Regulation of $[Ca^{2+}]_i$ homeostasis in MRP1 overexpressing cells

Catalin M. Filipeanu[a], Adriaan Nelemans[a], Robert Jan Veldman[b], Dick de Zeeuw[a], Jan Willem Kok[b,*]

[a] *Groningen University Institute for Drug Exploration (GUIDE), Department of Clinical Pharmacology, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands*
[b] *Department of Physiological Chemistry, University of Groningen, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands*

Received 15 March 2000

Edited by Maurice Montal

Abstract Regulation of capacitative Ca^{2+} entry was studied in two different multidrug resistance (MDR) protein (MRP1) overexpressing cell lines, $HT29^{col}$ and GLC4/ADR. MRP1 overexpression was accompanied by a decreased response to thapsigargin. Moreover, inhibition of capacitative Ca^{2+} entry by D,L-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) was abolished in MRP1 overexpressing cells. Both PDMP and the MRP1 inhibitor MK571 greatly reduced $InsP_3$-mediated $^{45}Ca^{2+}$ release from intracellular stores in HT29 cells. Again, these effects were virtually abolished in $HT29^{col}$ cells. Our results point to a modulatory role of MRP1 on intracellular calcium concentration ($[Ca^{2+}]_i$) homeostasis which may contribute to the MDR phenotype.

Key words: Multidrug resistance protein; Capacitative Ca^{2+} entry; HT29 cell; GLC4 cell; MK571; D,L-*Threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol

1. Introduction

Multidrug resistance (MDR) of cancer cells is often resulting in chemotherapy treatment failure. MDR can be conferred by P-glycoprotein (Pgp) or MDR protein (MRP1), both members of the ATP binding cassette (ABC) superfamily of transporter proteins, which are responsible for enhanced drug efflux from cells [1,2]. Apart from (over)expression of ABC transporters, other mechanisms may contribute to MDR, e.g. reduced sensitivity to apoptotic induction [3–5]. In this respect, the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is of importance. Intracellular Ca^{2+} is involved in regulation of many cellular processes including contraction, secretion and intracellular transport [6]. Moreover, $[Ca^{2+}]_i$ plays an important role in both necrotic and apoptotic cell death [7]. Two models have been proposed to explain the involvement of Ca^{2+} homeostasis in apoptosis [8]. In one, increases in $[Ca^{2+}]_i$ due to depletion of intracellular stores and possibly influx of Ca^{2+} across the plasma membrane act as a signal for apoptosis. This may lead to activation of Ca^{2+}-dependent catabolic enzymes of the apoptotic machinery. In the second, the emptying of intracellular Ca^{2+} stores triggers apoptosis, rather than increases in cytosolic Ca^{2+}. In this model, emptying of Ca^{2+} stores, such as endoplasmic reticulum and mitochondria, would result in loss of organelle membrane integrity, leading to release of apoptotic effectors. An important role for the endoplasmic reticulum Ca^{2+} homeostasis in the apoptotic process is clearly indicated by the observations that thapsigargin induces apoptosis through its persistent Ca^{2+} pool depletion [9], while bcl-2 represses apoptosis by regulating endoplasmic reticulum-associated Ca^{2+} fluxes [10,11]. In the present study, we show that compared to the respective parental cells, $[Ca^{2+}]_i$ regulation is altered in two entirely different MDR cell lines, $HT29^{col}$ colon carcinoma cells and GLC4/ADR small cell lung carcinoma cells. These MDR cells cell lines have in common their abundant overexpression of MRP1, resulting from selection with different cytotoxic agents, colchicine and doxorubicin, respectively. This indicates that in addition to enhanced drug efflux by MRP1, this ABC transporter may have a role in altering Ca^{2+} homeostasis, which in turn contributes to enhanced cell survival.

2. Materials and methods

2.1. Materials

Indo-1/AM was purchased from Molecular Probes (Eugene, OR, USA). $Ins(1,4,5)P_3$ and ATP were from Boehringer Mannheim GmbH (Mannheim, Germany), $^{45}CaCl_2$ (19.3 Ci/mol) was from Amersham (Bercks, UK), D,L-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) was purchased from Matreya (Pleasant Gap, PA, USA). Colchicine, thapsigargin and β-escin were from Sigma (St. Louis, MO, USA). MK571 was a kind gift of Prof. Dr. A.W. Ford-Hutchinson (Merck-Frost Inc., Kirkland, Canada).

2.2. Cell culture

The human colon adenocarcinoma cell line HT29 was cultured in Dulbecco's modified Eagle medium containing 25 mM glucose, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) heat-inactivated (56°C, 30 min) fetal calf serum. Cells were seeded in a density of 4×10^4 cells/cm^2 and maintained in a water-saturated atmosphere of 5% CO_2/95% air at 37°C. Medium was refreshed every 48 h and the culture was passed weekly in a 1:15 dilution. The in vitro colchicine-selected, MRP1 overexpressing, Pgp-negative subline $HT29^{col}$ was obtained and maintained as described [12]. The in vitro doxorubicin-selected, MRP1 overexpressing, Pgp-negative subline GLC4/ADR was cultured as described [13].

2.3. $[Ca^{2+}]_i$ measurements

Intracellular Ca^{2+} concentrations were determined as described previously [14,15]. After trypsinization, the cells were resuspended in Hanks' solution at $3–5\times10^7$ cells/ml for HT29 cells and 10^8 cells/ml for GLC4 cells. The cells were loaded with 2 μM indo-1/AM for 30 min at room temperature in the dark. Under these conditions, compartmentalization of the dye was minimal ($8.2\pm2.2\%$, $n=6$ for HT29 cells and $9.3\pm3.6\%$, $n=4$ for GLC4 cells) as judged from the ratio of fluorescence signals obtained after selective permeabilization of the plasma membrane (10 μM β-escin) and full permeabilization of

*Corresponding author. Fax: (31)-50-3632728.
E-mail: j.w.kok@med.rug.nl

Abbreviations: ABC, ATP binding cassette; $[Ca^{2+}]_i$, intracellular calcium concentration; MDR, multidrug resistance; MRP, multidrug resistance protein; PDMP, D,L-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol

PII: S0014-5793(00)01585-4

the cells (1% Triton X-100). Then the cells were washed twice by centrifugation and their fluorescence was measured in an Aminco–Bowman spectrophotometer, using 10^6 cells/ml. Measurements were performed at 22°C, with a single excitation wavelength (349 nm) and a dual emission wavelength (410 and 490 nm) at a frequency of 1 Hz. Thapsigargin responses were measured at the plateau phase, which represents capacitative Ca^{2+} influx. In Ca^{2+}-free conditions, this plateau was not reached (see also [14]).

2.4. $^{45}Ca^{2+}$ fluxes in permeabilized cells

The cells were plated 48–72 h before the experiment in 6 well plates (Costar) at a density of 5×10^6 cells/well. The experiments were carried out at room temperature (22°C), exactly as described previously [16]. The $^{45}Ca^{2+}$ release was represented as the fractional loss of $^{45}Ca^{2+}$ per min in the mono-exponential phase, representing the amount of $^{45}Ca^{2+}$ leaving the cell, normalized to the amount of labelled $^{45}Ca^{2+}$ present in the cell at that time.

2.5. Statistics

All experiments were performed in at least two different cell passages with $n>6$. The results are presented as mean ± S.D. When necessary, the statistical difference was tested using Student's *t*-test, considering $P<0.05$ significant.

3. Results

3.1. Thapsigargin is less effective in inducing $[Ca^{2+}]_i$ increases in MRP1 overexpressing cells

The two MRP1 overexpressing MDR cell lines HT29^{col} and GLC4/ADR did not differ in their basal $[Ca^{2+}]_i$ levels (121 ± 14 nM, $n=60$ and 46 ± 8 nM, $n=6$, respectively), as compared to the non-resistant parental cell lines HT29 (112 ± 15 nM, $n=60$) and GLC4 (51 ± 7 nM, $n=6$). Thapsigargin induced dose-dependent increases in $[Ca^{2+}]_i$ in both HT29 and HT29^{col} cells (Fig. 1, left panel). However, thapsigargin was less effective in increasing $[Ca^{2+}]_i$ in MRP1 overexpressing HT29^{col} cells, at concentrations above 10^{-8} M. A similar pattern was observed in GLC4 cells, where thapsigargin was again less effective in increasing $[Ca^{2+}]_i$ in the MDR cell line, GLC4/ADR (Fig. 1, right panel). These data show that overexpression of MRP1 is accompanied by an alteration of $[Ca^{2+}]_i$ regulation.

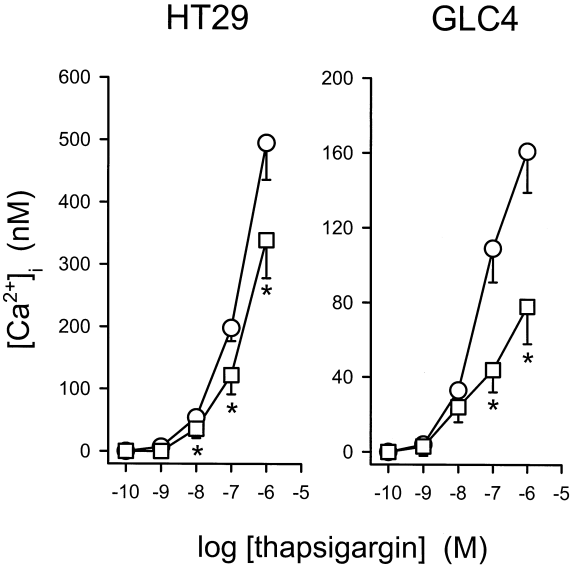

Fig. 1. Dose–effect response curves for thapsigargin in HT29 (left panel) and GLC4 cells (right panel), in non-resistant parental cells (○) and in cells overexpressing MRP1 (□), HT29^{col} and GLC4/ADR. The results are presented as the mean (± S.D.) maximal increase over basal levels ($n=6$), with a significance level of $P<0.05$.

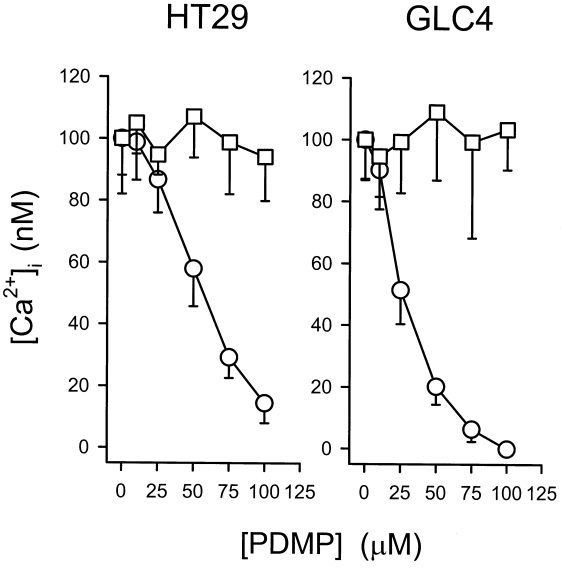

Fig. 2. Dose-dependent inhibition by PDMP of the thapsigargin (10^{-6} M)-elicited $[Ca^{2+}]_i$ increases in HT29 (left panel) and GLC4 cells (right panel). Measurements were performed in non-resistant parental cells (○) and MRP1 overexpressing HT29^{col} and GLC4/ADR cells (□). The results are presented as the mean percentage (± S.D.) of the thapsigargin response (in nM) at 0 μM PDMP (see Fig. 1), which was set at 100% ($n=6$).

3.2. PDMP does not inhibit capacitative Ca^{2+} influx in MRP1 overexpressing cells

We have recently demonstrated [14] that PDMP, an inhibitor of glucosylceramide biosynthesis, has three different effects on $[Ca^{2+}]_i$. (i) PDMP inhibited capacitative Ca^{2+} entry, (ii) PDMP elevated $[Ca^{2+}]_i$ under basal conditions and (iii) PDMP released Ca^{2+} from a thapsigargin-insensitive Ca^{2+} store. Therefore, PDMP serves as a valuable tool to analyze $[Ca^{2+}]_i$ regulation. Thus, we investigated if MRP1 overexpression, as observed in HT29^{col} and GLC4/ADR cells, modified the effects of PDMP on $[Ca^{2+}]_i$. In HT29^{col} cells, the PDMP-induced $[Ca^{2+}]_i$ increases above basal levels as well as the Ca^{2+} release from the thapsigargin-insensitive Ca^{2+} store were unchanged, as compared to the parental HT29 cells (Table 1). In contrast, in HT29^{col} cells, the inhibitory effect of PDMP on capacitative Ca^{2+} entry was abolished (Fig. 2). Again, this abolishment also occurred in MRP1 overexpressing GLC4/ADR cells (Fig. 2).

Table 1
Effect of PDMP on basal $[Ca^{2+}]_i$ and release from a thapsigargin-insensitive store in HT29^{col} cells

	Maximal $[Ca^{2+}]_i$ increase	Release from thapsigargin-insensitive Ca^{2+} store
HT29	135 ± 26	48 ± 15
HT29^{col}	119 ± 18	52 ± 12

Effects of PDMP on basal $[Ca^{2+}]_i$ in HT29 and HT29^{col} cells are reported as maximal increases above basal levels; PDMP-induced release of Ca^{2+} from a thapsigargin-insensitive store in HT29 and HT29^{col} cells was measured as described [14]. The results are presented as the mean ± S.D. ($n=6$).

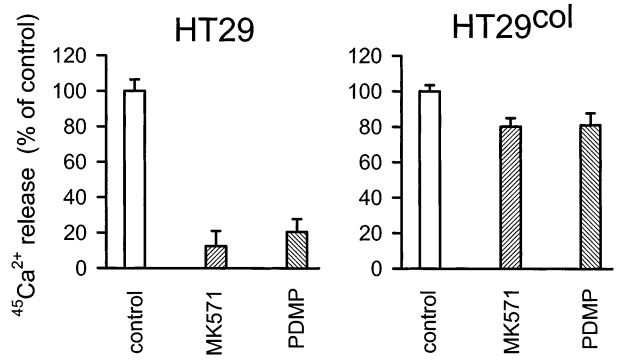

Fig. 3. Effects of MK571 and PDMP on $InsP_3$-mediated $^{45}Ca^{2+}$ release from permeabilized HT29 (left panel) and HT29^{col} (right panel) cells. The results are presented as the mean (±S.D.) percent of fractional loss of $^{45}Ca^{2+}$ (%/min) in control cells ($n=6$).

3.3. PDMP and MK571 do not inhibit $InsP_3$-mediated $^{45}Ca^{2+}$ release in MRP1 overexpressing cells

In order to investigate the relation between the altered Ca^{2+} homeostasis and the overexpression of MRP1 in HT29^{col} cells, we analyzed the effect on $[Ca^{2+}]_i$ regulation of MK571. This is an inhibitor of MRP1 function [17], which shares inhibitory properties with PDMP on intracellular membrane transport [14]. However, MK571 turned out to have a high intrinsic fluorescence that interfered with measurement of $[Ca^{2+}]_i$ using a variety of dyes (fura-2, indo-1, fluo-3). Therefore, we choose to compare its effects with those of PDMP on $^{45}Ca^{2+}$ fluxes in permeabilized cells. Neither MK571 (50 μM) nor PDMP (100 μM) modified basal $^{45}Ca^{2+}$ efflux. However, both drugs largely inhibited the $^{45}Ca^{2+}$ release elicited by 5 μM $InsP_3$ in HT29 cells (Fig. 3). In contrast, in MRP1 overexpressing HT29^{col} cells, MK571 as well as PDMP were ineffective inhibitors of the $InsP_3$-induced $^{45}Ca^{2+}$ efflux (Fig. 3).

4. Discussion

In the present work, we showed that overexpression of MRP1 is accompanied by changes in $[Ca^{2+}]_i$ homeostasis. This study was performed in two separate cell lines, both characterized by overexpression of the ABC transporter protein MRP1. GLC4/ADR is a well-established cell line, which was obtained from GLC4 small cell lung carcinoma cells by selection with doxorubicin. We recently described a novel MDR cell line, designated HT29^{col}, which was selected from the HT29 population with colchicine [12]. Resistance to colchicine was partly reversed by the MRP1 inhibitor MK571, which indicated that other mechanisms of resistance were operative in these cells. We showed that an altered sphingolipid composition in HT29^{col} cells may contribute to increased resistance, since glucosylceramide levels were enhanced similarly as observed in other MDR cells [12,18,19]. Moreover, such changes in sphingolipid composition were also observed to occur in GLC4/ADR cells. In the present study, a novel feature of both HT29^{col} and GLC4/ADR cells was discovered, which may contribute to the resistant phenotype. Although the basal levels of $[Ca^{2+}]_i$ were unchanged in both MDR cell lines, both MRP1 overexpressing cell lines displayed a reduced capacitative Ca^{2+} entry response to thapsigargin, as compared to their non-resistant counterparts. Persistent Ca^{2+} pool depletion by thapsigargin has been shown to result in apoptosis in HepG2 cells [9]. Furthermore, overexpression of the anti-apoptotic protein bcl-2 has been reported to maintain endoplasmic reticulum Ca^{2+} homeostasis and to reduce the Ca^{2+} response to thapsigargin [10,11]. Endoplasmic reticulum Ca^{2+} pool depletion is thus recognized to be of importance in triggering apoptosis. On the other hand, it has been reported that a reduced Ca^{2+} entry can facilitate apoptosis [20]. In this respect, the ability of PDMP to reduce capacitative Ca^{2+} entry may add to its potential as an anti-cancer drug. A role for glucosylceramide synthase inhibitors, like PDMP, in the treatment of cancer has been documented previously [21,22]. Our present results indicate an additional potential 'target' for this drug. The inhibitory effect of PDMP on $InsP_3$-mediated $^{45}Ca^{2+}$ release was mimicked by MK571, an inhibitor of MRP1 function. The effects of both drugs were virtually abolished in MRP1 overexpressing cells. Also the inhibitory effect of PDMP on thapsigargin-induced capacitative Ca^{2+} influx was abolished in MRP1 overexpressing cells. These data suggest that Ca^{2+} regulation is intimately linked to MRP1 expression and function. In parental, non-resistant HT29 and GLC4 cells, a low expression of MRP1 occurs [12,13], which is subject to efficient inhibition by MK571 (and possibly PDMP). MDR variants of these cells express high levels of MRP1, which may exert strong control of Ca^{2+} homeostasis. Apparently, this favors a situation in which on the one hand high cytosolic Ca^{2+} levels, and on the other hand complete emptying of intracellular Ca^{2+} stores, are prevented. The latter is achieved by preventing inhibition of capacitative Ca^{2+} influx, which is necessary to refill organelle stores after release from these stores is triggered. To our knowledge, this is the first study demonstrating the relationship between $[Ca^{2+}]_i$ homeostasis and MRP1 expression.

Acknowledgements: C.M.F. is a recipient of an Ubbo Emmius fellowship from Groningen University Institute of Drug Exploration (GUIDE).

References

[1] Bradshaw, D.M. and Arceci, R.J. (1998) J. Clin. Oncol. 16, 3674–3690.
[2] Smyth, M.J., Krasovskis, E., Sutton, V.R. and Johnstone, R.W. (1998) Proc. Natl. Acad. Sci. USA 95, 7024–7029.
[3] Hanahan, D. and Weinberg, R.A. (2000) Cell 100, 57–70.
[4] Valentini, A.M., Caruso, M.L., Armentano, R., Pirrelli, M., Rizzi, E., Lapenna, F. and Renna, L. (1999) Anticancer Res. 19, 3019.
[5] Mano, Y., Kikuchi, Y., Yamamoto, K., Kita, T., Hirata, J., Tode, T., Ishii, K. and Nagata, I. (1999) Eur. J. Cancer 35, 1214–1219.
[6] Berridge, M.J. (1998) Neuron 21, 13–26.
[7] Orrenius, S. and Nicotera, P. (1994) J. Neural Transm. Suppl. 43, 1–11.
[8] McConkey, D.J. and Orrenius, S. (1996) J. Leuk. Biol. 59, 775–783.
[9] Kaneko, Y. and Tsukamoto, A. (1995) Cancer Lett. 79, 325–335.
[10] Lam, M., Dubyak, G., Chen, L., Nunez, G., Miesfeld, R.L. and Distelhorst, C.W. (1994) Proc. Natl. Acad. Sci. USA 91, 6569–6573.
[11] He, H., Lam, M., McCormick, T.S. and Distelhorst, C.W. (1997) J. Cell Biol. 138, 1219–1228.
[12] Kok, J.W., Veldman, R.J., Klappe, K., Koning, H., Filipeanu, C.M. and Muller, M. (2000) Int. J. Cancer (in press).
[13] van der Kolk, D.M., de Vries, E.G.E., Koning, J.A., van den Berg, E., Muller, M. and Vellenga, E. (1998) Clin. Cancer Res. 4, 1727–1736.

[14] Kok, J.W., Babia, T., Filipeanu, C.M., Nelemans, A., Egea, G. and Hoekstra, D. (1998) J. Cell Biol. 142, 25–38.
[15] Filipeanu, C.M., de Zeeuw, D. and Nelemans, S.A. (1997) Eur. J. Pharmacol. 336, R1–R3.
[16] Sipma, H., van der Zee, L., van den Akker, J., den Hertog, A. and Nelemans, A. (1996) Br. J. Pharmacol. 119, 730–736.
[17] Renes, J., de Vries, E.G., Nienhuis, E.F., Jansen, P.L. and Muller, M. (1999) Br. J. Pharmacol. 126, 681–688.
[18] Lavie, Y., Cao, H.-t., Bursten, S.L., Giuliano, A.E. and Cabot, M.C. (1996) J. Biol. Chem. 271, 19530–19536.
[19] Lucci, A., Cho, W.I., Han, T.-Y., Giuliano, A.E., Morton, D.L. and Cabot, M.C. (1998) Anticancer Res. 18, 475–480.
[20] Jayadev, S., Petranka, J.G., Cheran, S.K., Biermann, J.A. and Barrett, J.C. (1999) J. Biol. Chem. 274, 8261–8268.
[21] Nicholson, K.M., Quinn, D.M., Kellett, G.L. and Warr, J.R. (1999) Br. J. Cancer 81, 423–430.
[22] Lavie, Y., Cao, H.-t., Volner, A., Lucci, A., Han, T.-Y., Geffen, V., Giuliano, A.E. and Cabot, M.C. (1997) J. Biol. Chem. 272, 1682–1687.