroblastoma x rat glioma NG108-15 hybrid cells. The results support the notion that, in these cells, the resting potential is mainly determined by the activity of both voltage-dependent K^+ channels and an electrogenic vacuolar-type H^+-ATPase.

Materials and Methods

The experiments were performed at room temperature or at 37°C on differentiated neuroblastoma x glioma NG108-15 cells. All methods of cell culture and electrophysiological recordings were as previously described (Rouzaire-Dubois & Dubois, 1990*a*). Cells were differentiated by first adding 2% dimethylsulfoxide to the culture medium for 72 hr and then 0.5 mM dibutyryl-cAMP for 1–5 days. Membrane current and potential were recorded with the whole-cell patch-clamp technique (Hamill et al., 1981). Standard external and internal solutions had the following composition (mM). External: 140 NaCl, 5 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 4-(2-hydroxyethyl)-l-piperazineethanesulfonic acid (HEPES). Internal: 140 KCl, 2 $MgCl_2$, 10 HEPES. External and internal pH were adjusted to 7.3 using NaOH. In some experiments, the external medium was buffered with HCO_3^-/CO_2 in place of HEPES. In these conditions, 25 mM NaCl was replaced equimolarly by $NaHCO_3$ and the solution was bubbled with 5% CO_2–95% air to maintain an external pH of 7.4. The cell under investigation was continuously superfused by control or test solutions. Agents used in this study were purchased from Sigma Chemical (Saint Quentin Fallavier, France) except bafilomycin A1 which was provided by Professor K. Altendorf (Universität Osnabrück, Germany).

Results

K^+ Sensitivity of the Resting Potential

Under standard conditions (K_o = 5 mM), the resting potential (V_R) was -60 ± 1 mV (n = 23). V_R was slightly more negative at 37°C (-65 ± 2, n = 5) or when the external medium was buffered with HCO_3^-/CO_2 (-67 ± 2, n = 18). In two-thirds of the cells, this value remained constant for at least 5 min after establishing the whole-cell configuration. A twofold increase in K_o induced a depolarization of 4 ± 1 mV (n = 8). When K_o was reduced to 1 mM, V_R was not noticeably changed (depolarization of 1 ± 2 mV, n = 6). The membrane input resistance (R_m) was calculated from the size of hyperpolarizations caused by 10 pA inward current pulses. Under standard conditions, R_m was 1.23 ± 0.07 GΩ (n = 13). When K_o was decreased to 1 mM or increased to 10 mM, the mean value of R_m was multiplied by 1.11 and 0.74, respectively.

Effects of K^+ Channel Blockers on the Resting Potential

The above results show that, under physiological conditions, the resting potential is not significantly controlled by the membrane permeability to K^+. However, since V_R and R_m are not totally independent of K_o, they also indicate that K^+ channels do contribute to determining the resting potential. Three types of K^+ channels have been described in NG108-15 cells, namely: delayed voltage-dependent, Ca^{2+}-activated and muscarine-sensitive channels (Brown & Higashida, 1988*a*; Rouzaire-Dubois & Dubois, 1989, 1990*b*; Robbins & Sim, 1990; Schäfer, Béhé & Meves, 1991). To see whether these K^+ channels control the resting potential, we used specific blockers. Ca^{2+}-activated K^+ channels are blocked by d-tubocurarine (dTC) (Brown & Higashida, 1988*a*, *b*). The external application of 0.2 mM dTC did not alter the resting potential. Similarly, external muscarine (10 μM) or metacholine (50 μM), known to block M channels (Brown & Higashida, 1988*a*) had no effect on the resting potential. In NG108-15 cells, it has been shown that external bradykinin induces a transient hyperpolarization followed by a prolonged depolarization. These effects result from, on one hand, an inositol 1,4,5-triphosphate-dependent release of stored Ca^{2+} into the cytoplasm and an activation of Ca^{2+}-dependent K^+ channels and, on

the other hand, the inhibition of M channels (Higashida et al., 1986; Brown & Higashida, 1988*b*). In our cells, the external application of 5 μM bradykinin induced a hyperpolarization of 9 ± 2 mV (n = 7) which lasted about 20 sec but was not followed by a depolarization.

The delayed K^+ current of differentiated NG108-15 cells is made up of two components (fast and slow) sensitive to tetraethylammonium (TEA) and 4-aminopyridine (4-AP) (Rouzaire-Dubois & Dubois, 1989, 1990*b*; Robbins & Sim, 1990). Apparently, these two components have similar sensitivities to TEA (Robbins & Sim, 1990), which at a concentration of 10 mM reduced the peak K^+ current recorded at large depolarizations to 15% of its control value. In contrast, fast and slow K^+ components have different sensitivities to 4-AP (Rouzaire-Dubois & Dubois, 1990*b*; Dubois & Rouzaire-Dubois, 1991; Rouzaire-Dubois, Gérard & Dubois, 1991). 4-AP (5 mM) decreases fast and slow currents to 5 and 15%, respectively. The external application of 5 mM 4-AP or 10 mM TEA induced depolarizations of 1 ± 1 mV (n = 5) and 5 ± 2 mV (n = 9), respectively. Since almost all K^+ channels are blocked by internal Cs^+, we made a series of experiments with Cs^+ in the place of K^+ in the pipette. Under voltage-clamp conditions, an outward rectifying current, which started to activate between −60 and −40 mV and had properties of a delayed K^+ current, was blocked by Cs^+ which left intact a linear leakage current. Under current-clamp conditions, a depolarization of 21 ± 3 mV (n = 8) developed within 3 to 5 min after establishing the whole-cell configuration with Cs^+ in the pipette.

Effects of Pump Inhibitors on Resting Potential

In agreement with Lichtshtein, Kaback and Blume (1979) and Miyake and Kurihara (1983*a*), the Na^+/K^+ pump inhibitor, ouabain (5 mM), had almost no effect on V_R (depolarization of 1 ± 2 mV, n = 4). Suspecting the existence of a vacuolar type H^+-ATPase in the membrane of neuroblastoma cells (*see* Introduction), we tested the effects on the membrane potential of three agents known to be inhibitors of this type of ATPase, namely: NO_3^-, N-ethylmaleimide (NEM) and bafilomycin A1 (BFA) (*see* Pedersen & Carafoli, 1987; Bowman, Siebers & Altendorf, 1988; Forgac, 1989).

These agents were added to the pipette medium at concentrations of 140 mM (NO_3^- in place of Cl^-), 10 μM (NEM), 1 or 10 μM (BFA). Figure 1 shows the effects of 1 μM BFA on the resting potential. In all cells tested, 3 to 7 min after establishing the whole-cell configuration, the membrane was depolarized by 18 ± 2 mV, n = 6 (NO_3^-), 15 ± 2 mV, n = 6 (NEM), 16 ± 2 mV, n = 16 (10 μM BFA) and 15 ± 2 mV, n = 10 (1 μM BFA) (Fig. 2). A similar effect of BFA was observed

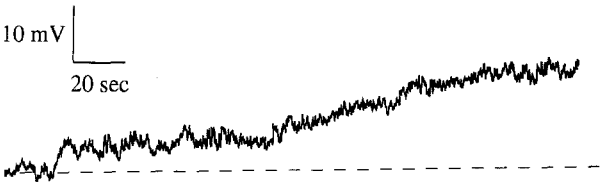

Fig. 1. Effect of bafilomycin on the resting potential. Under current-clamp conditions, BFA (1 μM), added to the pipette standard medium, induced a membrane depolarization which developed within 3–4 min. The resting potential was −75 mV some seconds after establishing the whole-cell configuration.

when the external medium was buffered with HCO_3^-/CO_2 (depolarization of 16 ± 2, n = 20). In contrast, the external application of 10 μM BFA for 3 to 5 min had no effect on the resting potential (n = 5). While these effects are likely mediated by an H^+-ATPase inhibition, we tested the possibility that they could be due to a K^+ channel blockade. Peak K^+ current was recorded during depolarizations of various amplitudes some seconds and 4 min after establishing the whole-cell configuration with 10 μM NEM in the pipette. Whereas the K^+ current was decreased at positive voltages, it was increased at negative voltages. In four different experiments, the K^+ current amplitude relative to its control value (determined some seconds after establishing the whole-cell configuration) was decreased by 24 ± 1% at +50 mV and increased by 9 ± 4% at 0 mV. Similar effects were observed with 10 μM BFA which decreased the K^+ current by 8 ± 2% at +50 mV and increased it by 13 ± 6% at 0 mV (n = 3). Under the same conditions, internal NO_3^- had no effect on K^+ current.

Taken together, the above results suggest that V_R is controlled by both a H^+-ATPase and K^+ channels. To confirm this view, we tested the effects on V_R of both internal Cs^+ and either BFA or NEM. Three to nine minutes after establishing the whole-cell configuration, the membrane was depolarized by 42 ± 2 mV (n = 8) with 140 mM Cs^+ and 10 μM BFA and by 45 ± 1 mV (n = 11) with 140 mM Cs^+ and 10 μM NEM in the pipette.

Effects of an Increased H^+ Influx

If, as suggested by the above results, a H^+ pump contributes to the genesis of the resting potential, a decrease in internal pH may increase the ATP-driven H^+ efflux and thus hyperpolarize the membrane. To transiently decrease the internal pH, we used the protonophore carbonylcyanide m-chlorophenylhydrazone (CCCP) (McLaughlin & Dilger, 1980). Under voltage-clamp conditions, the external application of 15 μM CCCP induced a current which was linear with voltage and reversed at the equilibrium potential for H^+ ions (Fig. 3). Moreover, a 10 sec application of CC-

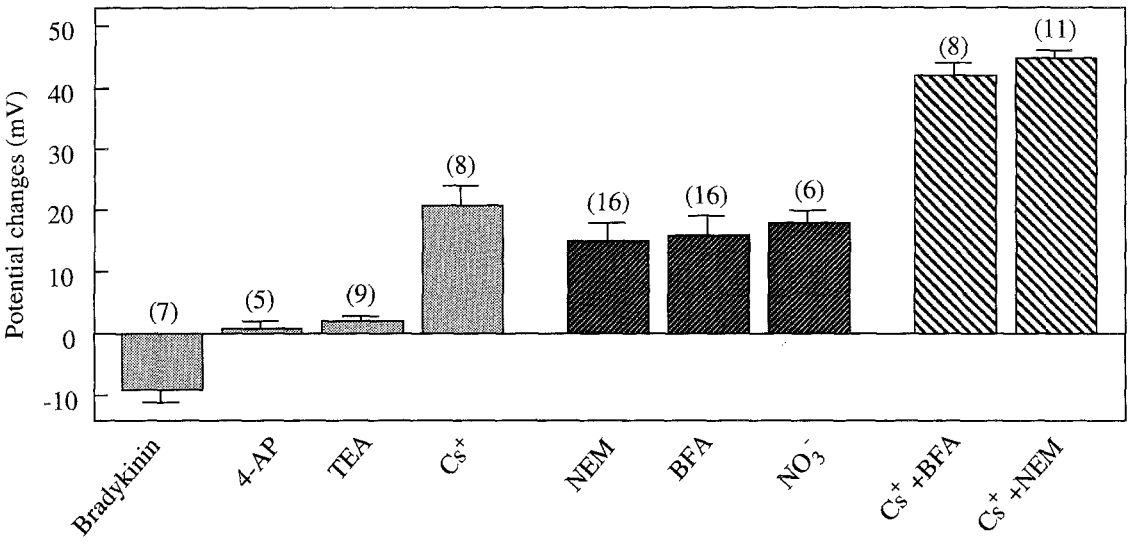

Fig. 2. Effects of various K^+ channels blockers and vacuolar H^+-ATPase inhibitors on the resting potential. Resting potential was measured before and during the external application of 5 μM bradykinin, 5 mM 4-aminopyridine (4-AP), 10 mM tetraethylammonium (TEA) and some seconds and 3 to 5 min after establishing the whole-cell configuration with 140 mM Cs^+ in place of K^+ in the pipette, 10 μM NEM, 10 μM BFA, 140 mM NO_3^- with K^+ as the major cation in the pipette or 10 μM NEM, 10 μM BFA with Cs^+ as the major cation in the pipette. Results are means ± SEM of several experiments (numbers in parentheses).

CP at −80 mV with $pH_o = pH_i = 7.3$ induced K^+ current modifications lasting for 3 min (Fig. 4). About 20 sec after the beginning of CCCP application, the K^+ current was reduced by 28 ± 7% at +50 mV and increased by 30 ± 9% at 0 mV ($n = 3$). These effects likely reflect a transient increase in internal H^+ concentration inducing a block of K^+ channels at positive voltages (Wanke, Carbone & Testa, 1979) and a shift towards negative voltages of the K^+ activation-voltage relationship resulting from the titration of internal fixed surface charges on the membrane (Chandler, Hodgkin & Meves, 1965). Under current-clamp conditions, CCCP induced a transient depolarization of 17 ± 1 mV ($n = 9$) followed by a prolonged hyperpolarization of 8 ± 1 mV ($n = 9$) (Fig. 5*A*). When CCCP was applied on BFA-treated cells, a maintained depolarization of 15 ± 1 mV ($n = 10$) was observed and the post-hyperpolarization was almost absent (2 ± 1 mV, $n = 10$) (Fig. 5*B*). What is more, the post-hyperpolarization was not observed in the presence of internal BFA, when the cells were repolarized to −60 mV before the CCCP application.

Discussion

The Resting Potential is Controlled by K^+ Channels

In agreement with previous reports on the same cells, the present results show that the resting potential of neuroblastoma cells is hardly sensitive to changes in external K^+ concentration around its physiological value or to K^+ channel blockers (Miyake & Kurihara, 1983*a*; Robbins & Sim, 1990; Rouzaire-Dubois & Dubois, 1991). These findings may suggest that K^+ channels are closed at the resting potential in the presence of low K^+ concentrations (Miyake & Kurihara, 1983*a*). However, we think that one cannot be so categoric and, on the basis of the following argumentation, we conclude that, if at the resting potential the open probability of K^+ channels is low, it is not nil.

That the resting potential is not fully independent of the K^+ concentration between 1 and 10 mM and the membrane input resistance decreased with K_o (*see also* Miyake & Kurihara, 1983*a*) suggests that, in these conditions, the membrane is not totally impermeable to K^+ ions. A remaining question is what type of K^+ channels are responsible for this permeability. The lack of effect on the resting potential of muscarine and metacholine may be due to the fact that the proper muscarine receptor is missing in NG108-15 cells (Robbins et al., 1992). However, that bradykinin, known to partially block M channels in NG108-15 cells (Schäfer et al., 1991; Robbins et al., 1992), does not depolarize the cells indicates that either I_M does not contribute to the resting potential genesis (*see also* Robbins et al., 1992) or that M channels are not expressed in our cells. Bradykinin induced a hyperpolarization which is likely due to the activation of Ca^{2+}-sensitive K^+ channels (Brown & Higashida, 1988*b*). However, the lack of effect of d-tubocurarine on the resting potential suggests

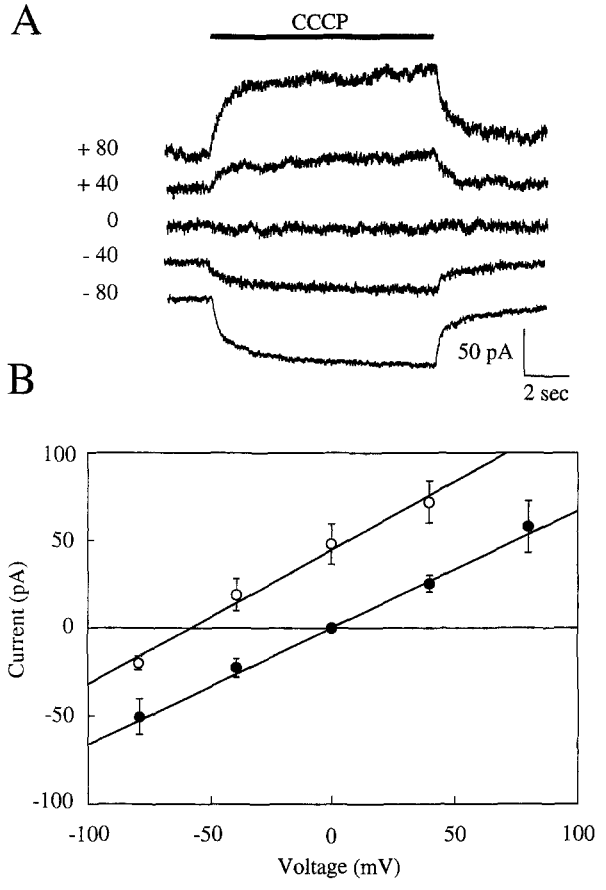

Fig. 3. H^+ current induced by CCCP. (*A*) Current responses to 10 sec external application of 15 μM CCCP at different potentials (numbers besides each trace) with $pH_o = pH_i = 7.3$. (*B*) CCCP-induced current-voltage relationships. The CCCP-induced current was recorded at different potentials with $pH_i = 7.3$ and $pH_o = 7.3$ (filled circles) or 8.3 (open circles). Mean of four experiments. Straight lines are linear regressions to the points. The reversal potential of the CCCP-induced current was −3 mV with $pH_o = 7.3$ and −58 mV with $pH_o = 8.3$.

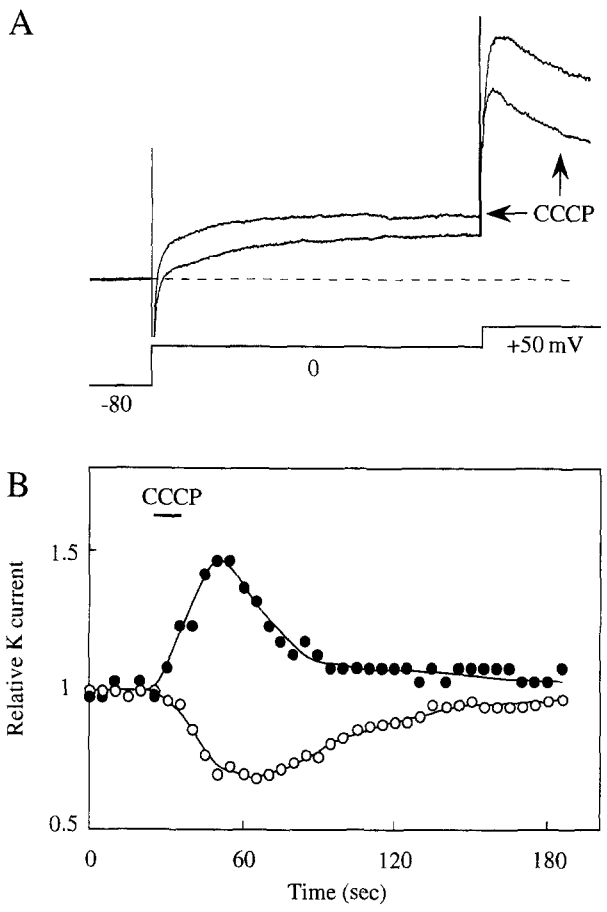

Fig. 4. Effects of CCCP on the peak K^+ current. (*A*) Superimposed traces of K^+ current recorded during depolarizations to 0 and +50 mV (pulse protocol below traces) before and during the application of 15 μM CCCP. The K^+ current was increased at 0 mV and decreased at +50 mV by CCCP. The initial inward current at 0 mV is a peak Na current partially represented in the figure. (*B*) Kinetics of CCCP-induced K^+ current change at 0 mV (filled circles) and +50 mV (open circles). The K^+ current was recorded as shown in *A* before, during and after the application of 15 μM CCCP.

that Ca^{2+}-sensitive K^+ channels are not activated in standard conditions. Internal Cs^+ significantly depolarized the membrane and, while having smaller effects, 4-AP and TEA slightly decreased the resting potential. Three explanations can be proposed to account for the differential effects on the resting potential of Cs^+ on the one hand and 4-AP and TEA on the other hand. (i) Whereas with 140 mM internal Cs^+ the voltage-dependent outward current is totally blocked at negative potentials, a noticeable fraction (about 15%) of this current is not blocked by 5 mM 4-AP or 10 mM TEA. The remaining current in the presence of 4-AP or TEA is increased by depolarization and thus contributes to maintaining the resting potential at negative values. (ii) The voltage-dependent K^+ current activated around the resting potential may be less sensitive to TEA and 4-AP than the K^+ current recorded during depolarization. (iii) Unspecified K^+ channels poorly sensitive to TEA or 4-AP may be active near the resting potential. In favor of this idea, it must be noted that NG108-15 cells possess two genes (NGK1 and NGK2) encoding for voltage-sensitive K^+ channels (Ito, Yokoyama & Higashida, 1992). When expressed in *Xenopus* oocytes, homomultimeric NGK1 channels are almost insensitive to TEA and begin to activate at potentials more negative than NGK2 channels. If homomultimeric NGK1 channels are present in neuroblastoma cells, they can account for the low sensitivity of V_R to TEA. Concerning 4-AP, two current phases can be recorded in NG108-15 cells (fast and slow) with the slow one being 18-fold less sensitive to 4-AP than the fast one (Rouzaire-Dubois & Dubois, 1989, 1990*b*). Whereas fast and slow K^+ current components contribute in almost equal

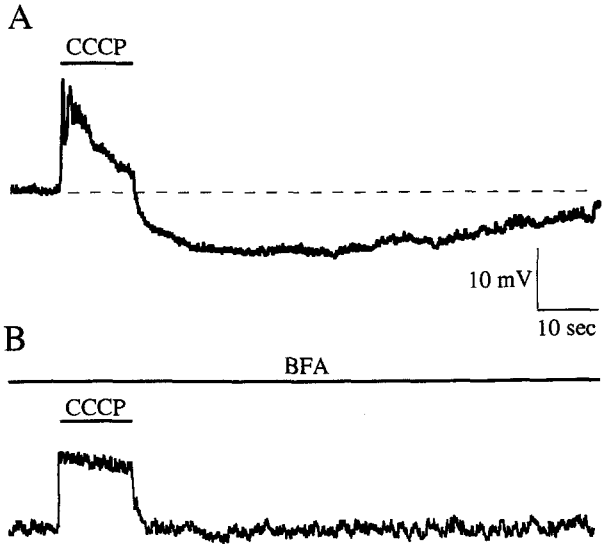

Fig. 5. Effects of CCCP on the membrane potential. (*A*) With the standard internal solution, CCCP (15 μM) induced a transient depolarization followed by a long-lasting hyperpolarization. The resting potential was −58 mV before CCCP. (*B*) When 10 μM BFA was added to the pipette solution, the CCCP-induced depolarization was not followed by a hyperpolarization. The resting potential was −46 mV before CCCP.

parts to the peak current recorded at large depolarizations, one does not know their respective contributions to the current at negative voltages. Furthermore, the K^+ channel block induced by 4-AP may depend on the membrane potential and the frequency of channels opening (Dubois & Rouzaire-Dubois, 1991; Choquet & Korn, 1992). This may lead to different effects of 4-AP on the K^+ current recorded during single voltage pulse and the K^+ current at the resting potential.

In summary, as in other excitable cells, the resting potential of neuroblastoma cells seems to be controlled by an outward K^+ current likely flowing through voltage-dependent K^+ channels and balanced by a leakage inward current. However, the small effects of K^+ channel blockers suggest that another system contributes to maintain the potential at negative values.

The Resting Potential is Controlled by a Vacuolar-Type H^+ Pump

In our experimental conditions, all ionic species except K^+ have Nernst potentials equal or positive to 0 mV. As a consequence, in addition to K^+ channels, an electrogenic pump should maintain the resting potential at negative values. Our results showing that the vacuolar H^+-ATPase inhibitors NEM, NO_3^- and BFA decrease the resting potential by about 15 mV with K^+ as the major internal cation and by about 40 mV with Cs^+ as the major internal cation, suggest that a vacuolar type H^+-ATPase should contribute to determining the resting potential. Previous authors who investigated the effects of NEM (1–10 mM) on the resting potential observed either no effect on crayfish axons (Shrager, 1977) or a gradual depolarization on lobster (Smith, 1958) and squid (Baumgold, Matsumoto & Tasaki, 1978) axons. However, since NEM is a sulfhydryl group reagent, it is not specific for the H^+ pump. Similarly, NO_3^-, which is added to the pipette medium at high concentrations may act on various systems controlling the resting potential. In contrast, BFA is considered to be a selective inhibitor of vacuolar H^+-ATPases. That it depolarizes neuroblastoma cells at relatively low concentrations and suppresses the hyperpolarization consecutive to the application of the protonophore CCCP strongly suggests that a vacuolar H^+-ATPase contributes to determining the resting potential of these cells. In the cell, vacuolar H^+-ATPases function at a number of endomembrane sites where they contribute to decreasing intraorganelle pH. Recent studies have also provided evidence that a vacuolar H^+-ATPase is present in the plasma membrane of macrophages (Swallow et al., 1990) where it contributes to the regulation of cytoplasmic pH by extruding H^+. Such an H^+-ATPase is likely also present in the plasma membrane of neuroblastoma cells where, by extruding H^+, it contributes to the resting potential genesis. It would be interesting to know if, as suggested by Martinez-Zaquilan et al. (1991), the existence of a plasma membrane vacuolar H^+-ATPase is a common feature of tumor cells, and if it contributes in these cells to the control of proliferation *via* a control of either the cytoplasmic pH or the membrane potential (*see* Dubois & Rouzaire-Dubois, 1993).

In conclusion, the present results indicate that the resting potential of NG108-15 neuroblastoma cells is controlled by both voltage-dependent K^+ channels and a vacuolar-type H^+ pump. As a corollary to this conclusion, V_R should be nil or positive in the presence of both internal Cs^+ and an H^+-ATPase inhibitor. In fact, under these conditions V_R is about −20 mV. This indicates that either K^+ channels and the H^+ pump are not fully blocked or another unspecified electrogenic pump also contributes to the resting potential.

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