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Polar/apolar compounds induce leukemia cell differentiation by modulating cell-surface potential

(membrane electric fields/tumor cell differentiation)

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ABSTRACT **The mechanism of action of polar/apolar inducers of cell differentiation, such as dimethyl sulfoxide and hexamethylene-bisacetamide, is still obscure. In this paper evidence is provided that their effects on murine erythroleukemia cells are modulated by various extracellular cations as a precise function of the cation effects on membrane surface potential. The interfacial effects of the inducers were directly measured on the charged electrode, showing that both dimethyl sulfoxide and hexamethylene-bisacetamide, at the effective concentrations for cell differentiation and within the physiological range of charge density, adsorb at the charged surface and produce a potential shift. A linear correlation was found between this shift and the inducer effects on cell differentiation. Besides offering a different interpretation of the mechanism of action of the inducers, these findings indicate that surface potential has a signaling function. They may also be relevant to cancer treatments based on tumor-cell commitment to terminal differentiation.**

Cell fate during embryogenesis and cell culture, as well as tumor progression, can be altered by a heterogeneous class of compounds, known as inducers (1). Of these, a category most widely effective on transformed cell lines and primary malignancies are the so-called polar/apolar inducers (2–6)—for example, dimethyl sulfoxide (DMSO) and hexamethylene-bisacetamide (HMBA). These compounds contain an apolar region linked to one or more polar molecular groups, so that their essential feature is a combination of hydrophobicity and a high dipole moment (5, 6), and their target is the plasma membrane (ref. 7 and references therein). Studies (7) based on the use of lipophilic cations indicated that the electrical potential across the plasma membrane was implicated in leukemia cell commitment to differentiation by DMSO and HMBA. To deepen this observation it was necessary to distinguish the contribution given to the true transmembrane potential (ϕ_m) by the resting potential (ϕ_{rest}) and the surface potentials at the intra- and extracellular side of the plasma membrane (ϕ_i and ϕ_e, respectively). In fact, voltage-dependent macromolecules located within the plasma membrane—for example, the voltage-dependent sodium channels of excitable cells—sense and are modulated by the sum $(\phi_i - \phi_e) + \phi_{rest} = \phi_m$. We present evidence that DMSO and HMBA act by modulating the surface potential, indicating a link between this potential and cell fate. This demonstration has potentially far-reaching implications for cell contact and cell-surface adhesion signaling, particularly during embryogenesis (8) and cancer invasiveness (9). These findings may have application in therapies that use polar/apolar inducers in beneficial treatment of cancer (3).

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MATERIALS AND METHODS

Cell Culture. Murine erythroleukemia cells (MELC; strain 745 A) were routinely cultured in RPMI 1640 medium/5% fetal calf serum (Flow Laboratories).

Cell Differentiation Tests. Benzidine-positive (B^+) cells and colonies were detected as described (7).

RNA Isolation and Blot Hybridization. Cytoplasmic RNA was prepared according to standard techniques (10). Twenty micrograms of RNA preparations was fractionated by electrophoresis in the presence of glyoxal and transferred to nylon membranes (Hybond-N; Amersham). RNA blots were hybridized with a ≃1.1-kb *Hin*dIII fragment of the pPK268 plasmid (11), containing an *Eco*RI restriction fragment of the mouse major β-globin gene and with a ≃0.5-kb *Kpn* I fragment of a chicken β-actin subclone. Probes were labeled to specific activities of $0.5–1 \times 10^9$ μg^{-1} by priming with random hexanucleotides in the presence of [^{32}P]dCTP. Hybridization occurred for 16 hr at 65°C. Blots were washed extensively in a 0.1× standard saline citrate/0.1% SDS solution at 65°C. Autoradiographic exposure was 3 hr.

Patch-Clamp Measurements of ϕ_{rest}. Patch-clamp measurements were taken in the current-clamp configuration (open circuit) according to Hamill *et al.* (12), using an amplifier Axopatch 1-D (Axon Instruments, Burlingame, CA).

Measurement of Intracellular K^+, Na^+, and Cl^- Concentrations. Measurements were done as reported (7).

Measurement of Inducer Adsorption and of Potential Shift at a Charged Interface. The interfacial behavior of DMSO and HMBA was studied at the charged mercury–aqueous solution interface in a described apparatus (13). The interfacial tension γ was determined as a function of polarization potential ϕ_M and inducer concentration c, at the temperature $T = 25°C$. From these measurements, the surface charge density and the adsorbed quantity of the inducer have been obtained by $\sigma_M(\phi_M, c) = (\partial\gamma/\partial\phi_M)_c$ and $\Gamma_M(\phi_M, c) = (\partial\gamma/\partial RT \ln c)_{\phi_M}$ (14) and then, by numerical inversion of the function $\sigma_M(\phi_M)$, $\Gamma_M(\sigma_M, c) = \Gamma_M(\phi_M(\sigma_M), c)$ has been obtained. The potential shift $\Delta\phi_M(\sigma_M, c)$ produced at the charged interface has been computed by numerical inversion of the function $\sigma_M(\phi_M, c)$ into $\phi_M(\sigma_M, c)$ and then subtracting the reference values $\phi_M(\sigma_M, 0)$.

RESULTS AND DISCUSSION

We used MELC as a model (15): their developmental program is blocked at a stage approximating the colony-forming cells for erythropoiesis (CFU-e), but it can be rescued by

Abbreviations: DMSO, dimethyl sulfoxide; HMBA, hexamethylene-bisacetamide; ϕ_m, transmembrane potential; ϕ_{rest}, resting potential; ϕ_i and ϕ_e, surface potential at the intracellular and extracellular side of the plasma membrane, respectively; B^+, benzidine-positive; MELC, murine erythroleukemia cell(s).

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polar/apolar inducers (6). Data in Fig. 1 show that MELC commitment to differentiation after rescue by polar/apolar inducers is a linear function of the sum of the monovalent extracellular cation concentration ($[Na^+]_e + [K^+]_e$) in the culture medium but is independent of cation species and osmolarity variations (see legend). After exposure for a given time to HMBA or DMSO at optimum concentrations, the number of differentiated cells, which had accumulated hemoglobin (B^+ cells) was directly proportional to extracellular $[Na^+]_e + [K^+]_e$ values, attaining the same value in medium where Na^+ was largely substituted with K^+, and vice versa

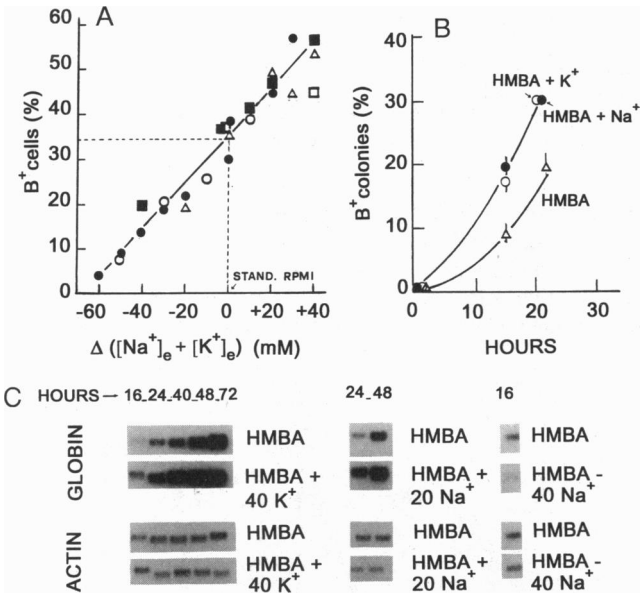

FIG. 1. (*A*) Modulation of DMSO- and HMBA-inducing activity by the sum of extracellular Na^+ and K^+ concentrations ($[Na^+]_e + [K^+]_e$). MELC were harvested from preparatory cultures attained at cell concentrations $<10^6$ cells per ml and seeded at 10^5 cells per ml into 24 multiwell plates with 1 ml of standard (STAND.) or reconstituted RPMI 1640 medium, containing various NaCl concentrations and KCl at one of the following concentrations: 5, 35, or 65 mM. When the sum NaCl plus KCl was lower than in the standard RPMI 1640 medium, mannitol was used to equalize the final osmolarity. When the sum was higher, the effects of hyperosmolarity were checked in samples containing mannitol. Concentrations of mannitol up to 80 mM were ineffective on the cell response to DMSO and HMBA. Hemoglobin-positive cells were scored by the benzidine test (16) (B^+ cells), after cell incubation at 37°C for 60 hr in DMSO (210 mM) or 48 hr in HMBA (5 mM). ○, DMSO, KCl 5 mM; ●, DMSO, KCl 35 mM; △, DMSO, KCl 65 mM; ■, HMBA, KCl 5 mM; □, HMBA, KCl 35 mM. Experimental values refer to five separate experiments and are expressed as percentage of B^+ versus total cells. Values in abscissa are the variations (Δ) of the sum $[K^+]_e$ plus $[Na^+]_e$ with respect to the value of this sum in the standard RPMI 1640 medium (5.4 + 138 = 143.4 mM). (*B*) Cation-enrichment of the culture medium accelerates MELC commitment to differentiation by HMBA at the single cell level. Cells were incubated for indicated times in 5 mM HMBA, either in standard RPMI 1640 medium (△), or in RPMI 1640 medium containing a 40 mM excess of NaCl (●) or KCl (○). At the end of incubation, commitment was assayed by transfer of cells to an inducer-free semisolid cloning medium, containing methylcellulose in standard RPMI 1640 medium (17). B^+ colonies and their size were scored at day 5 after cell seeding into the cloning medium. Values are means ± SEM of three separate samples. (*C*) Induction of globin mRNA expression in HMBA-treated cells is modulated by the extracellular cation concentration. Cells were incubated for the times indicated in 5 mM HMBA, either in standard RPMI 1640 medium, or in RPMI 1640 medium containing a cation excess (40 mM KCl or 20 mM NaCl), or in RPMI 1640 medium deprived of 40 mM NaCl (compensated with mannitol). At the end of incubation, total RNA isolation and blot hybridization were done as described.

(Fig. 1*A*). This cation-dependent modulation of the inducer activity affected early steps of cell commitment; in fact, Na^+ and K^+ equally potentiated the effects of a brief cell exposure to the inducers, revealed by scoring the percentage of B^+ colonies developed after cloning the cells in an inducer-free medium, containing standard Na^+ and K^+ concentrations (Fig. 1*B*). Consistently, addition of Na^+ and K^+ strongly accelerated the expression of β-globin mRNA in HMBA-treated cells, whereas this expression was retarded when the cation concentration was lowered as compared with controls (Fig. 1*C*). Neither increase nor decrease of extracellular cations varied the expression of actin mRNA.

Addition of extracellular cations did not modify either the intracellular concentrations of Na^+ and K^+ or ϕ_{rest} (Table 1). This potential was also unaffected by DMSO and HMBA, keeping constantly around −15 mV throughout incubation in the presence as in the absence of the inducers.

That the cation effect is independent of chemical species and causes no alterations of ϕ_{rest} is consistent with an effect on membrane surface potential (20). We developed a numerical algorithm to resolve in the most general case the diffuse layer equation from the Gouy–Chapman theory (21, 22); this enabled us to calculate ϕ_i and ϕ_e under various conditions, as a function of extracellular cation concentration (C) and valence (Z) (Fig. 2*A*). The membrane carries a negative charge resulting from the charged groups of phospholipids, glycolipids, and proteins with a surface density σ_{mi} and σ_{me} evenly distributed on the internal and external side, respectively. On both extra- and intracellular side of the lipid bilayer, a diffuse ion layer arises (of charge σ_e and σ_i), containing mainly cations. At a given temperature and bulk composition, the potential drop across the two diffuse layers (ϕ_e and ϕ_i, respectively) is only linked to the total charge contained in the corresponding layer (σ_e, σ_i), by the Grahame equation (14),

$$\sigma^2 = 2\,RT\varepsilon\textstyle\sum_n C_n[\exp(-z_nF\phi/RT) - 1], \qquad [1]$$

where Z_n and C_n are the valence and the bulk concentration of the ions in the external or internal medium, respectively; the other symbols have their usual meaning. The smeared transmembrane potential, computed by the plane condenser formula, is

$$\phi_m = -\frac{d}{\varepsilon_m}(\sigma_e + \sigma_{me}) = \frac{d}{\varepsilon_m}(\sigma_i + \sigma_{mi}), \qquad [2]$$

where d is the membrane thickness (assumed as 50 Å); ε_m is the membrane dielectric constant, assumed as twice the permititivity of free space, ε_o (23).

On the basis of Table 1, it is assumed that the potential difference between the internal and external bulk solution—that is, the resting potential (ϕ_{rest}), is not significantly affected by the inducers or the changes in extracellular cation concentration. Electroneutrality over the whole "trilayer system" σ_e, σ_m, σ_i, imposes

$$\sigma_e + \sigma_{me} + \sigma_{mi} + \sigma_i = 0, \qquad [3a]$$

whereas potential balance gives

$$\phi_m - \phi_{rest} = \phi_i(\sigma_i) - \phi_e(\sigma_e). \qquad [3b]$$

Recalling Eq. 2,

$$\phi_e(\sigma_e) - \phi_i(\sigma_i) - \frac{d}{\varepsilon_m}(\sigma_e + \sigma_{me}) = \phi_{rest}. \qquad [3c]$$

A single nonlinear equation can be obtained in the unique variable σ_e, substituting in Eq. 3c (from Eq. 3a) $\phi_i(\sigma_i) = \phi_i$

Table 1. Electrochemical equilibrium potential of K^+ and Na^+ and resting potential in MELC: Effects of adding cations or polar inducers to the standard medium

Addition (mM)	$[K^+]_i$, mM (*n*)	$[K^+]_e$, mM	E_k, mV	$[Na^+]_i$, mM (*n*)	$[Na^+]_e$, mM	E_{Na}, mV	ϕ_{rest}, mV (*n*)
None	145.4 ± 8.5 (4)	5.4	−88	16.6 ± 0.9 (4)	138	+57	−15.5 ± 2.7 (30)
KCl (40)	137.5 ± 5.8 (5)	45.4	−30	13.6 ± 2.5 (5)	138	+62	−9.4 ± 1.3 (14) NS
NaCl (40)	149.2 ± 10 (5)	5.4	−88	12.8 ± 2.5 (5)	178	+70	−15.5 ± 3.4 (6) NS
DMSO (210)	147.4 ± 11 (4)	5.4	−88	15.2 ± 4.1 (4)	138	+59	−16.9 ± 7 (12) NS
HMBA (5)							−14.4 ± 0.4 (15) NS

Reported values of potential were taken immediately after the rupture of the patch. All seals were >10 GΩ, and the input resistance of MELC (diameter = 8–12 μm) was ≈1 GΩ. Electrode resistances were 4–7 MΩ. External solutions: 140 (or 180) mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 (or 45) mM KCl, 10 mM Hepes, pH 7.34 (adjusted with NaOH). When needed, DMSO or HMBA was added. Internal solution: 125 mM KCl, 20 mM NaCl, 4 mM $CaCl_2$, 10 mM Hepes, 10 mM EGTA-K_2 (pCa 7), pH 7.32 (adjusted with KOH). NS, not significant (Student's *t* test for unpaired samples). Measurements were taken over the interval 1–48 hr of incubation and are means ± SEMs of the number of experiments listed in parentheses. Note that the ineffectiveness of NaCl on ϕ_{rest} is due to the fact that Na^+ permeability is normally very low in MELC (7). On the other hand, the lack of a significant depolarization after KCl addition can be explained by the fact that ϕ_{rest} is governed in these cells by Ca^{2+}-dependent K^+ channels and Cl^- channels, whereas the electrochemical equilibrium potential of Cl^- (E_{Cl}) is close to zero (18, 19). Under these conditions, these data can be used in the Goldman equation to calculate that, upon KCl addition, the depolarization produced by K^+ is compensated by the roughly equivalent hyperpolarization produced by Cl^-.

($-\sigma_e - \sigma_{me} - \sigma_{mi}$). In this expression ϕ_e and ϕ_i are the inverse of Grahame's equation, which is used twice, with two different sets of C_n values, for either the internal or the external medium. Solutions of Eq. 3c have been obtained numerically, for $\sigma_{mi} + \sigma_{me} = -20, -28\ \mu C/cm^2$ (corresponding to the range reported for biological membranes) (20, 24–26), either balanced on both sides of the membrane or slightly unbalanced (by 1–2 $\mu C/cm^2$): ε was assumed to equal 78, a value successfully used with measurements of the membrane electrostatic potential profiles despite theoretical

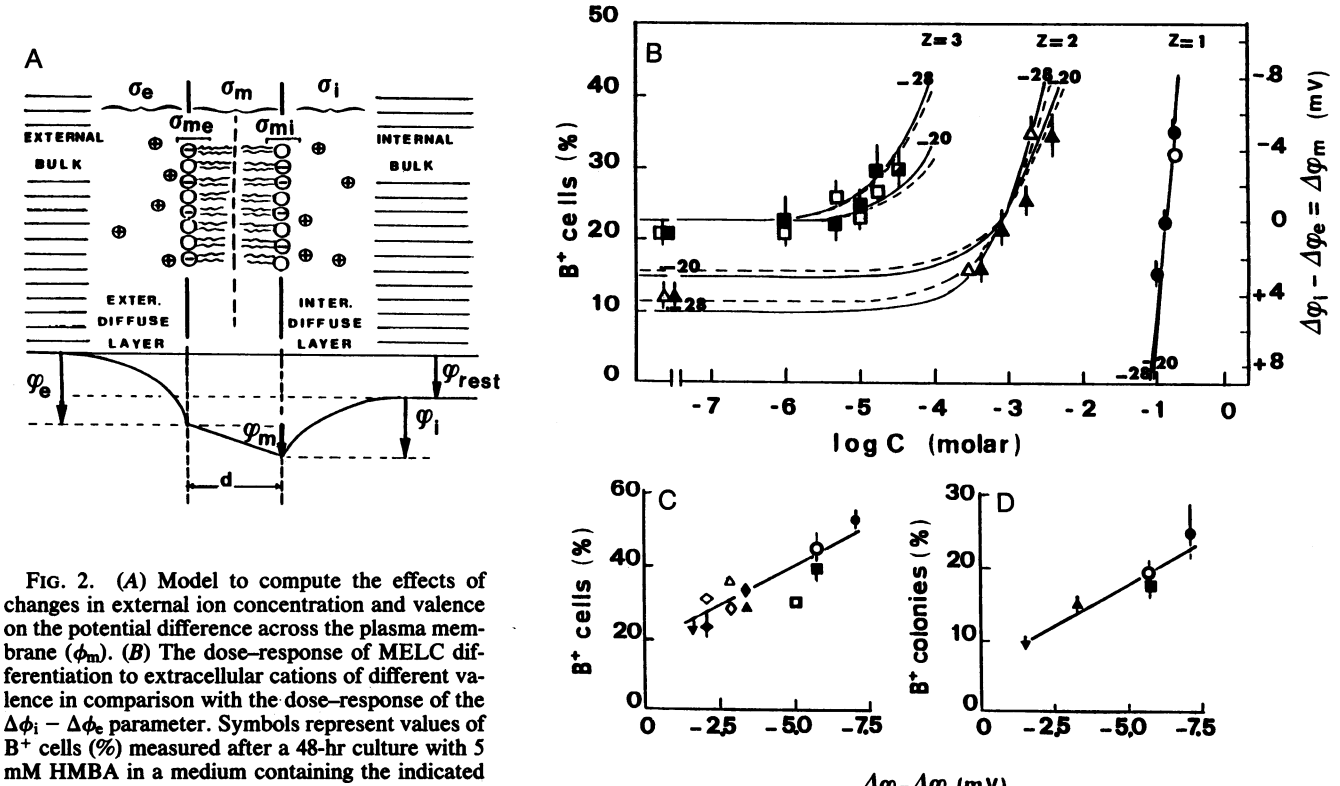

FIG. 2. (*A*) Model to compute the effects of changes in external ion concentration and valence on the potential difference across the plasma membrane (ϕ_m). (*B*) The dose–response of MELC differentiation to extracellular cations of different valence in comparison with the dose–response of the $\Delta\phi_i - \Delta\phi_e$ parameter. Symbols represent values of B^+ cells (%) measured after a 48-hr culture with 5 mM HMBA in a medium containing the indicated final concentration (*C*) of the varied cation with respect to the standard RPMI 1640 medium: ●, Na^+; ○, K^+; ▲, Mg^{2+}; △, Ca^{2+}; □, La^{3+}; ■, Fe^{3+}; ◪, $La^{3+} + Fe^{3+}$ (1/1). When Ca^{2+} and Mg^{2+} were omitted, $NaNO_3$ and $NaSO_4$ were added instead. Values are means ± SEM of two or three experiments. Cations were added to the standard RPMI medium as chloride salts. Curves represent $\Delta\phi_m = \Delta\phi_i - \Delta\phi_e$ obtained by solution of Eqs. 3, as a function of $\log_{10} C$, for the indicated values of parameter $\sigma_{mi} + \sigma_{me}$, either balanced (——) or with a constant difference $\sigma_{mi} - \sigma_{me} = -2\ \mu C/cm^2$ (– – –). Ionic composition of standard electrolyte solutions assumed for computations was as follows (A^-, anions; C^+, cations): Internal = C^+, 160 mM; C^{2+}, 0.8 mM; A^-, 161.6 mM. External = C^+, 136 mM; C^{2+}, 0.8 mM; A^{2-}, 6 mM; A^-, 125.6 mM (standard RPMI 1640 medium). (*C* and *D*) Whatever the cation addition, the differentiation indices (B^+ cells and B^+ colonies) of HMBA-treated cells are directly proportional to the ensuing negative shift of $\Delta\phi_i - \Delta\phi_e$. Experimental points refer to B^+ cells or colonies scored as illustrated in Fig. 1 *A* and *B* after exposure to 5 mM HMBA for 48 or 16 hr, respectively, in standard RPMI 1640 medium (▼) or RPMI 1640 medium containing the following cation excess: 40 mM NaCl (○); 40 mM KCl (■); NaCl 40 mM plus $LaCl_3$ 0.015 mM (●); $LaCl_3$ 0.005 mM (◇); 0.010 mM (◇); 0.015 mM (◆); $FeCl_3$ 0.005 mM (◂); 0.010 mM (△); 0.015 mM (▲); $CaCl_2$ 1 mM (□). Values are means ± SEM of two to five experiments (B^+ cells) or of three samples within the same experiment (B^+ colonies). In *C* the equation represented by the straight line is $B^+ = 17.98 - 4.32(\Delta\phi_i - \Delta\phi_e)$; $r = 0.90$, $n = 19$ and $P < 0.001$; for *D* $r = 0.97$, $n = 5$, and $P < 0.01$. Values of $\Delta\phi_i - \Delta\phi_e$ were calculated for $\sigma_{mi} = \sigma_{me} = -14\ \mu C/cm^2$ ($\sigma_{mi} + \sigma_{me} = -28\ \mu C/cm^2$).

objections (14, 24–28). Hence, $\phi_i - \phi_e$, together with ϕ_{rest} allows us to calculate ϕ_m for various values of the total surface charge density on the internal and external surface of the plasma membrane ($\sigma_{mi} + \sigma_{me}$), which was treated as a parameter to give the best fit of our experimental data.

We found that HMBA-induced commitment of MELC is enhanced by Na^+, K^+, Mg^{2+}, Ca^{2+}, La^{3+}, and Fe^{3+} (Fig. 2*B*), as is predicted by their effects on $\Delta\phi_i - \Delta\phi_e$ for $\sigma_{mi} + \sigma_{me}$ values ranging from -20 to -28 $\mu C/cm^2$ and without substantial influence by σ_{mi} versus σ_{me} unbalance up to 2 $\mu C/cm^2$: note that the B^+ parameters correlate precisely with $\Delta\phi_i - \Delta\phi_e$ (Fig. 2 *C* and *D*) over a 10^5-fold range of cation concentrations. Thus, the effects of cations during induction by polar/apolar inducers can be precisely ascribed to their effects on surface potential. Although cations alone can act as surface potential modifiers, within the concentrations used they were not potent enough to act themselves as surface potential-altering inducers (data not shown). However, they synergize with the polar/apolar inducers, so that the combined effect of cations and inducer is the sum of their contributions to the alterations in $\phi_i - \phi_e$. In fact (Fig. 3), a suboptimal dose of polar/apolar inducer produces a delay in MELC commitment that can be largely compensated by the addition of either Na^+ or K^+. This synergy indicates that the inducers are interchangeable with cations to determine the surface potential alterations required to cause maximal rates of commitment.

Our experiments indicated that polar/apolar inducers alter membrane surface potential, implying that the latter is a determining property of cell fate. The inducer capability to alter the surface potential was thoroughly analyzed by using an apparatus devised for studying polarized interfaces (13). Both HMBA and DMSO displayed a certain degree of hydrophobic adsorption at the electrode surface, which varied as a function of the charge density, σ_M, with a maximum around -10 $\mu C/cm^2$ (Fig. 4*A*). At this density, which corresponds to physiological values of σ_{mi} and σ_{me} (see Fig. 2*B*), the molecular adsorption of DMSO and HMBA at optimal doses for MELC differentiation was of the same order despite the 60-fold difference in the bulk concentration (300 versus 5 mM). This is clearly due to the different hydrophobicity of the two molecules. Being driven by short-range hydrophobic forces, adsorption of the electrically neutral molecules of inducers occurs in the immediate contact with the surface,

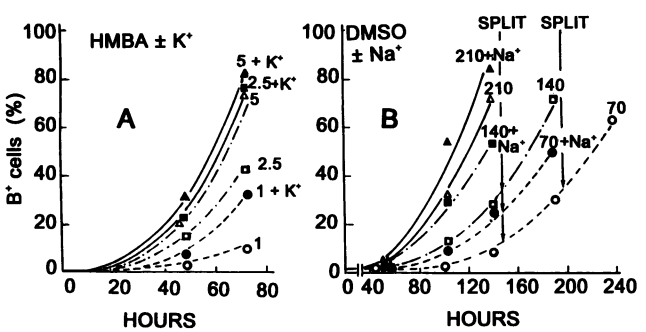

FIG. 3. Above a threshold concentration of inducers, DMSO and HMBA are interchangeable with cations for cell commitment. Cells were incubated for various times in the absence or in the presence of HMBA (*A*) or DMSO (*B*) at the indicated concentrations, in either standard RPMI 1640 medium or RPMI 1640 medium containing a 40 mM excess of KCl (*A*) or NaCl (*B*). ○, ●, 1 mM HMBA or 70 mM DMSO; □, ■, 2.5 mM HMBA or 140 mM DMSO; △, ▲, 5 mM HMBA or 210 mM DMSO. ○, □, △, standard RPMI 1640 medium; ●, ■, ▲, RPMI 1640 medium containing 40 mM excess of NaCl or KCl. Arrows indicate the times at which cultures were split 1:1 with fresh medium of the same composition. The lowering of the concentration of HMBA or DMSO below the optimum (5 mM and 210 mM, respectively) can be largely compensated by cation addition.

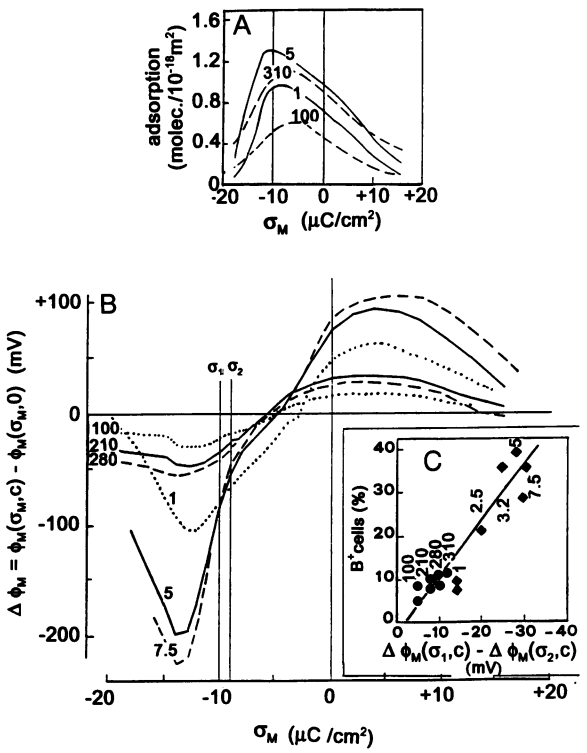

FIG. 4. (*A*) The interfacial adsorption of DMSO and HMBA as a function of the charge density and of inducer concentration. Measurements were done by dissolving the inducers at the indicated concentrations in 0.1 M KCl. ——, HMBA; – – –, DMSO. Numbers close to lines represent bulk concentration of the inducer (mM). (*B*) The surface potential shift ($\Delta\phi_M$) produced by DMSO and HMBA as a function of the electrode surface charge density. Lines represent, versus σ_M, the potential shifts produced by DMSO and HMBA at the indicated bulk concentrations (c), as compared with the corresponding potentials at $c = 0$—i.e., $\Delta\phi_M = \phi_M(\sigma_M, c) - \phi_M(\sigma_M, 0)$. · · ·, 100 mM DMSO or 1 mM HMBA; ——, 210 mM DMSO or 5 mM HMBA; – – –, 280 mM DMSO or 7.5 mM HMBA. (*C*) The difference in the potential shift produced by DMSO and HMBA at slightly different values of σ_M. For values of electrode charge density within the membrane physiological range, this difference is directly proportional to the differentiating activity of the inducers. The effects of inducers on the B^+ cell parameter was measured as reported in the legend to Fig. 1*A*, except that the inducers were simultaneously tested on the same cell preparation and the time of cell exposure was 48 hr for either DMSO or HMBA. Values in abscissa represent $\Delta\phi_M(\sigma_1, c) - \Delta\phi_M(\sigma_2, c)$, for $\sigma_1 = -10$, and $\sigma_2 = -9$ $\mu C/cm^2$, respectively. ●, DMSO; ◆, HMBA. The numbers next to the symbols represent the bulk concentrations of the inducer. The equation corresponding to the straight line is $B^+ = 3.04 - 1.296$ $[\Delta\phi_M(\sigma_1, c) - \Delta\phi_M(\sigma_2, c)]$; $r = 0.94$; $n = 14$.

altering the arrangement of water molecules and modifying the partial order that exists in the molecular monolayer adjacent to the surface even at zero surface charge density (29). This changes the polarization in this layer and shifts the surface potential (ϕ_M). In fact, as shown in Fig. 4*B*, both DMSO and HMBA shift ϕ_M, and the magnitude of this shift varied as a function of σ_M. This result indicates that the adsorption of inducers also changes the polarizability of the molecular layer in contact with the surface, altering the effective dielectric constant and the electric field produced by the surface charge. The whole shape of the curves representing the potential shift ($\Delta\phi_M$) versus σ_M is determined by the adsorption properties of the inducers, as well as by the interaction of their dipole moment with the electric field.

The following points should be stressed here: (*i*) qualitatively, the two inducers behave similarly, although HMBA is quantitatively much more effective than DMSO; (*ii*) both inducers give rise to a region in which $\Delta\phi_M$ is roughly

proportional to $\Delta\sigma_M$; and (*iii*) this region extends over the physiological range of the surface charge density (around -10 $\mu C/cm^2$). Adapting these data to the leukemia cells, one should recall that the inducers act in the cells after reaching the same concentration on both sides of the plasma membrane (2), expecting one of the two following possibilities: (*i*) $\sigma_{me} = \sigma_{mi} \simeq -10$ $\mu C/cm^2$, with a consequent potential shift equal on both sides ($\Delta\phi_i - \Delta\phi_e = 0$); and (*ii*) σ_{mi} and σ_{me}, although both around -10 $\mu C/cm^2$, differ in magnitude, producing a proportional $\Delta\phi_i - \Delta\phi_e$ and an almost equal shift of the transmembrane potential ϕ_m. Indeed (Fig. 4*C*), we found a good correlation between the effects of DMSO and HMBA on the B^+ cell parameter and the difference in $\Delta\phi_M$ produced by the inducers at slightly different σ_M values included within the physiological range (-8, -12 $\mu C/cm^2$) (Fig. 4*C*). To account for their synergy with extracellular cations, polar/apolar inducers should produce a $\Delta\phi$ with the same sign as that produced by cations—i.e., a negative shift of $\phi_i - \phi_e$ (see Fig. 2 *B* and *C*). This implies that σ_{mi} is greater in magnitude than σ_{me} by a quantity computable after dividing the slopes of the straight lines representing B^+ versus $\Delta\phi$ in Figs. 4*D* and 2*C*; this computation gives $\sigma_{mi} - \sigma_{me} = -0.3$ $\mu C/cm^2$.

In sum, the mechanism of action we propose for polar/apolar inducers is the following. The apolar portion of inducers ensures a certain degree of preferential adsorption at the membrane–solution interface. This adsorption produces a shift of the surface potential; the dependence of the surface potential upon the charge density is quite complex and is determined by the interplay of hydrophobic and dipolar electrical features. At the diffusion equilibrium of the inducer, even a slight difference between σ_{mi} and σ_{me} is determinant for the biological effects. When σ_{mi} is more negative than σ_{me}, a negative shift of $\phi_i - \phi_e$ ensues, changing ϕ_m an almost equal amount. This change is in the same direction as that caused by the addition of extracellular cations. We suppose that, as in other cases (30, 31), ϕ_m is sensed by a protein transduction mechanism, responsible for signaling cell commitment, which is located inside the plasma membrane and is responsive to alteration in ϕ_m of the order of 10 mV. Proteins of this sort have been identified (32, 33) and may also include GTP-binding proteins (34). Protein kinase C might be more or less directly implicated in this mechanism because activation of this kinase is associated with HMBA-inducing activity (35), whereas the surface potential contributes to this activation at the plasma membrane level (36). The voltage threshold for the signaling proteins can be approached by changes in $\phi_i - \phi_e$ produced by addition of extracellular cations; however, it can be superated only by potential shifts created either by the inducers at the optimum concentrations or by the synergic combination of lower doses of inducers with extracellular cation additions.

There are good *a priori* reasons for supposing that cell-surface potential may have a role in signal transduction (27, 37), particularly in cells that have relatively low resting membrane potentials, especially cancer cells (38), where even rather small changes in ϕ_m could cause a proportionally large change in the transmembrane electric field. Others have suggested that membrane surface potential changes of this sort may mediate some of the effects of cell–cell and cell–matrix adhesion (27, 39). Finally, there may also be a link with the striking phenomenon of gene expression regulated by the ionic composition of the extracellular medium (40). Any hypothesis of cell-surface-mediated determination of neoplastic transformation must now take into account the contribution of the cell-surface potential.

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