Investigation of the relationship between altered intracellular pH and multidrug resistance in mammalian cells

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Summary The intracellular pH of a number of multidrug resistant cell lines was compared with that of their parental lines using the fluorescent probe bis-carboxyethylcarboxyfluorescein. In four different cases, cells having 5-fold resistance or more exhibited an intracellular pH which was 0.10–0.17 units higher than that of the parental cell line. A CHO cell line, AB5, and its 180-fold resistant counterpart, CHC5, were further investigated with regard to the role of Na⁺/H⁺ antiport. The Na⁺/H⁺ antiport activity was greater at any intracellular pH for the CHC5 cells than the AB5 cells. To investigate the possible role of higher intracellular pH in multidrug resistance, the effect of several agents which are either known to reverse multidrug resistance or inhibit Na⁺/H⁺ antiport activity were examined. Verapamil was found to reverse multidrug resistance but had no effect on intracellular pH while amiloride, which acidifies the cytosol by blocking Na⁺/H⁺ antiport activity, did not cause reversal of drug resistance. In contrast to verapamil, treatment of CHC5 cells with cyclosporin A had a parallel effect on reversal of their drug resistant phenotype and a lowering of their intracellular pH to that of the sensitive cell level. However, cyclosporin was ineffective in either lowering the intracellular pH or reversing drug resistance in DC3F/ADX cells. Therefore, except for the effect of cyclosporin A on the CHC5 line, the effects of other agents on reversal of multidrug resistance and intracellular pH did not correlate with each other.

The development of multidrug resistance (MDR) is a major problem in cancer chemotherapy and could be one of the main reasons for treatment failure. Several differences between drug-sensitive and drug-resistant cell lines have been advanced to account for the phenomenon of multidrug resistance (Gerlach et al., 1986; Bradley, et al., 1988). A higher drug efflux and hence a lower drug accumulation in the resistant cells as compared to the sensitive cells is generally considered an important underlying cause of this resistance (Dano, 1973; Inaba et al., 1987). In order to study the biochemical basis for the phenomenon of multidrug resistance, we have focused on the regulation of the intracellular pH of several drug-sensitive and drug-resistant cell lines. Intracellular pH (pHᵢ) is higher in a drug-resistant human breast cancer cell line (Lyon et al., 1988) and was recently shown to increase in multidrug resistant cell lines derived from a human lung tumour (Keizer & Joenje, 1989). In the present manuscript, we have also observed this phenomenon in a number of different multidrug resistant cell lines. Further, to explore the relevance of this change in pHᵢ to the phenomenon of multidrug resistance, the effect of several agents which are known to cause reversal of MDR (verapamil, cyclosporin A) or to inhibit Na⁺/H⁺ antiport activity (amiloride) have been examined. Our results show that the effect of various agents on the reversal of MDR did not correlate well with changes in pHᵢ.

Materials and methods

Materials

Cyclosporin A was generously provided by Sandoz Pharmaceuticals Corp. Cyclosporin was dissolved in DMSO and diluted into aqueous media. The final concentration of DMSO was below 1%. Appropriate controls demonstrated that this vehicle did not affect the assays at the concentrations used. Vinblastine sulphate was from Aldrich Chemical Co. (Milwaukee, WI, USA). The fluorescent pH probe 2′,7′-bis-(2-carboxyethyl)-5-(and -6) carboxyfluorescein (BCECF) was purchased as its membrane-permeant acetoxymethyl ester from Molecular Probes Inc. (Eugene, OR, USA). Other biochemicals were from Sigma Chemical Co. (St Louis, MO, USA).

Cell lines and culture conditions

The origins of the Chinese hamster ovary (CHO), Chinese hamster lung (CHL) and HeLa cell lines have been described earlier (Bech-Hansen et al., 1976; Biedler & Riehm, 1970; Gupta, 1983 Gupta et al., 1988; Akiyama et al., 1985). The cells were grown in α-MEM medium supplemented with 7% fetal bovine serum at 37°C in a humidified incubator in an atmosphere of 95% air and 5% CO₂. The drug resistant phenotypes of most of the cell lines employed, except DC3F/ADX, do not show significant change upon growth in non-selective medium for 3–4 weeks, and hence these were routinely grown in the absence of any selective drug. The DC3F/ADX line, which shows partial reversion under these conditions, was routinely maintained in the presence of 10 µg ml⁻¹ of actinomycin D, and transferred to non-selective medium 3 days before any tests were performed.

Measurement of intracellular pH

Intracellular pH was measured with the pH-sensitive, intracellularly trapped fluorescent dye bis-carboxyethylcarboxyfluorescein (BCECF) (Rink et al., 1982). Cells were loaded with 1 µM acetoxy-methyl ester of BCECF for 20 min at 37°C, sedimented and resuspended in HCO₃⁻-free glucose saline solution (130 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.5 mM CaCl₂, 10 mM glucose) adjusted at different pHs with the following buffers: 20 mM Pipes (pH 6.1–6.9), Hepes (pH 7.0–7.5), Tricine (pH 7.6–8.2). After an incubation period of 30 min at 37°C, aliquots of 2 × 10⁵ cells were added to a cuvette containing Na⁺ buffer (10 mM glucose, 1 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 140 mM NaCl and 20 mM Hepes pH 7.3). Fluorescence is measured under continuous magnetic stirring and in a thermostated chamber, at 37°C, of a Perkin Elmer MFP 44 fluorescence spectrophotometer with excitation at 500 nm and emission at 525 nm, using 5 and 10 nm slits, respectively. Calibration of pH, versus fluorescence intensity was done by resuspending the cells in K⁺ buffer (similar to Na⁺ buffer with isoosmotic replacement of KCl for NaCl) and 2 µM nigericin. The extracellular pH, which under these conditions represents intracellular pH as well, is varied in steps while recording the fluorescence intensity (Thomas et al., 1979). Alternatively, the dye was released
using 0.1% Triton X-100 and the pH of the medium was changed stepwise by addition of small volumes (2 µl) of concentrated acid (1 M Mes) or base (1 M Tris) (Gristein et al., 1984). Both methods gave similar results and a linear relationship between fluorescence intensity and pH was observed in the range of 6.3–7.6.

**Measurement of Na⁺/H⁺ antiporter**

A suspension of 3 × 10⁵ cells, which had been loaded with BCECF/AM, was added to a cuvette containing a N-methylglucamine chloride solution (same as Na⁺ buffer with the iso-osmotic replacement of NaCl for N-methylglucamine chloride. Excitation and emission wavelengths were 500 and 525 nm, respectively. The K⁺/H⁺ ionophore, nigericin (2 µM), was added to the cells, and the acidification was terminated by removal of the ionophore with fatty acid-free bovine serum albumin as previously described (Gristein, 1988). The kinetics of acidification was not analysed. The desired pH was attained within two minutes after the addition of nigericin. Both the AB₂ and the CH₁C₅ cell lines could be acidified to pH 6.6 by this method. The amiloride-sensitive Na⁺/H⁺ exchange can be monitored fluorometrically by measuring the rate of recovery of pH, following the addition of 50 mM NaCl. The activity of the antiport was quantified from the calibrated fluorescence recording as the initial rate of Na⁺-induced change of pH, (in pH units min⁻¹). This assay is done in the absence of HCO₃⁻ and therefore does not measure HCO₃⁻/Cl⁻ exchange, which may occur in cell culture.

**Drug sensitivity test**

The effect of various agents on the reversal of the drug resistance was examined by determining the cloning efficiencies of the parental and resistant cell lines in the presence of different concentrations of either vinblastine or colchicine, in the absence and presence of the reversing drugs. In these experiments, which were generally carried out in 24-well tissue culture dishes, 0.5 ml of various dilutions of vinblastine (made at two times the final concentrations in growth medium) were added to duplicate wells of 24-well dishes. Generally, 12 different dilutions of the drug in addition to a control without any drug, were employed. The single cell suspensions of the cell lines were suitably diluted (based on cell count measurement done by Coulter counter), and 0.4 ml of these containing either 100 or 250 cells were added to the wells of 24-well dishes containing the drug dilutions. Different compounds, whose effect on drug reversal was examined, were then added to the wells in 0.1 ml of the growth medium. The experiments were carried out in parallel with and without the reversing agents. The stock solutions (10 mM) of verapamil and amiloride HCl were prepared in H₂O, while cyclosporin A (5 mM) was dissolved in DMSO. Before use, the stocks were diluted into the growth medium to give the desired final concentrations. The control dishes (i.e. without reversing agent) received an equivalent amount of the appropriately diluted solvent. At the concentrations employed, the various reversing agents do not show any significant toxicity towards the cell lines. The dishes were incubated for 6–8 days at 37°C in a 5% CO₂/95% air incubator. Subsequently, the dishes were stained for about 30 min with 0.5% methylene blue in 50% methanol and the number of colonies in each well was scored. From the average numbers of colonies observed in the presence of different drug concentrations, the D₉₀ values (i.e. drug concentrations which reduced cloning efficiency to approximately 10% of that in the absence of any drug) of different cell lines in the absence and presence of various reversing agents were determined. The degree of resistance of any cell line was determined from the ratio of D₉₀ values for the mutant versus parental cell lines. The sensitising effect of reversing agents was calculated from the ratios of D₉₀ values observed in the absence and presence of reversing drug(s).

Results

We examined the pH of several multidrug resistant cell lines. Highly resistant CH₁C₅ and DC3F/ADX cells maintained a pH that was about 0.15 ± 0.03 pH units above that of the parental cell line. Cells with a lower degree of resistance showed less difference in pH, compared to their drug-sensitive counterparts (Table 1). The higher values of pH for the CH₁C₅ resistant cell lines were observed regardless of extracellular pH (pH₆) (Figure 1).

Since a Na⁺/H⁺ exchange system could be involved in the control of pH, in these cell lines, we studied the ability of AB₂ and CH₁C₅ cells to recover from an intracellular acid load after incubation with nigericin (Figure 2). The cytoplasmic alkalinisation was completely inhibited by 100 µM amiloride, indicating that a Na⁺/H⁺ exchange system is active and does play an important role in controlling the pH in this cell line (Figure 2). Neither cyclosporin A nor verapamil had any effect on the rate of pH recovery after acid loading. The rate of recovery upon addition of NaCl was higher in the resistant than in the sensitive cells (Figure 3).

A number of drugs have been shown to sensitize multidrug resistant cells to cytotoxic agents. We measured the effects of several of these drugs on pH and on the sensitivity of cells to the cytotoxic effect of vinblastine. We also tested the effects of amiloride, a known inhibitor of Na⁺/H⁺ antiport, on the reversal of multidrug resistance. This was done with parental and drug resistant CHO and CHL cells. As seen from Table II, treatment of either AB₂ or DC3F cells with either 5–20 µM verapamil or 3 µM cyclosporin A sensitises them by a factor of up to about 10-fold towards vinblastine. This sensitisation, as shown recently (Gupta, 1988), is due to the fact that Chinese hamster cells display an intrinsic MDR phenotype, in comparison to human cells, which are reversed by these agents. Verapamil at the above concentrations also caused a dose-dependent reversal of vinblastine resistance in the two mutant cell lines. At the higher concentration, the cells became nearly as sensitive as the parental line in the presence of verapamil. However, in contrast to verapamil, cyclosporin A was effective in sensitising only the CH₁C₅

| Table I | pH of MDR cells |
|---------|----------------|
| **Cell line** | Reference | **Relative drug resistance** | **pH₉₀** |
| CH₁C₅ | Bech-Hansen et al. (1976) | CHO | Colchicine | 180 | 7.01 ± 0.03 (9) | 7.18 ± 0.03 (9) |
| DC3F/ADX | Biedler & Rehm (1970) | CHL | Actinomycin D | 2,500 | 7.02 ± 0.02 (6) | 7.16 ± 0.03 (6) |
| HeLa Pur⁺/⁴⁻ | Gupta et al. (1983) | Human | Puromycin | 50 | 6.95 ± 0.02 (3) | 7.05 ± 0.02 (3) |
| Tax⁺/⁻ | Gupta (1983) | CHO | Taxol | 8 | 7.03 ± 0.02 (2) | 7.07 ± 0.03 (2) |
| KB-Cl | Akiyama et al. (1985) | Human | Colchicine | 260 | 6.96 ± 0.02 (2) | 7.08 ± 0.02 (2) |

*Intracellular pH was measured with the fluorescent probe BCECF as indicated in Materials and methods. Values are the means ± s.e.m. of several experiments (indicated in parentheses).*
Figure 1 pH dependence on pH in CHO cells. pH as a function of pH in a drug-sensitive cell line, AB (O) and a drug-resistant cell line, CH5CS (●). The cells were pre-equilibrated in HCO3−-free media for 60 min at the indicated pH. Then the cells were loaded with BCECF/AM and the pH was measured as indicated in Materials and methods. Each point is the mean of triplicate determinations. Error bars represent s.d.

Figure 2 Measurement of Na+/H+ antiport activity. Cells were acidified with the addition of nigericin. Acidification was terminated with the addition of fatty acid-free albumin. Na+/H+ antiport activity was initiated with the addition of 50 mM NaCl (see Methods). Amiloride (200 μM) completely blocked the increase in pH, while addition of either cyclosporin A (20 μM) or verapamil (40 μM) with the NaCl had no effect on the recovery from acidification.

Discussion

We have shown that the pH of a number of different multidrug-resistant cell lines is higher than their parental counter-

Table II Effect of different agents on the relative drug resistance of various cell lines

| Compounds      | AB | CH5CS | DC3F | DC3F/ADX |
|----------------|----|-------|------|----------|
| Control (no addition) | 1.0 | 50.0  | 1.0  | 3000     |
| + Cyclosporin A (3 μM) | 0.1 (10) | 0.15 (330) | 0.1 (10) | 3000 (1) |
| + Verapamil (4 μM) | 0.35 (2.9) | 1.2 (42) | 0.30 (33) | 5.5 (545) |
| + Amiloride (200 μM) | 0.1 (10) | 0.4 (125) | 0.1 (10) | 0.5 (6000) |

*The experiments were done as described in Materials and methods. Assuming the D50 value of vinblastine for the parental sensitive cell lines (AB; 5 nM; DC3F; 3.5 nM) in the absence of any reversing agents to be 1, the relative resistance of the cell lines under different conditions are indicated. The numbers in parentheses show the fold sensitisation of the cell lines (as compared to the control lacking any sensitising drug) in the presence of indicated concentrations of the reversing agents. A fold sensitisation of 1 indicates no change in sensitisation. Similar results with these cell lines and agents have been obtained in at least two independent experiments.

Table III Effect of drugs on pHi and MDR

| Modifier                | pHi | pHi | Reversal MDR           |
|------------------------|-----|-----|------------------------|
| Chinese hamster ovary  |     |     |                        |
| cells                  |     |     |                        |
| AB                    | 7.01 ± 0.03 | 7.18 ± 0.03 | Complete reversal         |
| Cs A (20 μM)           | 6.98 ± 0.03 | 7.00 ± 0.02 | Complete reversal         |
| Verapamil (40 μM)      | 6.97 ± 0.02 | 7.15 ± 0.03 | Complete reversal         |
| Amiloride (200 μM)     | 6.95 ± 0.01 | 7.06 ± 0.02 | No effect                |
| Chinese hamster lung   |     |     |                        |
| cells                  |     |     |                        |
| DC3F                   | 7.02 ± 0.02 | 7.16 ± 0.03 | Complete reversal         |
| Cs A (20 μM)           | 6.97 ± 0.02 | 7.12 ± 0.02 | Complete reversal         |
| Verapamil (40 μM)      | 7.00 ± 0.01 | 7.13 ± 0.02 | Complete reversal         |
| Amiloride (200 μM)     | 6.93 ± 0.02 | 7.08 ± 0.03 | No effect                |

Intracellular pH was measured with the fluorescent probe BCECF. Reversal of MDR indicates the ability of the modifier to sensitize the cell line to the cytotoxic action of vinblastine. Values are the mean ± s.e.m. of triplicate determinations. Cs A is cyclosporin A.
part. The magnitude of the difference between the resistant and sensitive cell lines is related to the degree of resistance, with the most resistant cell lines showing the greatest alkalinisation of intracellular pH (Table I). However, there is no direct proportionality between the degree of resistance and pH, which is in contrast to a previous report which showed a linear relationship between resistance and pH, for a series of increasingly multidrug resistant variants of a human lung tumour cell line (Keizer & Joens, 1989). The lack of quantitative correlation between drug resistance and pH for different cell lines does not rule out a role for pH in resistance since there may be many differences among the different cell lines. However, as we will show below, there are a number of lines of evidence to demonstrate the lack of a consistent correlation between intracellular pH and multidrug resistance.

The observed increased activity of the Na+/H+ antiporter in one of the resistant cell lines (Fig. 3) is consistent with the hypothesis that this antiport mechanism is responsible for the higher pH found in resistant cells. However, blockade of this activity by amiloride does not reverse multidrug resistance (Table III). Of course the lack of effect of amiloride in the clonogenic assay is negative evidence and therefore not conclusive. It could be due, for example, to the metabolic instability of amiloride in the cell cultures used. In addition, however, cyclosporin A and verapamil, which reverse multidrug resistance, have no effect on Na+/H+ antiport activity. Therefore, the higher antiport activity observed in the CH8C5 drug resistant cells does not appear to be closely associated with the mechanism of their resistance. There are a number of possible causes for the increased Na+/H+ antiport activity in resistant cells, including increased expression of the antiporter, alteration in the pH dependence of antiporter activity or changes in the regulation of antiporter activity. The Na+/H+ exchange activity is activated by protein kinase C (Siffert & Akkerman, 1988). Protein kinase C activity is higher in several but not all multidrug resistant cell lines (Palayoor et al., 1987; Fine et al., 1988). It is possible that the alkalinisation of multidrug resistant cell lines is an indirect manifestation of a higher protein kinase C activity. It is also possible that the increased pH of multidrug resistant cells is not a result of changes in Na+/H+ antiport activity but rather to differences in metabolic activities between parental and resistant cell lines (Lyon et al., 1988). Further studies are required to determine the generality of the changes in Na+/H+ antiport activity with multidrug resistance and to determine the cause of such changes. However, the changes in Na+/H+ antiport activity appear independent of the mechanism of drug resistance and their contribution to the higher pH of resistant cells remains to be determined.

Although higher pH appears to be a general characteristic of all MDR cell lines examined, its relevance to the MDR phenotype is at present unclear. Amiloride acidifies the pH in both sensitive and resistant cell lines, but it does not cause any reversal of MDR. The pH difference between the parental and the resistant cell lines is maintained (although some what reduced in the case of CHO cells) even in the presence of amiloride (Table III), suggesting that Na+/H+ antiport may be less important for the maintenance of a higher pH in the resistant cells. Further, if the higher pH in the resistant cells was related to their MDR phenotype, then treatment with agents which cause reversal of the MDR phenotype should abolish the pH difference between sensitive and resistant cell line. However, such a correlation was not observed for verapamil, which caused complete reversal of vinblastine resistance in the two sets of cell lines without changing their pH. In the study of Keizer and Joens (1989) verapamil did lower the pH of resistant cells at concentrations greater than 4 μM. The cell lines used in that work had particularly high pH values for their degree of resistance and they showed greater acidification by verapamil than the resistant clones used in the present work. The origin of these differences is not known but it is clear that acidification of resistant cells is not a general property of verapamil. Furthermore, both the Na+/H+ ionophore, monensin, which would increase pH, and the K+/H+ ionophore, nigericin, which would decrease pH, increase drug accumulation in resistant cells (Sehested et al., 1988). This is another indication that there is no correlation between pH, and cell resistance. In contrast to verapamil, interesting results were obtained with cyclosporin A. It was observed that the concentrations of cyclosporin which completely reversed vinblastine resistance in CH8C5 cells had no observable effect on the DC3F/ADX cells (although it sensitised the parental DC3F cells by a factor of about 10). To our knowledge, this is the first report where such marked specificity (or differences) towards a reversing agent has been observed between two MDR cell lines. The observed difference between CH8C5 and DC3F/ADX cell in their response to cyclosporin A points to some important difference in the mechanisms leading to MDR phenotype in the two cell lines. Interestingly, and in contrast to verapamil, the reversal of drug resistance in CH8C5 by cyclosporin A was accompanied by a lowering of pH, to the same level as the sensitive AB, cells. However, cyclosporin A had no effect on the pH of the DC3F/ADX cells. It thus appears that the reversal of multidrug resistance by cyclosporin A is closely associated with a process which causes a lowering of pH. This process is not the inhibition of Na+/H+ antiport since we have shown that cyclosporin has no effect on this mechanism. However, the lowering of pH by cyclosporin is a collateral event, rather than being the mechanism of reversal of drug resistance by this agent. Further investigation of the mechanism by which cyclosporin A causes reversal of the MDR phenotype and affects pH, and the manner in which the two MDR cell lines examined differ should be of considerable interest.

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