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CIRCUMVENTION OF MULTIDRUG-RESISTANCE IN P388 CELLS IS ASSOCIATED WITH A RISE IN THE CELLULAR CONTENT OF PHOSPHATIDYLCHOLINE

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Abstract—In fura-2 stained drug-sensitive and multidrug-resistant P388 cells, 50 mM KCl failed to provoke an increase in the fluorescent signal, indicating that potential-dependent Ca^{2+} channels are not present in either cell line. Therefore the circumvention of drug-resistance by verapamil must be related to some other mechanism. In the present study, verapamil and two other circumventors of drug-resistance, tamoxifen and dipyridamole were found to induce an increase in the synthesis of phosphatidylcholine in multidrug-resistant but not in drug-sensitive cells. The relative resistance of multidrug resistant cells to permeabilization by digitonin indicates that the organization of the plasma membrane lipids in these cells must be different from the one occurring in drug-sensitive cells. Extended exposure of multidrug-resistant cells to verapamil negates the resistance to digitonin. This effect of verapamil reflects its ability to modify the lipid organization of the plasma membrane of multidrug-resistant cells. It is suggested that if the lipid composition of the cell membrane is altered by these drugs as was found for whole cells, the change could explain the increase in drug permeability.

The first reports on restoration of sensitivity to vinca alkaloids and Adriamycin®§ in MDR¶ cells, by verapamil, came from Tsuruo's laboratory [1, 2]. The rationale for these studies was based on experiments suggesting that MDR was the result of increased drug efflux, and on reports that verapamil, a well known blocker of slow membrane Ca^{2+} channels, inhibited the secretion of various hormones. Following these studies many other compounds were reported as potent circumventors of MDR [3]. However, the relationship between this activity and the ability to block potential dependent Ca^{2+} channels emerged as dubious. We have shown that restoration of drug-sensitivity in MDR cells by another potential dependent Ca^{2+} channel blocker, perhexiline, was not affected by manipulating the medium's Ca^{2+} concentration and that La^{3+}, a blocker of Ca^{2+} channels, did not restore drug-sensitivity in these cells [4]. Similar findings were recently reported for verapamil [5]. In a further study we demonstrated for a series of compounds, that there was no correlation between their ability to inhibit Ca^{2+} uptake by synaptic vesicles and their potency in restoring the sensitivity of MDR cells to Adriamycin [6]. Although verapamil and other potential dependent Ca^{2+} channel blockers failed to affect Ca^{2+} pools [7], accumulation [8] or entry [9, 10] in MDR cells, there are conflicting reports on the presence of potential dependent Ca^{2+} channels in such cells as determined by the indirect method of nitrendipine binding. While Fine *et al.* found no specific nitrendipine binding in Adriamycin-resistant Chinese hamster ovary cells [11], other investigators detected such binding in Adriamycin-resistant, but not in drug-sensitive, Friend leukemia cells [12]. In the present study we will describe our efforts to clarify the status of functional potential-dependent Ca^{2+} channels in drug-sensitive and MDR P388 cells.

Some other mechanisms were proposed for the reversal of MDR. Cornwell *et al.* found that verapamil inhibited the binding of vinblastine to membrane vesicles isolated from MDR KB cells [13, 14]. These membranes were shown to contain a large quantity of a specific 170 kDa glycoprotein, known now as P-glycoprotein. It was also demonstrated that verapamil blocked the photoaffinity labeling of this glycoprotein by a vinblastine analog [14]. On the basis of these findings the P-glycoprotein was suggested as the drug efflux pump operating in MDR cells. It was further proposed that verapamil reversed drug-resistance by blocking the activity of the P-glycoprotein. However, in a recent study, human MDR cells, that lack P-glycoprotein were sensitized to vincristine by verapamil as cells that do contain this membrane component [15].

Verapamil was also reported to enhance the cytotoxicity of immunotoxins and conjugates of epidermal growth factor with *Pseudomonas* sp. exotoxin toward certain human cancer cell lines [16, 17]. The authors indicated that the enhancement did not involve Ca^{2+} channels and suggested that it may have resulted from an increase in the cell membrane permeability. We have recently

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§ Adriamycin is a registered trademark of Farmitalia Carlo Erba.

¶ Abbreviations: PBS, phosphate buffered saline; RPMI 1640, Roswell Park Memorial Institute 1640 medium; MDR, multidrug-resistance; CHOL, cholesterol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; CL, cardiolipin; PS, phosphatidylserine; PI, phosphatidylinositol; HP-TLC, high performance thin layer chromatography; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration.

demonstrated that in verapamil pretreated MDR P388 cells the rate of Adriamycin passive entry was increased 5-fold compared to untreated cells [18]. It seems therefore not unlikely, that verapamil also increases the membrane permeability of MDR cells to Adriamycin. Another compound, shown to overcome MDR is cepharanthine [19]. Like verapamil, it also increased the cytotoxicity of the conjugate of epidermal growth factor with *Pseudomonas* sp. toxin toward HeLa cells [20]. The authors suggested that cepharanthine perturbed the cell membrane function by binding to phosphatidylserine. Many other membrane functions were reported to be perturbed by verapamil and its analogs in a Ca^{2+} independent manner [21, 22]. Interactions of verapamil with liposomes prepared from soybean, brain or pure phosphatidylcholine were also reported [23, 24]. In addition, the liposomes were found to bind a considerable amount of verapamil [23]. These findings suggested that verapamil may modify membrane permeability to a variety of compounds by affecting the cell membrane lipid structure. Although a direct effect of verapamil on the fluidity of phosphatidylcholine liposomes was not observed [23], it was still possible that verapamil may affect the cell lipid metabolism and consequently its membrane lipid composition. We have therefore studied whether exposure of drug-sensitive and -resistant P388 cells to verapamil does influence their lipid composition. As tamoxifen and dipyridamole also restore the sensitivity of MDR P388 cells to Adriamycin [6, 25], their ability to modify the lipid composition in these cells was also examined.

MATERIALS AND METHODS

Cell culture. ADR-sensitive and -resistant murine leukemia P388 cells were grown in culture as previously described [26]. Briefly, an inoculum of cells was transferred to fresh medium once every 4 days to maintain them in exponential growth. The growth medium was RPMI 1640 supplemented with 10% fetal calf serum, 10 μM 2-mercaptoethanol, 50 units/mL penicillin base and 50 μg/mL streptomycin, all purchased from Biological Industries (Beth Haemek, Israel).

Studies with FURA-2 stained cells. Cells were loaded with FURA-2 by incubation with the parent acetoxymethyl ester FURA-2/AM (Molecular Probes, Eugene, OR). Typically 8×10^7 cells were washed twice in solution A, composed of (mM) 120 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 Na-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and 5 glucose (pH 7.4). Then the cell suspension, in a volume of 1 mL, was incubated with FURA-2/AM (1 μM) for 60 min at 37°. FURA-2/AM diffuses across the cell membrane and is hydrolysed by esterases present in the cytoplasm, releasing the poorly permeant FURA-2. The fluorescence intensity of this dye depends on the concentration of free Ca^{2+} [27]. The extracellular dye was removed by washing the cells twice, first in solution A containing 1% bovine serum albumin (Calbiochem, Lucerne, Switzerland) and then in solution A without albumin. The cells were then resuspended in solution A at a density of 3×10^6–5×10^6 cells/mL. Continuous monitoring of FURA-2 fluorescence was carried out in a SPEX Flurolog spectrofluorometer (Spex Industries, Edison, NJ), equipped with a digital plotter and a microprocessor (DM1B). Excitation and emission wavelengths were set at 340 and 510 nm, respectively. A 434 nm cut-off long pass filter was used across the emission path to reduce scattered light. Chromaffin cells were isolated from bovine adrenal medulla glands and maintained in primary culture essentially as reported [28].

Evaluation of permeabilization by digitonin. Drug-sensitive and MDR P388 cells were grown in the absence or presence of 10 μM verapamil. After 4 days of culture, the cells were washed twice with solution A and then resuspended in solution A at a density of 6×10^6 viable cells/mL. After 10 min of preincubation at 37°, digitonin (E. Merck, Darmstadt, F.R.G.) was added to a final concentration of 5, 20 or 25 μM. After incubation of 2 min at 37°, viability was elevated by trypan blue staining.

Lipid extraction, separation and analysis. Extraction was carried out as described by Bligh and Dyer [29]. PBS washed cells were suspended in 1 mL of water and sonicated on ice for 3 min at 80 W in an Ultrasonics type 7 cell disruptor (Heat Systems, Plainview, NY). Four millilitres of chloroform : methanol 1:2 were added to the cell homogenate. After 1 hr incubation in a shaking bath at 37° 1.4 mL of water and 1.33 mL of chloroform were added and a phase separation was obtained by centrifugation for 5 min at 900 *g*. The upper phase was discarded and the lower phase was washed twice with chloroform : methanol : water 1:50:49. The lower phase was evaporated under a nitrogen stream and the lipid residue was stored at −20° until the lipids were separated and analysed. Immediately prior to lipid separation the lipid residue was dissolved in chloroform : methanol 1:1.

Lipid separation was carried out as described by Macala *et al.* [30] with minor modifications. Neutral and acidic lipids were separated on a Fractogel silica column (170–325 Mesh ASTM, E. Merck). The silica was washed four times in chloroform : methanol : Na-acetate 0.8 M, 30:60:8 and then with chloroform : methanol 1:1. The lipid extract was mounted on the column and the neutral lipids were eluted by washing the column with 4 mL chloroform : methanol 1:1. This procedure recovered >95% of free cholesterol (CHOL), phosphatidylethanolamine (PE), phosphatidylcholine (PC) and sphingomyelin (SM). After eluting the neutral lipids, the column was washed with 1 mL chloroform and then 4 mL chloroform : methanol : Na-acetate 3 M, 50:50:8. This wash changed the column charge and allowed the elution of cardiolipin (CL), phosphatidylserine (PS) and phosphatidylinositol (PI). This eluant was washed first with 1.37 mL of 0.9% NaCl in water, then 4 mL of chloroform : methanol : NaCl 0.9% in water 1:50:49 and twice with 4 mL chloroform : methanol : water 1:50:49. The eluants were evaporated to dryness under nitrogen stream and the residues stored at −20° until analysed. Immediately

prior to the lipid analysis, the residues were dissolved in chloroform : methanol 1 : 1.

The dissolved neutral lipids residue was applied to high performance thin layer chromatography (HP-TLC) plates of Silica Gel 60 (E. Merck) and the lipids were separated by chloroform : methanol : acetic acid : water 65 : 43 : 0.5 : 3.5. Cholesterol was isolated by a solvent system of acetic acid : diisopropylether : *N*-hexane 2 : 35 : 65. The dissolved acid lipids residue was applied to the same type of HP-TLC plates and the lipids were separated by chloroform : acetone : acetic acid : formic acid : water 60 : 60 : 4 : 10 : 3. Standards of these lipids (Sigma Chemical Co., St Louis, MO) were used for identification and quantitative measurements. The quantitative determination of the lipids was carried out as described by Fewester *et al.* [31]. In essence, the developed HP-TLC plates were sprayed with cupric acetate 3% in phosphoric acid 8%. The plates were then dried for 10 min at 180° and the density of the spots was determined at 545 nm in a densitometer (Quick Scan R&D, Helena Laboratories, Beaumont, TX).

Choline incorporation by P388 cells. ADR-sensitive and -resistant cells were incubated for 36 hr in the presence of 3×10^{-6} M verapamil, tamoxifen or dipyridamole. Then 2.5×10^7 PBS washed cells were incubated at 37° in 5 mL RPMI 1640 medium with 3.6 μCi [*methyl*-^{3}H]choline (80 Ci/mmol, Amersham, Buckinghamshire, U.K.). After the incubation the cell suspension was ice-cooled, centrifuged for 10 min at 220 *g* and the cell pellet was washed twice with ice-cold PBS. The lipids were extracted from pellets of 8.7×10^6 cells as described above, then mounted on HP-TLC plates and developed in the solvent system used above for neutral lipids. PC spots were visualized by iodine vapor. The spots were scraped and transferred into vials with scintillation fluid and the radioactivity counted.

RESULTS

Figure 1 shows that KCl (50 mM) failed to increase FURA-2 fluorescence in both ADR-sensitive and -resistant P388 cells, indicating that the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) of either cell line remained unchanged upon addition of the depolarizing agent. In contrast, challenging bovine medullary chromaffin cells with high K^+ induced a prompt increase in $[Ca^{2+}]_i$, which subsequently decreased slowly over a time course of minutes. Clearly, the rise in $[Ca^{2+}]_i$ in the chromaffin cells is due to activation of voltage-dependent Ca^{2+} channels [28].

To examine the possibility that the leukemic cells might have been already depolarized and therefore insensitive to further depolarization, we have incubated the cells with the K^+ ionophore valinomycin (1 μM). In the presence of valinomycin, the cells should become hyperpolarized at the K^+ equilibrium potential. Figure 1 shows, however, that this pretreatment did not affect the insensitivity of the leukemic cells to K^+ depolarization. Identical results were obtained by incubating the cells with valinomycin for 30 min prior to the addition of KCl.

It might be argued that the lack of effect of depolarization on $[Ca^{2+}]_i$ reflects the inability of the current technique to detect $[Ca^{2+}]_i$ changes. We have therefore tested for the effects of ionomycin and found that this Ca^{2+} ionophore (1–10 μM) induced a prompt and steady increase in the $[Ca^{2+}]_i$ in both ADR-sensitive and -resistant P388 cells (Fig. 1). Previously we have found that in both P388 cell lines digitonin, at a concentration of 100 μM, permeabilized 100% of the cells (unpublished data). In the presence of ionomycin, the $[Ca^{2+}]_i$ remained unchanged upon addition of digitonin (100 μM), indicating saturation of FURA-2 fluorescence with respect to $[Ca^{2+}]_i$ (data not shown). In contrast to the response observed in cells in solution A, in a Ca^{2+} free medium containing ethyleneglycolbis-(aminoethylether)tetra-acetate (EGTA), the addition of ionomycin did not result in increased fluorescence in either ADR-sensitive or -resistant P388 cells (data not shown), indicating that the increase in FURA-2 signal was not caused by the addition of ionomycin *per se* but by its effect on Ca^{2+} entry. Verapamil up to a concentration of 100 μM did not affect the FURA-2 fluorescent signal measured in ADR-sensitive or -resistant cells suspended in solution A, nor did it affect the change in the fluorescence signal occurring after the addition of ionomycin (data not shown).

As indicated above when $3–5 \times 10^6$/mL drug-sensitive or MDR P388 cells were incubated with 100 μM digitonin, all the cells were permeabilized. However, at lower concentrations, MDR cells were found to be permeabilized by digitonin to a much smaller extent than drug-sensitive cells (Table 1). As shown in Table 1, while 2 min incubation with 20 and 25 μM digitonin resulted in 70 and 92% increase in trypan blue staining in drug-sensitive cells respectively, the corresponding values for MDR cells were 32 and 76%. Exposing the growing MDR cells to 10 μM verapamil, resulted in the loss of the ability to resist permeabilization by digitonin. When MDR cells were exposed for only 10 min to verapamil, even a 10-fold higher concentration of the drug failed to alter their resistance to digitonin. Exposing growing drug-sensitive P388 cells to verapamil did not affect their sensitivity to digitonin.

Previously we have found that a 15-fold increase in the sensitivity of MDR P388 cells to Adriamycin was obtained in the presence of 3×10^{-6} M tamoxifen. To obtain a similar increase in sensitivity by dipyridamole or verapamil their concentration had to be raised to 1×10^{-5} M. In spite of these differences, the lipid composition of both cell lines was examined after a 36 hr incubation at 37° with these compounds at an equal concentration of 3×10^{-6} M. The results obtained in MDR P388 cells are given in Table 2. The only significant change common to all three compounds is the increase in PC. Additional significant changes were found in MDR cells that were incubated with dipyridamole, namely, an increase in PS and a decrease in PE contents. In addition, in MDR cells that were incubated with verapamil a marked, although not significant, increase in PE content was observed. Compared to MDR cells that were not exposed to drugs, the PC/SM content ratio in these cells was increased 13% by verapamil or dipyridamole and

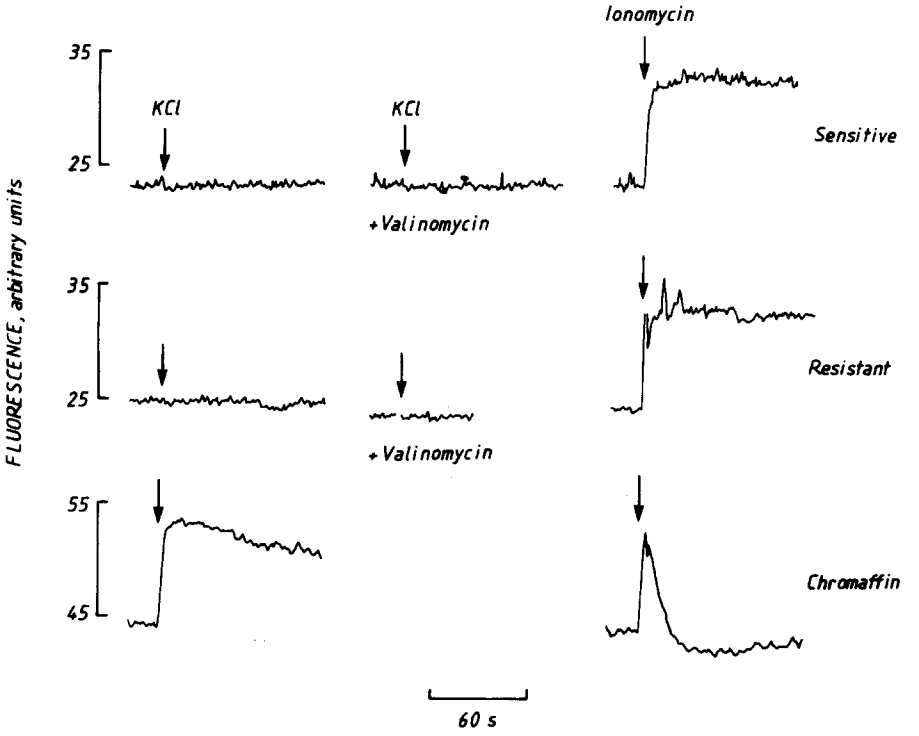

Fig. 1. Effects of KCl (50 mM) and ionomycin (1 μM) on the intracellular free Ca^{2+} of drug-sensitive (top traces) and multidrug-resistant (middle traces) P388 cells and of chromaffin cells (bottom traces). Changes in the intracellular free Ca^{2+} were followed by measuring the intracellular FURA-2 fluorescence. In the centre column of traces, valinomycin (1 μM) was added 5 min prior to KCl.

Table 1. The effects of verapamil on the ability of digitonin to permeabilize drug-sensitive and multidrug-resistant P388 cells

	Digitonin (μM)		
	5	20	25
P388	5.68*	1.78	0.47
P388 grown with verapamil (10 μM)	5.52	1.62	0.55
MDR-P388	6.10	4.10	1.46
MDR-P388 grown with verapamil (10 μM)	5.62	0.14	0.10
MDR-P388 cells exposed to 100 μM verapamil for 10 min	—	4.10	2.16

* $\times 10^6$ unstained cells/mL. Mean of two determinations. SD was always <10% of the mean. The initial density of unstained cells was 6×10^6/mL.

38% by tamoxifen. The only significant differences in lipid composition between drug-sensitive and drug-resistant cells were found in the contents of PC and SM which in the sensitive cells were (mean ± SD): 3.55 ± 0.20 and 1.00 ± 0.08 $\mu g/1 \times 10^6$ cells respectively. Following incubation of drug-sensitive cells with 3×10^{-6} M tamoxifen, verapamil or dipyridamole, no significant change in the cell lipid composition could be detected (data not shown). As shown in Table 2, tamoxifen, the most active MDR circumventor used in this study, caused a reduction in the SM content. However, compared to the control cells, the difference was significant only at a level of $P < 0.05$. In another experiment where MDR cells were incubated with tamoxifen for 12 hr, the content of SM was 0.86 ± 0.12 $\mu g/1 \times 10^6$ cells. As shown in Table 2, incubating MDR cells for 36 hr

Table 2. Lipid composition of multidrug-resistant P388 cells after incubation for 36 hr with 3×10^{-6} M verapamil, tamoxifen or dipyridamole

	$\mu g/1 \times 10^6$ cells (mean ± SD)			
	No drug	Verapamil	Tamoxifen	Dipyridamole
PC	2.96 ± 0.29	3.56 ± 0.16*	3.40 ± 0.15*	3.40 ± 0.20*
PE	2.40 ± 0.61	3.14 ± 0.50	2.64 ± 0.37	1.33 ± 0.21*
SM	1.23 ± 0.08	1.31 ± 0.15	1.02 ± 0.10	1.26 ± 0.14
PI	1.04 ± 0.24	1.05 ± 0.16	1.17 ± 0.15	1.22 ± 0.14
PS	0.54 ± 0.20	0.64 ± 0.19	0.68 ± 0.24	1.02 ± 0.20*
CL	0.61 ± 0.15	0.64 ± 0.20	0.49 ± 0.10	0.56 ± 0.16
CHOL	1.12 ± 0.24	1.00 ± 0.10	1.00 ± 0.44	1.19 ± 0.22

* Different from 'no drug' at a significance level of $P < 0.01$.

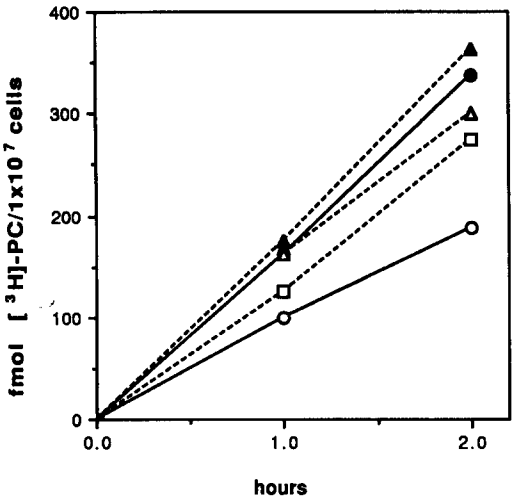

Fig. 2. Rate of incorporation of [^{3}H]choline into phosphatidylcholine in drug-sensitive (—●—) and multidrug-resistant P388 cells with and without (—○—) preincubation for 36 hr with 3×10^{-6} M verapamil (--□--), tamoxifen (--▲--) or dipyridamole (--△--). Each data point is the mean of three measurements. The SD values were <10% of the means.

with verapamil or dipyridamole did not result in significant change in the SM content. However, after incubation of MDR cells with these drugs for 12 hr, the content of this phospholipid was 0.96 ± 0.05 and 1.04 ± 0.08 μg/1 $\times 10^6$ cells, respectively. It seems therefore that verapamil, tamoxifen and dipyridamole induce a significant reduction in SM content in MDR P388 cells, however, the change is transitory in spite of a continued drug exposure.

One manner in which these drugs could have induced an increase in the PC content might have been by increasing the PC synthesis. We have therefore studied in both P388 cell lines the effect of a 36 hr preincubation with 3×10^{-6} M verapamil, tamoxifen or dipyridamole, on the rate of incorporation of [^{3}H]choline into the cell's PC pool. As shown in Fig. 2, all three drugs indeed induced in the MDR P388 cells an increased rate of PC synthesis. As was already reported previously [32], Fig. 2 also shows that the rate of PC synthesis in drug-sensitive P388 cells is significantly higher than that observed in multidrug-resistant cells. However, in drug-sensitive P388 cells this rate was not significantly affected by a 36 hr preincubation with these drugs (data not shown).

DISCUSSION

The results obtained with FURA-2 stained cells indicate that both drug-sensitive and MDR P388 cells are devoid of potential-dependent Ca^{2+} channels. These results are in accordance with the inability to demonstrate voltage-gated Ca^{2+} current by the patch-clamp technique in two other MDR cell lines [33, 34] and the failure of verapamil to block Ca^{2+} entry into MDR cells [7–10]. Therefore the capability of verapamil and other compounds [3] to restore drug-sensitivity in these cells must be mediated via some other mechanism(s).

Cass *et al.* showed that circumvention of MDR resistance by verapamil could be demonstrated only after extended exposure to the drug [15]. In a previous study we found that the enhancement of Adriamycin entry by verapamil developed over a considerable time period [18]. This time course was in a sharp contrast to the almost instantaneous blockade of the potential-dependent Ca^{2+} channels in excitable tissues. Furthermore, in that study it was shown that after incubating MDR P388 cells in the presence of verapamil, the activation energy of the passive entry process of Adriamycin was reduced to a level similar to the one measured in drug-sensitive P388 cells. It was suggested that the shift in this energy reflects a structural alteration in the domain of the cell membrane through which Adriamycin enters the cell. Passive diffusion of drugs, having a molecular weight of ~600, into cells is generally assumed to be mediated through the lipid domain of the plasma membrane. It therefore seemed plausible that the reduced entry rate of Adriamycin found in MDR cells (compared to that measured in drug-sensitive cells) might be related to difference in the membrane lipid composition. The lipid composition of whole cell and isolated plasma membranes has been studied in a variety of drug-sensitive cells and their MDR sublines [35–44]. In most of these studies differences in the content of some lipid components were found between the drug-sensitive and the MDR cells. However, it seems that there was no universal pattern of changes associated with the occurrence of MDR, rather the MDR associated lipid changes appear to be dependent on the cell type or the drug used for selecting MDR cells. The influence of each lipid constituent of the cell membrane on its permeability to drugs is not yet clearly known and apparently an increased drug permeability could be achieved by modification of various membrane lipids. It seems therefore plausible that when a cell population is exposed to cytotoxic compounds, that enter cells by passive diffusion, only cell variants with one of an assortment of alterations in the lipid composition of their plasma membrane, resulting in reduced drug permeability, will survive.

The results presented indicate that the major differences in lipid composition between drug-sensitive and MDR P388 cells were in the PC and SM content and that an extended incubation with 3×10^{-6} M verapamil, tamoxifen or dipyridamole induced a rise in the content of PC in the MDR- but not in the drug-sensitive P388 cells. Furthermore, it seems that among these compounds, the most potent circumventor of MDR, tamoxifen, induced the largest increase in the content of this phospholipid. An increase in PC content was also observed in human platelets that were incubated with dipyridamole [45]. Furthermore, similarly to the observed effects of dipyridamole on MDR cells, the drug also induced in the human platelets a reduction in SM and an increase in PE content. Many cationic amphiphilic drugs were reported to alter the

metabolism of phospholipids and cause, in a variety of cell types, changes in the lipid composition [46–51]. It is rather interesting that most of these compounds were also reported as potent circumventors of MDR [3]. Although, triparanol, a structural analogue of tamoxifen, was reported to induce cellular increase in PC [52], we are not aware that such an effect was described previously for tamoxifen or verapamil. It remains to be shown whether these compounds induce an increase in PC content in MDR cells selected from other cell types. The lack of effect of verapamil, tamoxifen and dipyridamole on the sensitivity to Adriamycin and on the PC content of drug-sensitive P388 cells tends to support the suggestion that there is an association between the ability to circumvent MDR and the induction of an increased PC synthesis.

The extent of reflection of changes in lipid composition of the plasma membrane by those induced by the MDR circumventors in whole cells, may indicate more directly on their relationship to the increase in drug permeability. While exploring techniques to selectively permeabilize the plasma membranes in both P388 cell lines, in our FURA-2 studies, we found inadvertently that MDR P388 cells resisted permeabilization by digitonin to a greater extent than did drug-sensitive cells. Digitonin, which forms very insoluble complexes with free cholesterol [53], is widely used to selectively permeabilize eukaryotic plasma membranes because the molar ratio of free cholesterol to phospholipid in these membranes is significantly greater than that of intracellular membranes [54]. The differential sensitivity to digitonin was somewhat surprising as differences in free cholesterol could not be demonstrated between drug-sensitive and MDR P388 cells [26, 32]. It must therefore be concluded that the plasma membrane's lipid microenvironment in which the cholesterol is located, is different in these cell lines. After exposure to verapamil, while there is no change in the free cholesterol content, MDR cells lose their resistance to digitonin. As this effect of verapamil on MDR cells was obtained only after extended exposure to the drug, it could be concluded that verapamil affects the plasma membrane lipid microenvironment indirectly, presumably by its effect on the cell's lipid metabolism as reflected by the induced changes in the whole cell lipid composition.

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