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EFFECT OF MEMBRANE POTENTIAL ON THE CELLULAR UPTAKE OF 2-*N*-METHYL-ELLIPTICINIUM BY L1210 CELLS

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Abstract—Some quaternary ammonium derivatives of ellipticine are active antitumor drugs on both experimental and human tumors. Because of their positive charge, the cellular uptake of these molecules is expected to be influenced by the electric membrane potential. Experimental variations of the potential were produced by changing the external potassium concentration and the potassium permeability by the addition of valinomycin. Using the fluorescent lipophilic cationic dye 3,3-dihexyloxacarbocyanine iodide, the L1210 cell membrane potential was estimated at −35 mV by flow cytometric analysis, and the same technique was then used to study the effects of the membrane potential variations on 2-*N*-methyl-ellipticinium (NME) cellular uptake. Our results show that indeed NME uptake depends on the cell membrane potential, which might then influence its pharmacological properties.

The DNA intercalating drug NMHE* has been recently introduced in cancer chemotherapy [1–3]. This is the first example of a quaternary ammonium derivative active as an antitumoral drug. Because of their positive charge, which is generally expected to influence their membrane permeability, the cellular uptake of such molecules can be expected to be affected by the membrane potential. In previous work [4], we studied the kinetics of uptake of NMHE and NME, a fluorescent analogue, by sensitive and resistant cells. Both cell types were found to concentrate these drugs equally by a process which does not involve an active transport. On the other hand, it is known that the cellular uptake of positively charged molecules may be influenced by the electric membrane potential. Indeed, lipophylic cationic dyes, such as the cyanine $DiOC_6(3)$, equilibrate across the cell membrane as a function of the membrane potential, and are used to measure this potential [5].

In eukaryotic cells, two factors exert a major contribution in the establishment of the membrane potential: (i) the ionic chemical potential gradient across the membrane, (ii) the ion permeabilities. Among them, potassium ion is frequently most important because K^+ permeability is usually higher than Na^+ permeability. Therefore, the membrane potential can be experimentally modified by changing the external potassium concentration, and the potassium permeability by the addition of valinomycin [6]. Using the fluorescent dye $DiOC_6(3)$, the resulting potential variations were determined by flow cytometric analysis.

The same technique was used in this work to evaluate the L1210 cells membrane potential and to study the effects of this potential on the cellular uptake of the fluorescent ellipticinium derivative, NME. Our results show that indeed the NME cellular uptake depends on the membrane potential, which therefore might influence its pharmacological activity.

MATERIALS AND METHODS

Cells and growth conditions. L1210 cells were maintained in RPMI 1640 with 10% fetal calf serum (Gibco, Grand Island, New York), 60 μM β-mercapto ethanol, 2 mM glutamine, 100 IU/ml penicillin and 50 μg/ml streptomycin. The suspension cultures were kept in exponential phase, and the cells had a doubling time of about 12 hr. The cells used in all experiments, except that corresponding to Fig. 3, had a viability of more than 95% as checked by trypan blue exclusion.

Drugs. NME, the structure of which is shown in Fig. 1, was a generous gift from Dr J. Chenu (Sanofi Recherche, Toulouse, France). The cyanine dye $DiOC_6(3)$ was purchased from Eastman and valinomycin from Sigma. All chemicals were of reagent grade and obtained from commercial sources.

Estimation of membrane potential variations. A cell suspension (3×10^5/ml in protein free RPMI 1640) was incubated either with $DiOC_6(3)$ (5 or 50 nM final concentration) or with NME (1 or 20 μg/ml final concentration), at 19° or 37° and for the indicated times. Among the possible ionophores, valinomycin has been shown to provoke the highest potential variations [5]. When used, the ionophore was added to the cell suspension at a final concentration of 6 μM, simultaneously with either $DiOC_6(3)$ or NME.

After incubation with NME at 20 μg/ml or $DiOC_6(3)$ at 50 mM, the samples were analysed with a 4800 cytofluorograf (Bio/Physics Systems) coupled to a 2100 Distribution Analyzer (Bio/Physics Systems). When the cells were treated with NME at 1 μg/ml or $DiOC_6(3)$ at 5 nM, the analysis required equipment with a higher sensitivity and was carried out on a FACS 440 (Becton–Dickinson). Control

* Abbreviations used: NME, 2-*N*-methyl-ellipticinium acetate; NMHE, 2-*N*-methyl-9-hydroxy-ellipticinium acetate; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; VAL, Valinomycin.

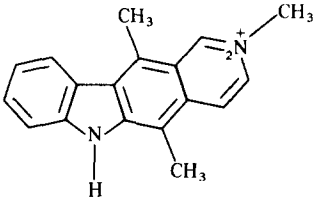

Fig. 1. NME Structure. Counter ion is acetate.

experiments with $DiOC_6(3)$ were performed together with the corresponding NME treatment and analysed on the same equipment. Apparatus settings (laser power, fluorescence detector gain and pulse height analyser display) were kept constant for all samples treated with the same drug in an experiment. After excitation at 448 nm from an argon ion laser source, the green fluorescence emitted by intracellular $DiOC_6(3)$ or NME were selected by a cut-off filter over 530 nm.

Determination of the cell-membrane potential of L1210 cells. As discussed below, when the external K^+ concentration varies in the presence of valinomycin, the membrane potential varies with the K^+ equilibrium potential.

Exponentially growing cells were centrifuged and resuspended in a solution containing glucose 1 g/l, $CaCl_2$ 1.4 mM, $MgSO_4$ 0.8 mM, and NaCl at a concentration such as to be equal to 150 mM when added to the KCl concentration. Various KCl concentrations, ranging from 4.5 to 145 mM, were added to this solution. After incubation for 15 min with $DiOC_6(3)$ 50 nM and valinomycin 6 μM, the cellular fluorescence was determined on the 4800A Cytofluorograf as described above.

RESULTS

Determination of the membrane potential by flow cytometric analysis on L1210 cells

The electric membrane potential results from both the unequal distribution of several ions across the membrane, and the different permeabilities of the membrane to these ions. If the permeability to one specific ion is made much greater than that of all the others, then the membrane potential reaches the equilibrium potential of this ion. Valinomycin, a small circular polypeptide, specifically chelates the potassium ion and then drastically increases the K^+ membrane permeability. Therefore, in the presence of valinomycin, the membrane potential (inside-outside, ΔE) is given by the following Nernst equation:

$$\Delta E = -\frac{RT}{F} \ln \frac{[K^+]_i}{[K^+]_o}$$

where $[K^+]_i$ and $[K^+]_o$ are respectively the inside and outside K^+ concentrations.

It has been shown [5] that the fluorescent dye $DiOC_6(3)$ can be used to measure the variations of the membrane potential by flow cytometric analysis. At the physiological potassium concentration (5.4 mM) valinomycin provokes a membrane hyperpolarization which results in an increased uptake of $DiOC_6(3)$. The insert to Fig. 2 shows that, in the presence of the antibiotic, the $DiOC_6(3)$ fluorescence distribution is shifted after 15 min from channel 28, in the control, to channel 69.

On the other hand, a minor peak of fluorescence appears at channel 10 in the presence of valinomycin. This peak indicates that a fraction of the cell population does not respond to the effect of the antibiotic. When the cell population is enriched in dead cells, this fraction correspondingly increases. Indeed (Fig. 3 shows a typical experiment), a direct correlation was observed between the level of this fraction and the percentage of Trypan blue-stained cells. We then conclude that this fraction is constituted of dead cells.

$DiOC_6(3)$ uptake was then determined at different external potassium concentrations, in the presence of valinomycin. As shown in Fig. 2, the fluorescence intensity decreases when the K^+ concentration is increasing. As provided by the Nernst equation, when $[K^+]_o$ tends to $[K^+]_i$, the membrane potential vanishes. The non-linear response observed here resembles that previously described for TPP^+ [7].

From the above results, it was possible to deduce the L1210 cells membrane potential. By flow cytometric analysis, it was found that the amount of $DiOC_6(3)$, retained by the cells in the absence of valinomycin (channel 28, insert to Fig. 2) is equal to

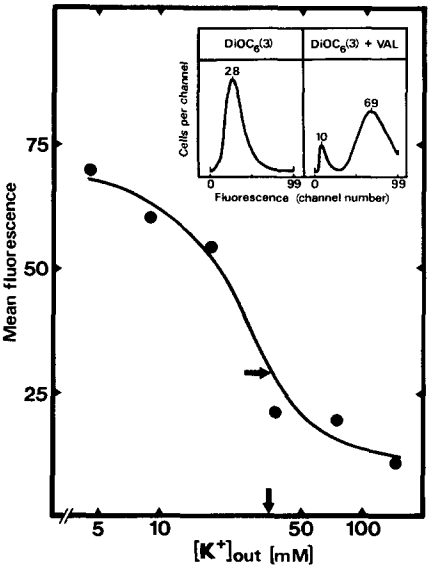

Fig. 2. Variations of the $DiOC_6(3)$ fluorescence as a function of the external KCl concentration. L1210 cells were incubated for 15 min in the presence of $DiOC_6(3)$ 50 nM, valinomycin 6 μM and increasing KCl concentrations. Cellular fluorescence was measured with the 4800 cytofluorograf. The maximum of the fluorescence peak was taken as the mean fluorescence. Insert: $DiOC_6(3)$ fluorescence in the presence or absence of valinomycin. Cells were incubated with $DiOC_6(3)$ 50 nM in protein-free RPMI 1640 (KCl 5.4 mM), with or without valinomycin 6 μM. The fluorescence of the cells in normal, valinomycin-free medium (channel 28) is indicated by the horizontal arrow and allows to define the corresponding external potassium concentration which gives the same fluorescence in the presence of valinomycin (vertical arrow).

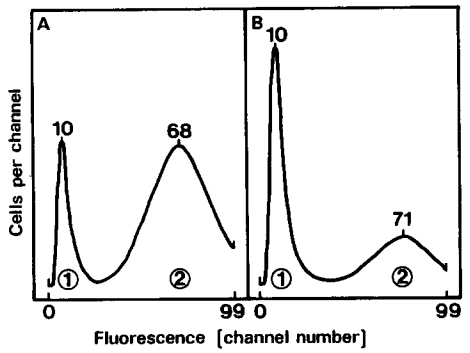

Fig. 3. Relationship between cell viability and $DiOC_6(3)$ fluorescence. L1210 cells either at the end of the exponential growth (A), or at late stationary phase (B) were incubated with $DiOC_6(3)$ 50 nM and valinomycin 6 μM in protein-free RPMI 1640. Cell fluorescence was determined on the 4800 cytofluorograf. In each case, peaks 1 and 2 areas were determined and the cell viability was estimated by trypan blue staining of the dead cells.

$$A: \frac{1}{1+2} \times 100 = 16;\ \%\ \text{stained cells } 18$$

$$B: \frac{1}{1+2} \times 100 = 47;\ \%\ \text{stained cells } 45.$$

the amount retained by cells treated with valinomycin in the presence of about 35 mM K^+ (Fig. 2). Thus the membrane potential is the same in both conditions. The membrane potential of the valinomycin-treated cells can then be computed from the Nernst equation provided that $[K^+]_i$ is known. When the fluorescence of valinomycin-treated cells is equal to that of the dead cells, the membrane potential is assumed to be zero, and $[K^+]_i$ is equal to $[K^+]_o$. The fluorescence intensity in dead cells corresponds to channel 10 (insert to Fig. 2, and Fig. 3) which corresponds on Fig. 2 to a $[K^+]_o$ of 145 mM. From these concentrations, we calculated an average membrane potential of about −35 mV, which is in the range of values reported for other eukaryotic cells [8–11].

Effect of membrane potential variations on the uptake of NME

NME cellular uptake was first measured as a function of the external concentration. Kinetics of NME uptake by the L1210 cells at 1 μg/ml and 20 μg/ml were determined (results not shown), and it was observed that after 2 hr incubation the drug uptake nearly reached the equilibrium. The amount of drug taken up by the cells in these conditions was determined by flow cytometric analysis. Figure 4 shows that the NME cellular uptake linearly increased with the external concentration up to about 10 μg/ml. At higher values, a second phase suggesting that the system was close to saturation was observed. This result is in agreement with previous experiments in which NME uptake by a Chinese hamster cell line was determined by high performance liquid chromatography [4]. In that case, NME uptake appeared as a saturable process. It was then necessary, for reasons discussed below, to study the effect of membrane potential at two external concentrations: one, 1 μg/ml, being in the linear part of the curve (Fig. 4); the other, 20 μg/ml. approaching the saturation of the system.

Figure 5 shows the effect of valinomycin on the uptake of $DiOC_6(3)$ (5 nM) and NME (1 μg/ml) at different times. At both 19° and 37°, the amount of $DiOC_6(3)$ taken up by the cell population in the control increased up to 1 hr of contact, and remained essentially at the same level after 2 hr. The addition of valinomycin provoked at both temperatures a shift of the fluorescence peaks towards higher values which was almost identical at different times.

In order to clearly position the different peaks on the fluorescence scale, it was necessary to use different settings of the fluorescence detector gain at 19° and 37°. As a result, Fig. 5 does not emphasize the difference of the amounts of $DiOC_6(3)$ or NME taken up by the cells at these temperatures. In fact, the amounts of $DiOC_6(3)$ and NME retained by the cells were respectively about 1.5- or 2-fold higher at 37° than at 19°. Our previous experiments on NME cellular uptake [4] are in agreement with this result.

At both temperatures, in the absence of valinomycin, NME uptake progressively increased with time (Fig. 5). This slow uptake was already observed on Chinese hamster cells [4]. At 19°, addition of valinomycin provoked an increment of the amount of drug retained by the cells which increased with time. At 37°, a comparable effect of valinomycin was observed. However, the fluorescence shift at 1 hr almost reached the same amplitude as that at 2 hr.

At 20 μg/ml, corresponding to the cell saturation, there was no detectable effect of valinomycin on the uptake of the drug (results not shown). This result will be discussed later.

These results show that, in non-saturating conditions (1 μg/ml), the NME uptake is influenced by the cell membrane potential similarly to the $DiOC_6(3)$, although at a slower rate of equilibration.

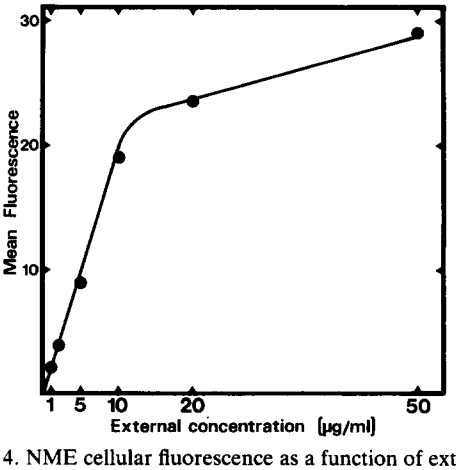

Fig. 4. NME cellular fluorescence as a function of external NME concentration. L1210 cells were incubated for 2 hr at 37° in protein-free RPMI 1640 in the presence of increasing NME concentrations. NME fluorescence was measured with the FACS 440. The maximum of the fluorescence peak was taken as the mean fluorescence.

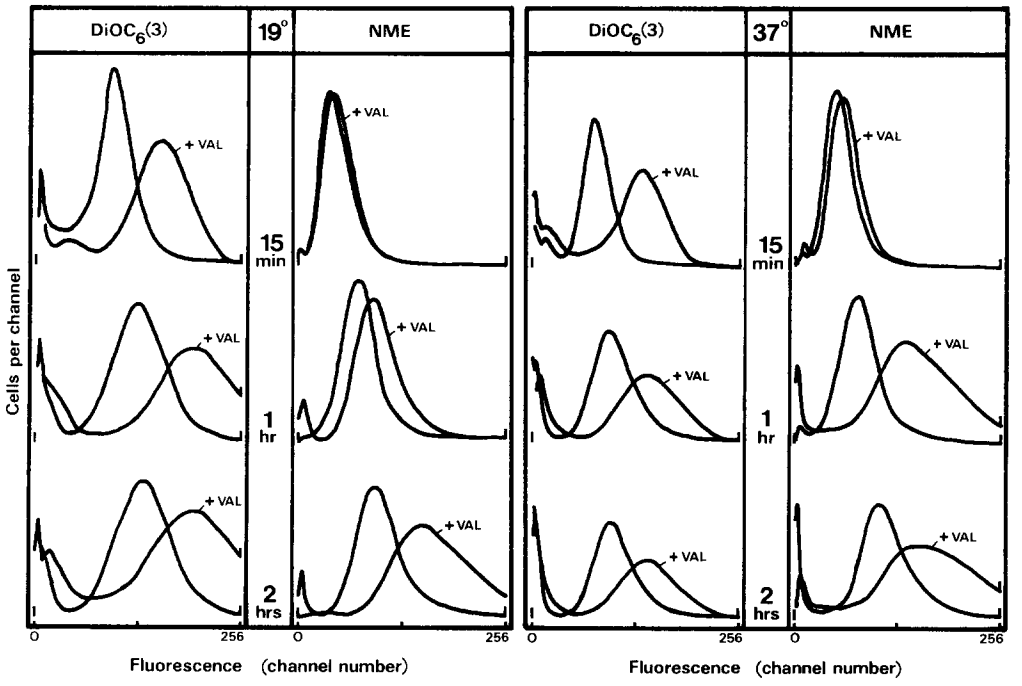

Fig. 5. Effect of valinomycin on $DiOC_6(3)$ and NME uptake as a function of time, at 19° and 37°. L1210 cells were incubated for the indicated times, either at 19° or 37°, with $DiOC_6(3)$ (5 nM) or NME (1 μg/ml) in the presence or absence of valinomycin. Cell fluorescence determination were carried out on the FACS 440.

Finally, it was verified that NME by itself had no effect on the uptake of $DiOC_6(3)$, in the presence or absence of valinomycin, thus demonstrating that it did not change the membrane potential of the treated or untreated cells.

DISCUSSION

The cellular uptake of most antitumor drugs appears to be critical for their activity against malignant cells, as demonstrated for example by studies on actinomycin D [12], daunomycin [13] or methotrexate [14]. When these drugs, or some analogues, are fluorescent, flow cytometric analysis might be a very appropriate method to study the drug uptake at the level of the individual cell. For example, flow cytometric studies of intracellular adriamycin in single cells have been reported [15]. However, among the possible limitations of the technique is the fact that the fluorescence of the drug could depend on the intracellular distribution and its eventual binding to some receptors.

It has been shown that the cationic fluorescent dye $DiOC_6(3)$ equilibrates across the plasma membrane according to the electric membrane potential, and can be used to estimate the variations of this potential by flow cytometry [5]. Our results show that the L1210 cells are well suited for this analysis because the distribution of the $DiOC_6(3)$ cellular fluorescence is quite narrow, indicating a relatively homogeneous distribution of cell membrane potential. The −35 mM value, thus obtained for L1210 cells in this work, is in the same range as that of other determinations on non-excitable eukaryotic cells, at the same temperature [16].

In valinomycin-treated cells, the $DiOC_6(3)$ fluorescence appeared to be bi-modal. The position of the low fluorescent peak was not affected by the variations of the external K^+ concentration in the presence of valinomycin (results not shown). This indicates that, in a fraction of the cell population, the ion gradient has collapsed. Flow cytometry determined that this cellular fraction was unresponsive to valinomycin-induced hyperpolarization. Should the cell capacity to maintain an ionic gradient be correlated to the cell viability, then a possibly early event associated with cell death might be easily detected by this technique. Such a technique would be specially useful in the study of pharmacological agents acting at the membrane level.

The effect of membrane potential on the total uptake of a cationic drug such as NME is expected to be influenced by the ability of this drug to bind to extracellular sites on the plasma membrane and to intracellular sites [17]. Indeed, the drug retained by the cell can be distributed in three different compartments: (i) The external binding to the plasma membrane. The variation of this fraction does not follow the Nernst equation. (ii) The free drug in the cell cytoplasm. This fraction depends directly on the membrane potential, according to the Nernst law (iii) The drug bound to intracellular sites, which is at equilibrium with the free cytoplasmic fraction. For this latter compartment, the dependence on the membrane potential is more complex.

Assuming that the drug binds to intracellular sites according to a simple equilibrium process, it could

be accounted for by the Scatchard equation:

$$\frac{r}{c} = K(n-r)$$

where r, n, c, K are the number of occupied sites per cell, the total number of intracellular sites per cell, the free intracellular drug concentration and the apparent binding constant respectively. When $r \ll n$, this equation reduces to:

$$r = Knc$$

meaning that, in this case, the amount of bound drug is directly proportional to the free concentration. Therefore, when the membrane potential varies, the amount of bound drug directly follows the variations of the free drug which itself depends on the membrane potential according to Nernst equation. When saturation is reached (r close to n), the concentration of the bound drug becomes almost independent of the concentration of the free drug c. If the concentration of the intracellular bound drug is much lower than the free drug concentration, the variations of total drug uptake remain apparently under the control of membrane potential. In contrast, when the concentration of the intracellular bound drug is much higher than the free drug concentration, then the variations of total drug uptake become almost independent of the membrane potential.

In experiments with NME at 1 μg/ml, the total drug uptake was dependent on membrane potential. At this concentration, it is clear that the intracellular sites are far from being saturated (Fig. 4). At 20 μg/ml, the NME uptake was found independent of the membrane potential. At this concentration, the intracellular binding sites are nearly saturated (Fig. 4). Besides, direct measurements of the total quantity of radioactive NME retained by the cells showed a 350-fold overconcentration of the drug inside the cells. This overconcentration, which did not result from an active transport [4], implies the presence of numerous intracellular binding sites.

Although the distribution of the drug in the various intracellular compartments might be much more complicated than this simple model, it is clear that our results are consistent with such model. Some subcellular organelles are also known to exhibit transmembrane potentials which in turn may influence the drug distribution. Indeed, mitochondria [18] and lysosomes [19] have been shown to concentrate cationic dyes as a consequence of this potential. An eventual role of these organelles in NME intracellular concentration remains to be studied.

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