Drug Development Section
Cancer Research Campaign
Laboratory
Institute of Cancer Research
Clifton Avenue
Sutton, Surrey SM2 5PX, U.K.

MARTIN G. ROWLANDS*
JOHN HOUGHTON
MICHAEL H. BAKER

REFERENCES

1. Waight ES, The cycloheptane, cyclo-octane and macrocyclic groups. In: *Rodd's Chemistry of Carbon Compounds*, Vol. IIB (Ed. Coffey S), pp. 347–423. Elsevier, London, 1970.
2. Peng R, Zhu X-Y and Yang S, Induction of rat liver microsomal cytochrome P-450 by muscone (3-methylcyclopentadecanone). *Biochem Pharmacol* **35**: 1391–1394, 1986.
3. Baker MH, Foster AB, Leclercq F, Jarman M, Rowlands MG and Turner JC, Effect of ω-trifluorination on the microsomal metabolism of ethyl and pent-1-yl *p*-nitrophenyl ether. *Xenobiotics* **16**: 195–203, 1986.
4. Ryan KJ, Biological aromatization of steroids. *J Biol Chem* **234**: 268–272, 1959.
5. Graves PE and Salhanick HA, Stereoselective inhibition of aromatase by enantiomers of aminoglutethimide. *Endocrinology* **105**: 52–57, 1979.
6. Hochberg RB, Vander Hoeven TA, Welch M and Lieberman S, A simple and precise assay of the enzymatic conversion of cholesterol into pregnenolone. *Biochemistry* **13**: 603–609, 1974.
7. Uzgiris VI, Whipple CA and Salhanick HA, Stereoselective inhibition of cholesterol side chain cleavage by enantiomers of aminoglutethimide. *Endocrinology* **101**: 89–92, 1977.
8. Foster AB, Jarman M, Leung C-S, Rowlands MG and Taylor GN, Analogues of aminoglutethimide: selective inhibition of cholesterol side chain cleavage. *J Med Chem* **26**: 50–54, 1985.
9. Liang T, Casceiri MA, Cheung AH, Reynolds GF and Rasmusson GH, Species differences in prostatic steroid 5α-reductases of rat, dog and human. *Endocrinology* **117**: 571–579, 1985.

* Author to whom correspondence should addressed.

Biochemical Pharmacology, Vol. 37, No. 11, pp. 2293–2297, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

Interactions of cardiac glycosides with cells and membranes. Therapeutic and toxic doses of ouabain acting on sodium and calcium pumps in plasma membranes

(*Received* 17 *November* 1987; *accepted* 7 *January* 1988)

According to the currently accepted mechanism for cardiac glycoside action, theses agents bind to and inhibit the action of the sodium pump [1–3]. Following several reports of data that are inconsistent with sodium pump inhibition mechanism, attention has been directed toward alternative explanations [4–6]. One possible mode is via the sarcolemmal calmodulin-regulated calcium pump, which has been implicated in the control of cellular Ca^{2+} [7–12]. The present communication describes the biphasic response to ouabain of two membrane-bound enzymes, Na^+–K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase in erythrocyte membrane vesicles, which serve as a good model system. Our data suggest that nanomolar concentrations of ouabain stimulate both Na^+–K^+-ATPase activity and calmodulin-mediated Ca^{2+}–Mg^{2+}-ATPase with concomitant inhibition of the "basal" Ca^{2+}–Mg^{2+}-ATPase activity. The possible involvement of the calcium pump mediated via cardiac glycosides in the regulation of intracellular Ca^{2+} concentrations is proposed.

Materials and methods

Human erythrocyte membrane vesicles were prepared by hemolysis in either (a) hypotonic media without Ca^{2+} [13] or in the presence of 1 mM EGTA [14], or (b) isotonic media containing 0.3 g/l saponin, 0.2 mM EGTA and Ca^{2+}. Whereas at $(Ca^{2+})_f < 0.1\ \mu M$ only "basal" Ca^{2+}–Mg^{2+}-ATPase could be detected, during hemolysis, at $(Ca^{2+})_f > 1\ \mu M$, Ca^{2+}–Mg^{2+}-ATPase activity that was stimulated by calmodulin was found [14, 15]. ($(Ca^{2+})_f$ is the concentration of free unbound Ca^{2+}.)

Na^+–K^+-ATPase and Ca^{2+}–Mg^{2+}-ATPase activities were assayed by measuring the release of inorganic phosphate [16] in an isotonic medium containing 1 mM EGTA ± $CaCl_2$ (1 mM Ca^{2+} in the presence of EGTA yielded $(Ca^{2+})_f = 14\ \mu M$ [17]); and ± ouabain in increasing concentrations. The effects of endogenous or exogenous calmodulin (derived from bovine brain) were measured by adding either 100 μg/ml compound 48/80 [18] or, 0.5 μM calmodulin and 10–100 μM Ca^{2+}. The enzyme was preincubated with increasing concentrations of ouabain either in the absence or presence of calmodulin. A similar procedure was followed with compound 48/80. Incubations at 37° for up to 60 min gave a linear response. The reaction was terminated by placing the tubes into liquid air. The P_i released was determined by the malachite green–molybdate complex assay, measuring color development at 1° during a 30 min period [19]. The activities of both enzymes were calculated according to Raess and Vincenzi [16] and expressed in mU/mg protein. Protein content was determined in the presence of 0.01% sodium dodecyl sulfate according to Lowry *et al.* [20].

The values are presented as means ± SEM. Significance of the differences between means was checked by Student's *t*-test, and gave values of less than 0.05.

Results and discussion

Upon treatment with calmodulin, vesicles prepared from human erythrocyte membranes exhibited a bimodal response to ouabain: nanomolar concentrations of ouabain stimulated Na^+–K^+-ATPase and inhibited "basal" Ca^{2+}–Mg^{2+}-ATPase, whereas micromolar concentrations had the opposite effect (Fig. 1). Calmodulin (0.5 μM) led to a fivefold increase in the "basal" Ca^{2+}–Mg^{2+}-ATPase activity. Nanomolar concentrations of ouabain further stimulated this activity by 160%. Micromolar concentrations of ouabain seemed to inhibit this "calmodulin-activated" Ca^{2+}–Mg^{2+}-ATPase (Fig. 2). Na^+–K^+-ATPase did not respond to calmodulin at these concentrations. Compound 48/80, 0.1–0.4 μM [18], a potent and specific antagonist of "calmodulin-dependent" cellular activities,

inhibited Na^+–K^+-ATPase. However, this inhibitor did not abolish the biphasic response of Na^+–K^+-ATPase to ouabain (0.1 nM to 0.1 mM) (Fig. 3). Gietzen *et al.* have shown that Ca^{2+}–Mg^{2+}-ATPase in erythrocyte membranes was almost completely inhibited by compound 48/80 at a concentration of 100 μg/ml. By contrast, 300 μg/ml compound 48/80 only inhibited erythrocyte Na^+–K^+-ATPase

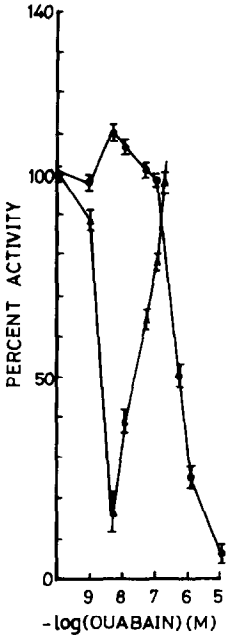

Fig. 1. Effects of ouabain on Na^+–K^+-ATPase and "basal" Ca^{2+}–Mg^{2+}-ATPase in human erythrocyte membranes. The figure shows results of 4 experiments as means ± SEM with significance of differences between means <0.05. Control values (100%) for Na^+–K^+-ATPase (●) and "basal" Ca^{2+}–Mg^{2+}-ATPase (▲): 4.8 and 3.2 mU/mg protein, respectively.

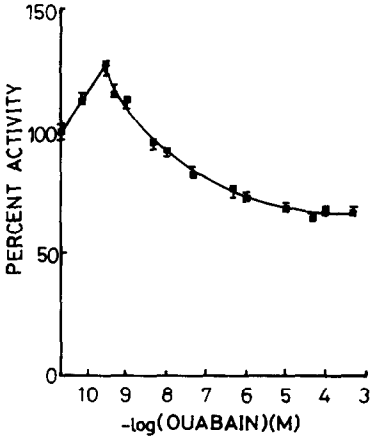

Fig. 2. Effects of ouabain on "calmodulin-activated" Ca^{2+}–Mg^{2+}-ATPase in human erythrocyte membranes. The results from 3 experiments (mean ± SEM with significance of differences between means <0.05). Calmodulin was added at a final concentration of 0.5 μM and Ca^{2+} at 50 μM.

activity by 25% [18]. Table 1 summarizes some of the properties of Ca^{2+}–Mg^{2+}-ATPase prepared from erythrocyte membranes by various hemolysis procedures, which were reflected in differences in permeability and calmodulin retention. Vesicles in a medium containing EGTA are permeable to and retain less calmodulin [14, 15].

At very low Ca^{2+} (i.e. less than 0.1 μM), the vesicles exhibited "basal" Ca^{2+}–Mg^{2+}ATPase activity. However, at Ca^{2+} concentrations greater than 1 μM, Ca^{2+}–Mg^{2+}-ATPase activities were higher and approached the values obtained in the presence of optimal concentrations of added calmodulin and Ca^{2+}. The activities of Na^+–K^+-ATPase and Ca^{2+}–Mg^{2+}-ATPase in calmodulin-depleted vesicles are shown in Table 2. Compound 48/80 led to inhibition of Na^+–K^+-ATPase— a result that was not expected (cf. Ref. 18).

Table 1. Ca^{2+}–Mg^{2+}-ATPase in human erythrocyte membranes prepared by various methods

Method of preparation	Enzymatic activity (mUnits/mg protein)	
	Control	+Calmodulin
1. Isotonic hemolysis + saponin ($(Ca^{2+}) < 0.1$ mM)	41 ± 2	68 ± 1.5
2. Isotonic hemolysis + saponin ($(Ca^{2+}) \geq 1$ mM)	52 ± 2	—
3. Hypotonic hemolysis (A)	10.7 ± 1.1	55 ± 3
Hypotonic + EGTA (B)	5.0 ± 1.3	50 ± 2
Hypotonic + EGTA (C)	13.6 ± 1.5	57 ± 2
Hypotonic + EGTA (D)	10.1 ± 1.1	—
(D) + Compound 48/80(E)	8.8 ± 0.7	—

Membranes were prepared according to Klinger *et al.* [15] (preps. 1 and 2) or according to Raess and Vincenzi [16] (prep. 3(A)); or according to Downes and Michell [14] (preps. 3(B–D)). In 3(B) during assay, $(Ca^{2+})_f$ was <0.1 mM in "control" and 2 μM in "+ Calmodulin" (0.5 μM). In 3(C) $(Ca^{2+})_f$ was <0.1 mM in "control" and 0.1 mM in "+ Calmodulin" (0.5 μM). In 3(D) $(Ca^{2+})_f$ was in "control" and "+ Compound 48/80" 7–10 μM.

The results are means ± SEM of 4 separate experiments, with significance of differences between means <0.05.

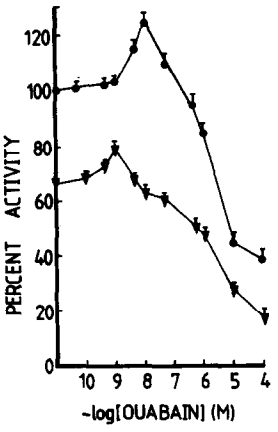

Fig. 3. Effects of compound 48/80 and ouabain on Na^+–K^+-ATPase in human erythrocyte membranes. Na^+–K^+-ATPase was titrated with 0.1 nM to 0.1 mM ouabain in absence (●) and presence (▼) of 100 μg/ml of Compound 48/80. For further details cf. Materials and Methods. The results of 3–4 experiments are shown as means ± SEM with significance of differences between means <0.05. Control value (100%): 37 mU/mg protein.

Lüllmann *et al.* [8, 9] found that inside-out vesicles from cardiac sarcolemma displayed "maximal" calcium transporting capacity and responded to inotropic and toxic doses of ouabain in a very similar manner to the erythrocyte calmodulin-reloaded membrane vesicles in the present study. In these experiments, the sarcolemmal vesicles were isolated from guinea-pig or cat hearts that had been perfused with ouabain prior to homogenization [8, 9]. These sealed inside-out vesicles were impermeable to externally added ouabain or digitoxin. In vesicles prepared in the presence of low concentrations of Ca^{2+} (i.e. 0.2 μM), the Ca^{2+} pump activity was enhanced by up to 10-fold as compared to those prepared in the Ca^{2+}-free medium containing EGTA [8]. Sarcolemmal vesicles from hearts perfused with inotropic doses of ouabain (i.e. 0.3 μM) displayed higher rates of calcium transport than those prepared from hearts that had been perfused with toxic doses (i.e. 1 μM) [8, 9]. Lüllmann *et al.* suggested that the increased Ca^{2+} transport in vesicles prepared from hearts perfused with inotropic doses of ouabain was caused by calmodulin retention.

Sarcolemmal vesicles are generally difficult to prepare, contain a sodium pump, a sodium–calcium exchanger and a calmodulin-regulated calcium pump [10, 11, 21]. On the other hand, human erythrocyte membranes are easy to prepare and exhibit a sodium pump and a calmodulin-activated calcium pump in the absence of the Na^+–Ca^{2+} exchanger [10–12, 14, 15]. Therefore, we used human erythrocyte membrane vesicles—a simpler and less complex model system—in order to test Lüllmann's hypothesis.

Calmodulin is bound more tightly to cardiac sarcolemma than to the erythrocyte membranes. Consequently dissociation of calmodulin from sarcolemma would be expected to be more difficult. However, hypotonic treatment, immediately followed by a hypertonic wash in presence of EGTA and by several hypotonic washes in the absence of EGTA led to depletion in calmodulin [10, 14]. There are several differences between the experiments with human erythrocyte membranes reported in this paper and the studies reported by Lüllmann *et al.* In the latter investigations in cardiac sarcolemma, the inotropic "low" and toxic "high" concentrations of ouabain were 0.3 μM and 1 μM, respectively [8]. In erythrocyte membrane vesicles, "low" and "high" refer to nanomolar and micromolar concentrations of ouabain, respectively (Fig. 1). However, the sarcolemmal vesicles retained ouabain during the homogenization in concentrations much lower than those of the medium. The low concentrations may only represent the molecules of ouabain bound to the receptors on the inner surface of the inside-out vesicles, which were originally on the outer surface of the membrane. In permeable erythrocyte membrane vesicles, the ouabain concentrations given are those of the external medium.

The studies done by Lüllmann *et al.* [8, 22] or those reported in this paper do not imply that ouabain has direct effects on the calcium pump, i.e. the Ca^{2+}–Mg^{2+}-ATPase. All the other investigations on Ca^{2+}–Mg^{2+}-ATPase were carried out on intact membranes with the addition of millimolar concentrations of ouabain to inhibit Na^+–K^+-ATPase. However, the effects of ouabain have not previously been tested in purified Ca^{2+}–Mg^{2+}-ATPase preparations [10–13]. In the present study, the effect of ouabain at nanomolar concentrations was also examined for the first time. However, more rigorous studies are required in order to determine the direct effects of ouabain on highly purified Ca^{2+}–Mg^{2+}-ATPase. Huang and Askari reported the presence of a Ca^{2+} activated para nitrophenylphosphatase in a highly purified Na^+–K^+-ATPase preparation from dog's kidney medulla [23]. The enzyme was completely inhibited by μM concentrations of ouabain and the inhibitory response to ouabain decreased by increasing the Ca^{2+} concentrations from 50 to 500 μM. Therefore, these effects may occur at sites that are in close proximity to one another, as primarily manifested in intact membranes but also in certain purified enzymatic preparations, which explain the

Table 2. Na^+–K^+-ATPase and Ca^{2+}–Mg^{2+}-ATPase in human erythrocyte membranes

Additions	Na^+–K^+-ATPase	Ca^{2+}–Mg^{2+}-ATPase
	mUnits/mg protein	
Mg^{2+}, Na^+, K^+, EGTA	38.8 ± 1.2	—
Mg^{2+}, Ca^{2+}, Na^+ (or K^+)	—	56 ± 2
+ Ouabain (0.1 mM)	13.5 ± 1.1	—
Compound 48/80 (100 μg/ml)	23.0 ± 2	31 ± 1.5
Ouabain + Compound 48/80	11.9 ± 1.1	24 ± 1
Calmodulin (0.5 μM)	39.5 ± 2	175 ± 3
Ouabain + Calmodulin	—	167 ± 3

Membranes were prepared in hypotonic medium containing 1 mM EGTA [14]. Enzymatic activity of Na^+–K^+-ATPase and Ca^{2+}–Mg^{2+}-ATPase was assayed in a final volume of 1 ml containing (in mM): Tris (50), pH 7.0; NaCl (80); KCl (30); $MgCl_2$ (3); ATP (3); EGTA (3); $CaCl_2$ (0.1) and membrane protein (0.1–0.3 mg). The results represent means ± SEM of 4 experiments with significance of differences between means with <0.05.

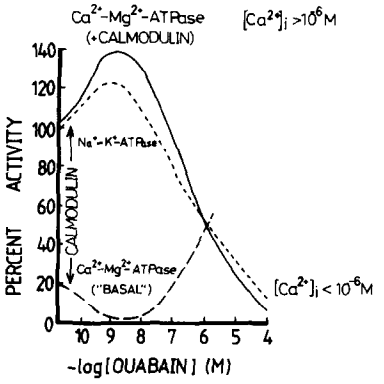

Fig. 4. Proposed biphasic action of ouabain on sodium and calcium pump enzymes (see text).

various effects of ouabain on Na^+–K^+-ATPase or, either direct or indirect on Ca^{2+}–Mg^{2+}-ATPase or both of these. Caution should be exercised in applying the conclusions from these experiments with erythrocyte membranes to other cellular systems, particularly to myocytes. However, it is tempting to speculate that Ca^{2+} at concentrations below 1 μM, enhance the inhibition of "basal" Ca^{2+}–Mg^{2+}-ATPase by inotropic doses of ouabain, since calmodulin alone does not bind to or stimulate the calcium pump (Fig. 4). Under these circumstances, the steady state pumping rates of calcium are low and cause a retention of $(Ca^{2+})_i$. At Ca^{2+} levels above 1 μM, calmodulin binds to Ca^{2+} and the complex formed becomes attached to the sarcolemmal calcium pump, thereby increasing the "basal" activity by several-fold [24]. At this point, nanomolar concentrations of ouabain further stimulate the pump, increase its turnover rate, accelerating the removal of cellular Ca^{2+} with the decrease of $(Ca^{2+})_i$ to below 1 μM. The Ca^{2+}–calmodulin-ATPase complex then dissociates with the return of Ca^{2+}–Mg^{2+}-ATPase activity to "basal" values and, the cycle continues in this fashion. Whether this proposed mechanism provides only a partial explanation of the mode of cardiac glycoside action or such a process serves an auxilliary or supplementary role remains to be established.

In summary, nanomolar concentrations of ouabain were found to stimulate Na^+–K^+-ATPase and "calmodulin-dependent" Ca^{2+}–Mg^{2+}-ATPase but inhibited the "basal" (calmodulin-independent) Ca^{2+}–Mg^{2+}-ATPase in erythrocyte membrane vesicles. These data indicate that the interactions between the plasma membrane sodium and calcium pumps, which might play a role in the regulation of intracellular calcium levels, may occur.

Acknowledgements—These studies were supported in part by a grant from the Chief Scientist, Ministry of Health.

Laboratory for Myocardial Research
Institute of Biochemistry
The Hebrew University—Hadassah Medical School
Jerusalem 91010, Israel

MICHAEL HELLER

REFERENCES

1. Akera T, Effects of cardiac glycosides on Na^+,K-ATPase. In: *Cardiac Glycosides*, Vol. 56, (Ed. Greeff K), Part I, pp. 287–336, Springer, Berlin, 1981.
2. Schwartz A, Lindenmayer GE and Allen JC, The sodium–potassium adenosine triphosphatase: Pharmacological, physiological and biochemical aspects. *Pharmacol Rev* **27**: 3–134, 1975.
3. Smith TW, Entman EM, Friedman PL, Blatt CM and Marsh JD, Digitalis glycosides: mechanisms and manifestations of toxicity. *Prog Cardiovas Dis*, part II, **26**: 495–523, 1984.
4. Heller M, Interactions and mode of action of cardiac glycosides with cultured heart cells. In: *Heart Cell in Culture*, Vol. III (Ed. Pinson A), pp. 1–20. CRC Press, Boca Raton, 1987.
5. Ghysel-Burton J and Godfraind T, Stimulation and inhibition of sodium pump by cardioactive steroids in relation to their binding sites and their inotropic effect on guinea pig atria. *Br J Pharmacol* **66**: 175–184, 1979.
6. Godfraind T, Subclassification of cardiac glycosides receptors. *Basic Res Cardiol* **79**: Suppl. 27–34, 1984.
7. Lüllmann H and Peters T, Action of cardiac glycosides on the excitation–contraction coupling in heart muscle. *Prog Pharmacol* **2**: 5–57, 1979.
8. Lüllmann H, Peters T and Preuner J, Mechanism of action of digitalis glycosides in the light of new experimental observations. *Eur Heart J* **3**: Suppl. D 45–51, 1982.
9. Lüllmann H, Peters T, Prillewitz HH and Ziegler A, Cardiac glycosides with different effects in the heart. *Basic Res Cardiol* **79**: Suppl. 93–101, 1984.
10. Caroni P and Carafoli E, The Ca^{2+} pumping ATPase of heart sarcolemma. *J Biol Chem* **256**: 3263–3270, 1981.
11. Penniston JT, Plasma membrane Ca^{2+}-ATPase as active Ca^{2+}-pumps. In *Calcium and Cell Function*, Vol. IV, (Ed. Cheung WY), pp. 99–149. Academic Press, New York, 1983.
12. Schatzmann HJ and Vincenzi, FF, Calcium movements across the membrane of human red cells. *J Physiol (Lond)* **201**: 369–395, 1969.
13. Heller M, Hanahan DJ, Erythrocyte membrane-bound enzymes. ATPase, phosphatase and adenylate kinase in human, bovine and porcine erythrocytes. *Biochim Biophys Acta* **255**: 239–250, 1972.
14. Downes P and Michell RH, Human erythrocyte membranes exhibit a cooperative calmodulin-dependent Ca^{2+}-ATPase of high calcium sensitivity. *Nature (Lond)* **290**: 270–271, 1981.
15. Klinger R, Wetzker R, Fleischer I and Frunder H, Effect of calmodulin, Ca^{2+} and Mg^{2+} on the $(Ca^{2+} + Mg^{2+})$-ATPase of erythrocyte membranes. *Cell Calcium* **1**: 229–240, 1980.
16. Raess BU and Vincenzi FF, A semi-automated method for the determination of multiple membrane ATPase activities. *J Pharmacol Methods* **4**: 273–283, 1983.
17. Fabaiato A and Fabiato F, Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *J Physiol (Lond)* **75**: 463–505 (1979).
18. Gietzen K, Adamczyk-Engelmann P, Wuthrich A, Konstantinova A and Bader H, Compound 48/80 is a selective and powerful inhibitor of calmodulin-regulated functions. *Biochim Biophys Acta* **736**: 109–118, 1983.
19. Lanzetta PA, Alvarez LJ, Reinach PS and Candia OR, An improved assay for nanomole amounts of inorganic phosphorous. *Anal Biochem* **100**: 95–97, 1979.
20. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the folin phenol reagent. *J Biol Chem* **119**: 265–275, 1951.
21. Lamers JMJ and Stinis JT, Inhibition of Ca^{2+}-dependent protein kinase and Ca^{2+}/Mg^{2+} ATPase in cardiac sarcolemma by the anti-calmodulin drug calmidazolium. *Cell Calcium* **4**: 281–294, 1983.

22. Kliem U and Preuner J, Inhibition of an active calcium transport in cardiac sarcolemma by ouabain. *Naunyn Schmiedeberg's Arch Pharmacol* **313**: R41, 1980.
23. Huang WH and Askari A, Interaction of Ca^{2+} with $(Na^+ + K^+)$-ATPase-properties of the Ca^{2+}-stimulated phosphatase activity. *Arch Biochem Biophys* **231**: 287–292, 1984.
24. Kristensen BI, The effect of Mg^{2+} and calmidazolium on the calmodulin binding to inside-out vesicles of the human red cell membrane. *Biochim Biophys Acta* **862**: 457–460, 1986.

Biochemical Pharmacology, Vol. 37, No. 11, pp. 2297–2299, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

Effects of phorbol esters on doxorubicin transport systems

(*Received* 15 *June* 1987; *accepted* 3 *December* 1987)

When neoplastic cells are selected for resistance to a single anti-tumor agent, the resulting sublines are sometimes cross-resistant to a variety of other agents of apparently unrelated structure. This phenomenon is termed "multi-drug resistance" (MDR*), and it has been described in several reports and recent reviews [1–5]. Cells exhibiting the MDR phenotype have several characteristics: a new membrane "P-glycoprotein" having a molecular weight of approximately 170 kD; cross-resistance to anthracyclines, actinomycin D, Vinca alkaloids and certain other natural or synthetic products; and an outward drug-transport system which limits accumulation of cytotoxic agents. Outward transport may be mediated by the 170 kD glycoprotein which bears a close resemblance to transport proteins in bacterial systems [6, 7]. This transport system can be inhibited by a variety of drugs including calcium-channel antagonists [8–11] and calmodulin inhibitors [12, 13].

A suggestion that phosphorylation of "P-glycoprotein" is involved in the maintenance of MDR was provided by *in vitro* studies [14–16]. While P-glycoprotein phosphorylation can be promoted by calcium-channel antagonists, calmodulin inhibitors and phorbol esters, sites of phosphorylation can differ, depending on the stimulus [17]. In one cell line, treatment with phorbols resulted in decreased phosphorylation of the 170 kD glycoprotein [18]. The role of phorbol ester-induced promotion of P-glycoprotein phosphorylation is not yet clear. Treatment of one drug-sensitive tumor cell line with a phorbol ester promoted vincristine resistance but there was no phorbol-induced alteration in drug responsiveness in a vincristine-resistant subline [19].

In this study, we examined the effects of several phorbol esters on anthracycline transport and cytotoxicity in the P388 murine leukemia cell line and an anthracycline-resistant subline, P388/ADR. The properties of these cell lines have been described before; P388/ADR cells exhibit the pertinent characteristics of the MDR phenotype [20, 21].

Cells were grown in sealed flasks using Fischer's medium (GIBCO, Grand Island, NY) supplemented with 10% horse serum. [^{14}C]DNR (30 Ci/mol) was provided by the Division of Cancer Treatment, NCI. [20-^{3}H(N)]-Phorbol,12-13-dibutyrate (550 Ci/mol) was purchased from New England Nuclear, Boston, MA. The 4α- and 4β-phorbol didecanoate esters were obtained from the Sigma Chemical Co., St. Louis, MO. Incubations were carried out in growth medium buffered with 20 mM HEPES at pH 7.2.

Cell cultures were exposed to 3–200 nM concentrations of different phorbol esters for 10 min to 24 hr, at 37°, and then suspended at a density of 7 mg (wet weight)/ml in growth medium buffered with 20 mM HEPES at pH 7.4. This medium contained the same levels of phorbol esters as were present during the first incubation. The cells were then treated with 0.1 μM [^{14}C]DNR for 0.3 to 90 min at 37°. In some studies verapamil (5–20 μM) was present during the second incubation. Drug exodus studies were carried out after 90-min incubations; cells were suspended in fresh medium (containing phorbol esters if these were present in the first incubation), and intracellular anthracycline concentrations were measured over an additional 60 min at 37°. Cytotoxicity of DNR was measured by a clonogenic assay [22] after a 2-hr exposure to phorbol esters, followed by a 1-hr treatment with specified anthracycline levels. Colonies were counted after 7 days. Cloning efficiency of P388 cells was approximately 80%.

We observed a 40% inhibition of steady-state DNR uptake by P388 cells upon treatment with 4β-phorbol ester for 120 min (Table 1). This effect was not seen after shorter (10–30 min) or longer (8–24 hr) incubations with the phorbol ester. The optimal phorbol concentration was 10 nM; we observed no effect at 3 nM, and no additional effects on DNR uptake were observed when the phorbol concentration was increased. Treatment with phorbol for 10 min to 24 hr had no effect on DNR accumulation by P388/ADR. The (inactive) 4α-phorbol ester did not alter the time-course of DNR accumulation by either cell line. Inhibition of DNR uptake by the 4β-phorbol ester (in P388 cells) was not reversed by 5–25 μM verapamil (Table 1).

Treatment with 10 nM 4β-phorbol ester for 2 hr protected P388 cells from the cytotoxic effects of a 1-hr exposure to DNR (Table 2). But when the time of exposure to DNR was increased to 24 hr, the 2-hr phorbol treatment did not alter DNR toxicity (data not shown).

The effect of phorbol esters on DNR accumulation by P388 cells appears to derive from inhibition of uptake, rather than on activation of an outward transport system (Fig. 1). Even at the earliest time points measured, uptake of DNR in phorbol-treated P388 cells was impaired. Accumulation of DNR by phorbol-treated P388 cells was not enhanced by use of glucose-free medium containing 10 mM sodium azide, nor was DNR efflux from P388 cells altered by treatment with the phorbol ester (Fig. 1). In contrast, uptake of anthracyclines by P388/ADR cells was inhibited by azide, an effect traced to inhibition of energy-dependent outward drug transport [20, 21].

To examine the relative numbers of phorbol ester binding sites on P388 vs P388/ADR cells, we incubated cultures for 2 hr with 10 nM labeled phorbol dibutyrate. Binding was proportional to phorbol concentration and reached a steady-state at this time point. Under these conditions, the binding of labeled phorbol was 2-fold greater in P388 cells, but increasing the level of 4β-phorbol ester to 20 nM did not affect any result described above.

* Abbreviations: ADR, adriamycin; DNR, daunorubicin; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; and MDR, multi-drug resistance.