Pergamon

Biochemical Pharmacology, Vol. 50, No. 3, pp. 337–346, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)00147-6

PROTEIN KINASE C INHIBITORS REDUCE PHORBOL ESTER-INDUCED RESISTANCE TO METHOTREXATE IN CHINESE HAMSTER OVARY CELLS

VÉRONIQUE NOÉ and CARLOS J. CIUDAD*

Biochemistry Unit, School of Pharmacy, University of Barcelona, Barcelona, 08028 Pedralbes, Spain

(*Received* 16 *September* 1994; *accepted* 15 *March* 1995)

Abstract—Phorbol 12-myristate 13-acetate (TPA) increases the number of colonies surviving methotrexate (MTX) exposure in a dose-dependent manner upon short incubation with Chinese hamster ovary (CHO) cells. Seventy percent of the isolated colonies showed increased copy number for the *dihydrofolate reductase* gene. EGTA prevents the increase in resistance triggered by TPA. Calcium ionophore A23187 and angiotensin II also increase this resistance, suggesting that calcium is involved in this process. Protein kinase C (PKC) from CHO cells is rapidly activated by TPA, A23187 and angiotensin II. PKC inhibitors, 1-(5-Isoquinolinylsulphonyl)-2-methyl-piperazine (H-7), glycyrrhetinic acid, staurosporine and calphostin C decrease the generation of resistant colonies to MTX upon incubation with TPA. However, 5 nM staurosporine on its own increases resistance to MTX while having the ability to translocate CHO PKC. *In vitro*, H-7, staurosporine and calphostin C inhibit PKC activity translocated by TPA incubation with CHO cells. We conclude that PKC, the activity of which is dependent on calcium and phospholipids, is part of the pathway that leads to development of increased resistance to MTX. Thus, inhibition of PKC prevents the appearance of this resistance. Our results suggest the possibility of using non-toxic PKC inhibitors as resistance modulators in MTX chemotherapy.

Key words: methotrexate; dhfr; PKC; TPA; calphostin C; staurosporine

Drug resistance is a serious problem associated with cancer chemotherapy. This resistance can be produced by mutation or gene amplification of the locus encoding for the target of chemotherapy, by a decrease in the transport of the drug or by development of the MDR phenotype [1] in which the drug is pumped outside the cell at higher than the normal rate.

In chemotherapy using MTX†, the appearance of resistant clones which present decreased transport of this drug [2], mutation of the dhfr protein [3, 4], or gene amplification of the *dhfr* locus [5] have been observed. Using different cell lines it is possible to obtain resistant colonies simply by incubating the cells with stepwise concentrations of MTX. However, the number of resistant colonies can be greatly enhanced if cells are pretreated with a number of different agents before the MTX selection is applied. This category includes phorbol esters such as TPA [6, 7] and vasopressin [8].

Taking as a basis the original observation by Varshavsky that TPA enhances resistance to MTX, mainly by gene amplification, and considering that TPA activates PKC, we decided to explore the possibility that PKC might be involved in the pathway causing this kind of resistance. In this report, we confirm that TPA increases the number of resistant colonies in CHO K1 cells, and demonstrate that both calcium ionophore A23187 and angiotensin II produce the same effect. We also document that the above agents activate CHO protein kinase C. Moreover, we show that H-7, glycyrrhetinic acid, staurosporine and calphostin C, which inhibit PKC *in vitro*, are able to decrease the number of resistant colonies to MTX upon cell incubation with TPA.

* Corresponding author. Tel. +34-3-402-4522; FAX +34-3-402-1896; email: cciudad@farmacia.far.ub.es.

† Abbreviations: TPA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; H-7, 1-(5-Isoquinolinylsulphonyl)-2-methylpiperazine; dhfr, dihydrofolate reductase; MTX, methotrexate; CHO, Chinese hamster ovary; PBS, phosphate buffered saline; PCR, polymerase chain reaction; SDS, sodium dodecyl sulphate; dNTP, deoxyribonucleotides.

MATERIALS AND METHODS

Materials. TPA, ethylene glycol-bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid, calcium ionophore A23187, angiotensin II; protein kinase inhibitors H-7, 18-*O*-β-glycyrrhetinic acid, staurosporine, calphostin C, trypsin, phenylmethylsulphonyl fluoride, leupeptin, aprotinin, Nonidet P-40, histone type III-S, phosphatidyl serine and 1-oleoyl-2-acetyl-rac-glycerol were from Sigma Chemical Co. (St. Louis, MO, U.S.A.) or Sigma Química (Madrid, Spain). Paraffin wax (melting temperature 58–60°) was from Fluka. Proteinase K and oligonucleotides were purchased from Boehringer Manheim. Taq DNA polymerase, Ham's F12 cell culture medium and selective F12 medium lacking glycine, hypoxanthine and thymidine (−GHT) as well as fetal calf serum were from GIBCO/BRL. Foetal calf serum used in the selective medium was dialysed against PBS for three days (2 changes per day) all at 4°. [γ-^{32}P] ATP and [α-^{32}P]dATP, both 3000 Ci/mmol, were from Amersham, and methotrexate came from Almirall laboratories (Barcelona, Spain).

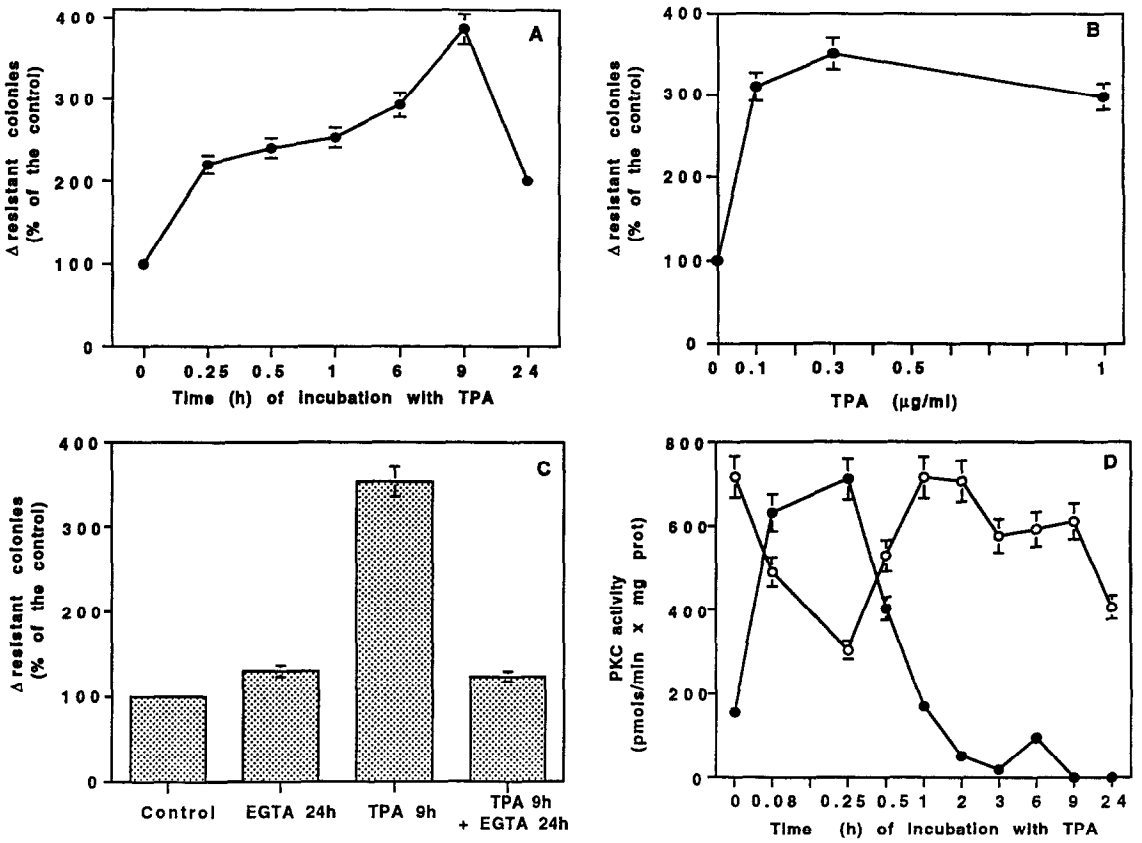

Fig. 1. (A) CHO K1 cells were incubated with 0.3 μg/mL TPA for the indicated times. Then, medium lacking glycine, hypoxanthine and thymidine (−GHT) was replaced and after 15 hr MTX (3×10^{-7} M) was added and renewed once a week until appearance of resistant colonies which were stained and counted. The 100% control value corresponds to a basal resistance frequency of 4×10^{-5} with respect to the population of cells present before addition of MTX. (B) Concentration dependence of the effect of TPA in increasing resistant colonies to MTX. Cells were incubated for 9 hr with the indicated concentrations of the phorbol ester. Other conditions as in Fig. 1A. (C) Effect of EGTA on TPA-induced resistance to MTX. Cells were pre-incubated with 0.5 mM EGTA for 30 min before addition of 0.3 μg/mL TPA (9 hr). Then, the medium was replaced maintaining the same concentration of EGTA for 15 hr. At this point selective medium (−GHT) including 3×10^{-7} M MTX was added. Other conditions as in Fig. 1A. (D) Time course of the effect of TPA on PKC activity from CHO cells. K1 cells were incubated with 0.3 μg/mL TPA for the indicated periods of time and then assayed for PKC activity in both membrane (●) and cytosolic (○) fractions as described in the Materials and Methods sections. Results are the mean ± SE of five experiments (A, B and D) or three experiments (C).

Cell lines. CHO cells were used throughout this study. K1 cells bearing two copies of the *dhfr* gene were obtained from Dr Lawrence A. Chasin (Columbia University, NY, U.S.A.). These cells were grown as monolayers [9] in Ham's F12 medium containing 7% foetal calf serum, and passaged with the aid of 0.075% trypsin.

Colony formation assay. Cells were plated (2×10^5 per 10 cm dish) in 10 mL of Ham's F12 medium containing 7% foetal calf serum. After 14 hr, the cells were incubated with the effectors. At the end of the incubation, the medium was removed and replaced with Ham's F12 medium lacking glycine, hypoxanthine and thymidine (for *dhfr* selection) containing 5% dialysed foetal calf serum. Following an expression time of 15 hr, MTX 3×10^{-7} M was added. Even though the minimum time needed to observe the cytotoxic response to MTX is between 2 and 3 days, we applied MTX pressure until the development of visible colonies (16–21 days). Both the medium and the MTX were renewed once a week. The surviving colonies were fixed with 2% formaldehyde, stained with crystal violet and counted. Resistance is expressed as the percentage of the number of colonies surviving selection with MTX after treatment with the effectors in relation to the number of resistant colonies obtained with MTX alone.

Determination of the dhfr *copy number from single resistant colonies.* The *dhfr* copy number was determined by quantitative PCR in resistant colonies to MTX using the method of Noé *et al.* [10] with slight modifications. The colonies (approx. 100 cells) were picked using cloning rings and 0.075% trypsin,

Table 1. *dhfr* copy number of methotrexate-resistant colonies

Clone	Optical density (arbitrary units)		*dhfr/aprt* optical density ratio	*dhfr* copy number per diploid cell
	dhfr	*aprt*		
WT (control)	100	100	1	2
MTX resistant				
1	124	28	4.4	8.8
2	213	49	4.3	8.6
3	205	48	4.3	8.6
4	98	24	4	8
5	148	75	2	4
6	64	41	1.5	3
7	96	47	2	4
8	118	18	6.5	13
9	73	40	1.8	3.6
10	99	23	4.3	8.6
11	106	48	2	4
12	109	8	14	28
13	115	20	6	12
14	53	48	1.2	2.4
15	92	74	1.2	2.4
16	88	20	4.4	8.8
17	198	169	1.2	2.4
18	48	3.5	13	26
19	68	40	1.7	3.4
20	52	42	1.2	2.4
21	31	21	1.4	2.8
22	43	18	3.3	6.6
23	102	110	0.9	1.8
24	27	7	3.8	7.6
25	78	57	1.3	2.6

CHO cells were incubated with TPA (0.3 μg/mL) for 9 hr, and 15 hr later subjected to selection with 3×10^{-7} M MTX for 3 weeks. Well-isolated resistant colonies were picked and used as starting material for quantitative PCR determination of the *dhfr* gene copy number. Autoradiographies were quantified by image analysis and the results normalized using the *aprt* signal as the internal control.

and resuspended in ice-cold PBS. The cells were centrifuged at $10,000 \times g$ for 5 min and after discarding the supernatant, resuspended in 20 μL of lysis buffer (20 mM NaCl, 1 mM EDTA, 0.1% SDS, and 50 mM Tris-HCl, pH 8) plus 2 μL of 10 mg/mL proteinase K. They were then incubated for 15 min at 55°, vortexed vigorously and incubated for 15 min at the same temperature. Finally, the mixture was incubated for 5 min at 100° and after cooling, 2 μL of this mixture was used for PCR amplification.

Each 50 μL PCR reaction contained: the cell mixture (2 μL), 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM dNTPs, 1.25 μCi of [α-^{32}P]-dATP, 1 unit of Taq DNA polymerase, 500 ng of each of two primers and 20 mM Tris-HCl, pH 8.4. The primers used for *dhfr* amplification were 5′-CCTGTTAACGC-AGTGTTTCTC-3′ inside intron 1 and 5′-TCCC-ACGGGAGACTTCGCACT-3′ within intron 2. For *aprt* the oligonucleotides were 5′-TCACG-AGCCAGCAAGGCGTT-3′ within intron 1 and 5′-ACGCAGTACTCATCCAGGGT-3′ within intron 2. The *aprt* gene was used as the internal control in the PCR to normalize the results. The PCR for both genes, *dhfr* and *aprt*, was performed in the same tube using hot-start with the aid of paraffin wax. The PCR was performed for 35 cycles after denaturation for 1 min at 94°. Each cycle consisted of denaturation for 1 min at 92°, primer annealing for 1 min at 59°, and primer extension for 1 min at 72°, using a MJ Research thermocycler equipped with peltier system and temperature probe. Ten microlitres of each PCR sample were electrophoresed on a 5% polyacrylamide (30:1 acrylamide:bis)/1 X TBE gel [11]. The gels were dried and subjected to autoradiography using Kodak X-ray films to show the amplified DNA products. The quantification of the intensity of the radioactive bands was carried out by image analysis (Vilbert Lourmat). Results are expressed as the optical density (OD) of the *dhfr* gene signal relative to that of the *aprt* gene signal used as reference.

Subcellular fractionation and PKC activity assays. Cytosolic and membrane PKC was prepared and PKC activities assayed as follows: CHO K1 cells (5×10^6) were washed twice in ice-cold PBS and homogenized (20 passes) in 0.7 mL of buffer A (2 mM EDTA, 0.5 mM ethylene glycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid), 20 μg/mL leupeptine, 2 μg/mL aprotinine, 1 mM phenylmethylsulphonyl fluoride, 1 mM dithiothreitol

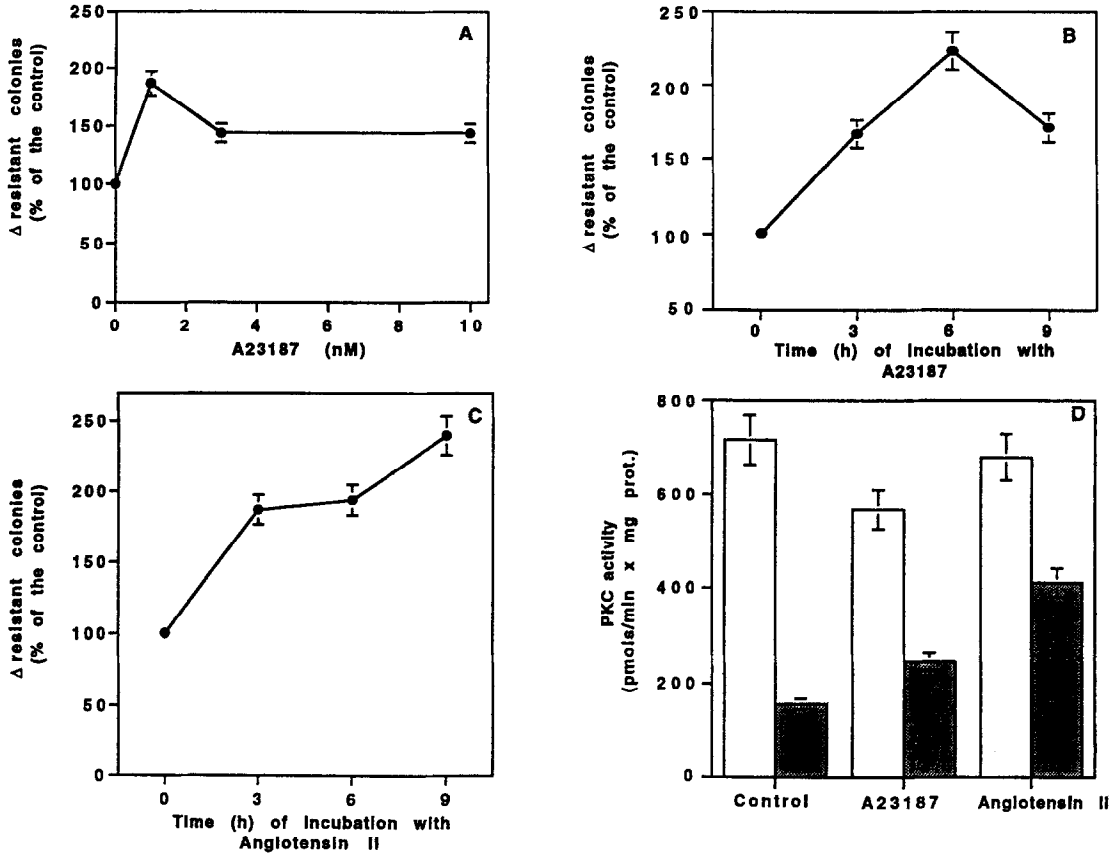

Fig. 2. Concentration (A) and time dependence (B) of the effect of calcium ionophore A23187 on the generation of resistant colonies to MTX. The ionophore was added for 9 hr or at 1 nM, respectively. (C) Time dependence of the effect of angiotensin II. The peptide was added for the indicated times at the concentration of 1 μM. Other conditions as in Fig. 1A. (D) Cytosolic (empty bars) and membrane (dotted bars) PKC activity of CHO cells incubated with 1 nM A23187 or 1 μM angiotensin II for 15 min. Results are the mean ± SE of three experiments.

and 20 mM Tris/HCl, pH 7.5) in a glass Potter Elvehjem homogenizer. The homogenate was centrifuged at 100,000 *g* for 30 min and the supernatant designated as the cytosolic fraction. The pellet was washed once in 0.7 mL buffer A and then resuspended in 100 μL of buffer A containing 0.1% Nonidet P-40. The suspension was rocked for 30 min and then centrifuged at 100,000 *g* for 30 min, all at 4°. The resulting supernatant was designated as the membrane fraction.

PKC activities were determined in both cytosolic and membrane fractions by measuring the transfer of ^{32}P from [γ-^{32}P]ATP to histone III-S. The reaction mixture (60 μL) contained the enzymatic source (20 μL), 24 μg histone III-S, 10 mM magnesium acetate, 50 μM [γ-^{32}P]ATP (200–400 cpm/pmol) and 20 mM Tris/HCl, pH 7.5, in the presence of either 1 mM EGTA, or 0.5 mM $CaCl_2$, 3.5 μg phosphatidylserine and 0.7 μg 1-oleoyl-2-acetyl-rac-glycerol. Following incubation for 4 min at 30°, the reaction was stopped by spotting 50 μL of the reaction mixture onto phosphocellulose P81 Whatman® chromatography papers (2 × 2 cm) and immediately placing them in 75 mM ice-cold phosphoric acid. The papers were washed twice in the same solution, dried and counted in an LKB scintillation counter after addition of 5 mL Biogreen. PKC activity was calculated by subtracting the incorporation of phosphate in the absence of Ca^{2+} and phospholipids to that in the presence of these effectors.

One unit of enzymatic activity is defined as the amount of enzyme that catalyses the transfer of 1 pmol of phosphate from ATP to histone per min at 30°.

Protein was determined by the Bradford method [12] with bovine serum albumin as standard so as to calculate PKC specific activity.

RESULTS

Effect of TPA, ionophore A23187 and angiotensin II on the resistance to MTX and on PKC activity in CHO cells

Incubation of K1 CHO cells with 0.1 μg/mL TPA for a week increased by 3.5-fold the number of colonies surviving a concentration of MTX of 3×10^{-7} M, which alone gave a value of basal resistance frequency of the order of 10^{-5}. TPA

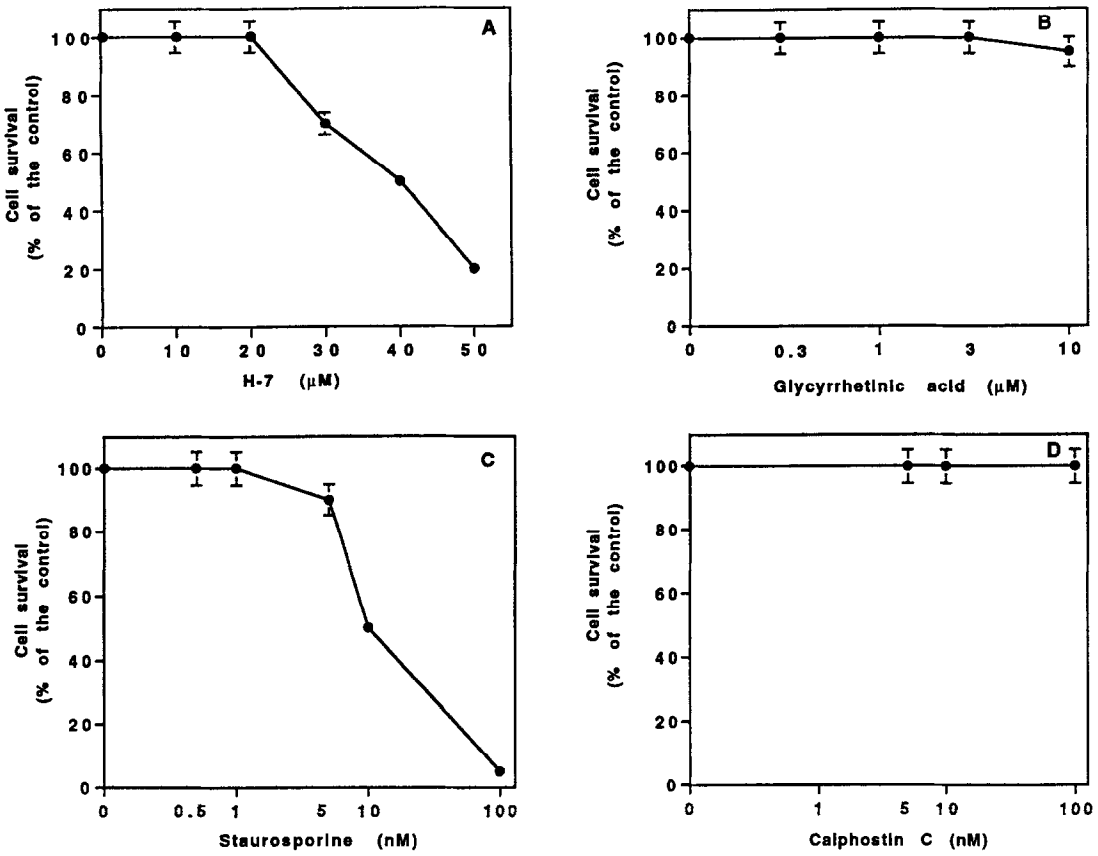

Fig. 3. Effect on cell survival of incubation with PKC inhibitors H-7 (A), glycyrrhetinic acid (B), staurosporine (C) or calphostin C (D). PKC inhibitors were maintained in the culture medium for 24 hr and fresh F12 medium was then added until appearance of colonies which were stained and counted. Results are the mean ± SE of two separate experiments.

increases cellular PKC activity after provoking its translocation to membranes. Therefore, the results described earlier by Varshavsky [6] and reported in this study could be explained by an increase in the activity of this kinase. However, given that in most cell types PKC is proteolysed as a result of permanent TPA incubation, it is not possible to distinguish whether the increase observed in the number of colonies could be due to an early translocation and activation of the enzyme or to its down regulation after the prolonged (1 or 3 weeks) incubation with the phorbol ester in these experiments. For that reason, we studied the time dependence of the increase in drug resistant colonies by TPA. In Fig. 1A it is shown that the number of MTX-resistant colonies was already increased upon 15 min–1 hr of incubation with TPA (0.3 μg/mL), reaching a peak value at 9 hr and decreasing slightly thereafter. The number of drug resistant colonies to a fixed concentration of 3×10^{-7} M MTX was also dependent on the concentration of TPA used (Fig. 1B). The maximal response to TPA (9 hr) was found at 0.3 μg/mL. However, the presence of MTX during the incubation with TPA did not increase the effectiveness of the phorbol ester. Moreover, the presence of actinomycin D or cycloheximide during the 15 hr period before exposure to MTX did not block the effect of TPA.

As in 3T6 cells, the mechanism underlying this resistance in CHO cells also appears to be gene amplification. This was established by determining the copy number for the *dhfr* gene in the resistant colonies by quantitative PCR analysis using oligonucleotides for intron sequences and taking the *aprt* gene as reference. It was found that more than 70% of the isolated resistant colonies contained increased copy number, with an average of seven copies of the *dhfr* gene (Table 1). Cell distribution throughout the cell cycle after incubation with TPA for 9 hr was also checked to determine if activation of PKC could slow down or arrest cell growth so that cells were not as sensitive to the cytotoxicity of MTX. The results obtained by flow cytometry revealed no significant changes versus control cells growing exponentially (data not shown).

Given that some PKC isoforms are calcium-dependent [13], we tested the action of EGTA on cells incubated with 0.3 μg/mL TPA for 9 hr. EGTA (0.5 mM) was added to the cell medium 30 min before incubation with the phorbol ester and

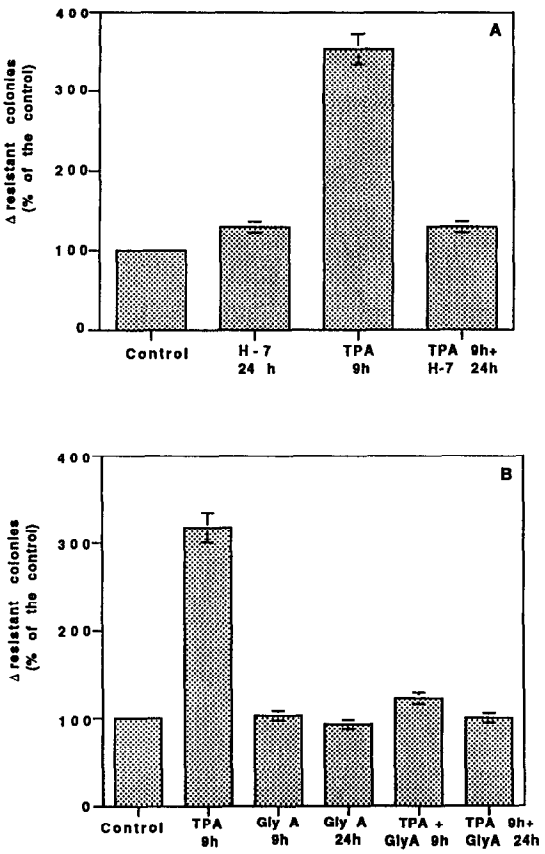

Fig. 4. Effect of PKC inhibitors H-7 and glycyrrhetinic acid on TPA-induced resistance to MTX. This phorbol ester (0.3 μg/mL) was incubated with the cells for 9 hr in the absence or the presence of 20 μM H-7 (A) or 10 μM glycyrrhetinic acid (Gly A) (B) for 24 hr. The inhibitors were added 30 min before TPA. In B, glycyrrhetinic acid was also tested for 9 hr. Other conditions as in Fig. 1A. Results are the mean ± SE of three separate experiments.

maintained for a total period of 24 hr. As can be observed in Fig. 1C, the specific calcium chelator was able to reduce the number of drug resistant colonies previously increased by the effect of TPA. We also tested whether agents that cause calcium mobilization could also increase drug resistance to MTX. We used A23187 as calcium ionophore; as is shown in Fig. 2A this ionophore at concentration of 1 nM clearly increased the number of resistant colonies to MTX. The effect of A23187 was dependent on the time of incubation (Fig. 2B). Angiotensin II, which acts through a calcium mechanism, was also able to increase resistance to MTX when incubated at 1 μM for different periods of time (Fig. 2C). Treatment of the cells with either TPA, A23187 or angiotensin II did not cause any change in the number of cells present after the 15 hr expression period before exposure to MTX.

Given the possible relationship between PKC activation and the generation of resistant colonies, we needed to know the time dependence of PKC translocation by TPA in CHO cells. However, to our knowledge there were no such data with respect to CHO cells. For that reason, we studied the effect of TPA, calcium ionophore A23187 and angiotensin II on CHO PKC activity.

As shown in Fig. 1D, TPA (0.3 μg/mL) was able to translocate and activate PKC in CHO cells after short incubations (5–15 min) and after 24 hr had depleted PKC in the membrane fraction and reduced the activity found in the cytosol by 60%. We then assayed PKC activity upon treatment of CHO cells with 1 nM ionophore A23187 or 1 μM angiotensin II. These agents were also able to activate PKC after 15 min of incubation, albeit to a much lesser extent than with TPA (Fig. 2D). The activation of PKC by both agents was transient, and after 3 hr of incubation the activity returned to basal levels. These results supported the possibility that the increase in the number of MTX-resistant colonies by TPA, A23187 and angiotensin II could have been triggered by an early activation of PKC, as it began subsequent to translocation and activation of the enzyme.

Effect of PKC inhibitors on MTX resistance produced by TPA and on PKC activity in CHO cells

We first determined the concentration of each PKC inhibitor that would not affect cell survival after 24 hr incubation (Fig. 3). The inhibitors were added 30 min before TPA (0.3 μg/mL, 9 hr) and upon removal of the phorbol ester, added for a further 15 hr to maintain PKC inhibition. At this point selection with MTX (3×10^{-7} M) was applied until appearance of resistant colonies. The medium and MTX were renewed once a week.

The first inhibitor used was H-7 [14] which, even if it is not completely specific for PKC, has been used for this purpose [15, 16]. H-7 at a concentration of 20 μM (Fig. 4A) was able to significantly decrease the number of resistant colonies to MTX provoked by TPA. Then, we assayed the effect of glycyrrhetinic acid, a compound of low toxicity found in the natural product licorice and reported [17] to inhibit purified PKC *in vitro*. Glycyrrhetinic acid at 10 μM (Fig. 4B) was also able to reduce the number of resistant colonies produced by the effect of TPA. Staurosporine is a potent inhibitor of PKC although its use in cell incubation has to be kept to a maximum concentration of 5 nM. Higher concentrations of staurosporine led to apoptosis (Fig. 3C), in agreement with the observations by Falcieri [18]. Staurosporine (5 nM) (Fig. 5A) caused a decrease in the number of resistant colonies developed by TPA in CHO cells. However, although staurosporine was effective in counteracting this resistance, this inhibitor on its own, at 5–10 nM, was also able to behave as an agonist increasing the number of resistant colonies. We studied this effect in more detail and found it to be dose- and time-dependent (Figs. 5A and B). The effect of staurosporine on the generation of resistant colonies could be diminished by the action of EGTA (0.5 mM) (Fig. 5C). Since staurosporine has been reported to have the ability to translocate PKC to membranes [19, 20], we checked for translocation of PKC by staurosporine in CHO cells. We found that in these cells, staurosporine alone (5–10 nM) was able to translocate PKC, showing a two-fold increase in the activity bound to membranes, and that

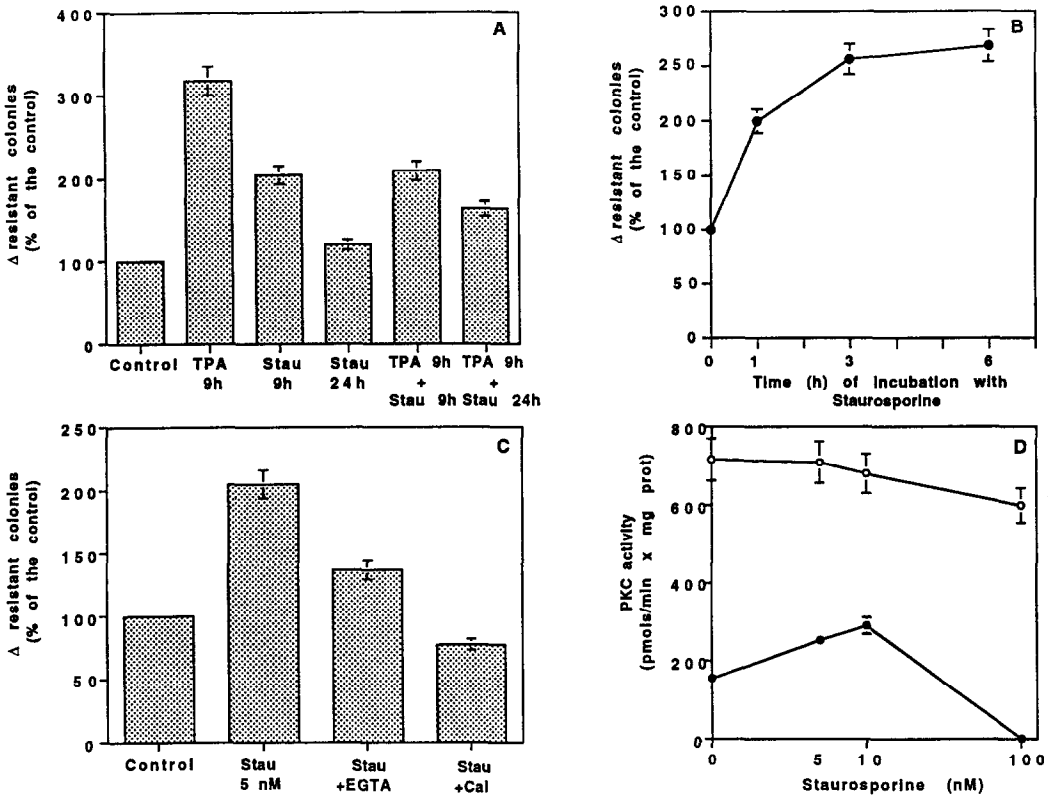

Fig. 5. Effects of staurosporine. (A) Counteraction by staurosporine (Stau) of TPA-induced resistance to MTX. K1 cells were incubated with TPA (0.3 μg/mL) for 9 hr in the absence or in the presence of 5 nM staurosporine either for 9 or 24 hr. Staurosporine was added 30 min before TPA. (B) Time dependence of the effect of staurosporine treatment on resistance to MTX. The inhibitor was added for the indicated periods of time at the concentration of 10 nM. (C) Effect of EGTA or calphostin C on staurosporine-induced resistance to MTX. Staurosporine (5 nM) was added for 9 hr in the presence of EGTA at a concentration of 0.5 mM or calphostin C at a concentration of 0.1 μM for 24 hr. EGTA or calphostin C were added 30 min before staurosporine. Other conditions as in Fig. 1A. (D) Dose-response of the effect of staurosporine on PKC activity in membrane (●) and cytosolic (○) fractions from CHO cells. Staurosporine was incubated with the cells for 30 min at the indicated concentrations. Results are the mean ± SE of three separate experiments.

membrane-associated activity was completely inhibited when 100 nM staurosporine was used (Fig. 5D).

In order to circumvent the agonist effect of staurosporine, we used another highly specific PKC inhibitor: calphostin C [21]. This inhibitor alone did not cause an increase in the number of resistant colonies to MTX and reduced those produced by cell incubation with TPA (Fig. 6A) to basal levels without altering the capability of this phorbol ester to translocate PKC (data not shown). Calphostin C did not translocate PKC and was able to inhibit the basal activity bound to the membrane fraction upon cell incubation (Fig. 6B).

Moreover, cell pre-incubation with 10^{-7} M calphostin C completely blocked the ability of staurosporine to increase the number of resistant colonies (Fig. 5C). We also determined, *in vitro*, the effect of different protein kinase inhibitors on PKC activity in membrane extracts from CHO cells incubated with TPA. The three PKC inhibitors tested (H-7, staurosporine and calphostin C) inhibited PKC activity translocated by TPA, in a dose-dependent manner (Table 2). It is worth noting that 5–10 nM staurosporine was able to partially inhibit (20–30%) TPA-translocated PKC activity.

Finally, we explored the potential ability of calphostin C to decrease resistance to MTX in the absence of TPA. Due to the low frequency of resistant colonies found at 3×10^{-7} M MTX, we decreased the dose to 10^{-7} M and 2×10^{-7} M MTX to sensitize the method. Under these conditions, the presence of 3×10^{-7} M or 10^{-6} M calphostin C for 6 days, which did not affect cell survival, led to a significant decrease in the basal resistance to MTX (Fig. 6C) [22].

DISCUSSION

The reduction of drug resistance during cancer chemotherapy constitutes in itself a kind of chemotherapy since it is of vital importance to the success of the treatment. Our experimentation ties in with the efforts to study the biochemical pathways

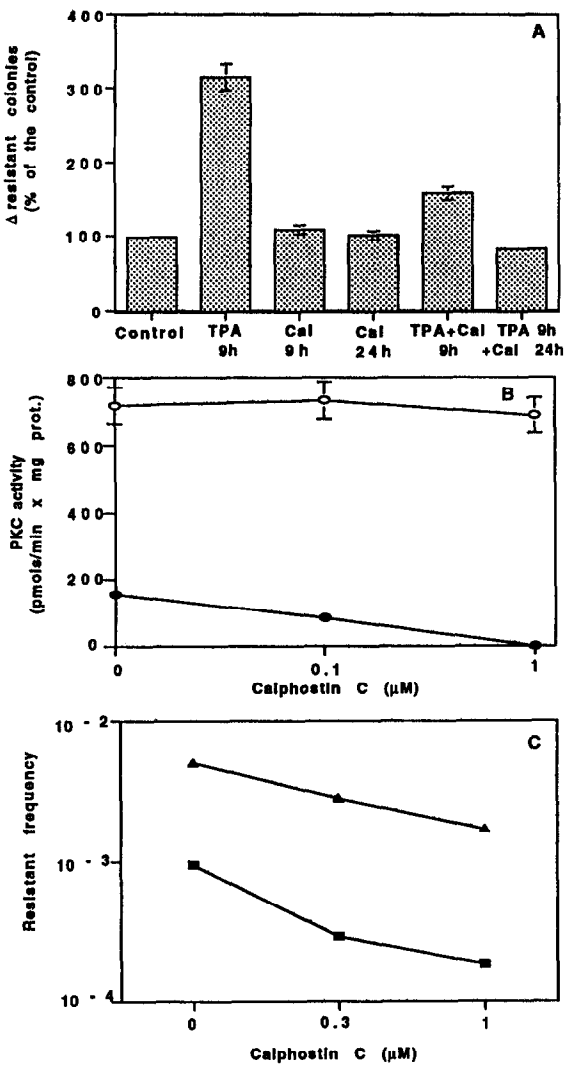

Fig. 6. Effects of calphostin C. (A) Effect of calphostin C on TPA-induced resistance to MTX. This phorbol ester (0.3 μg/mL) was incubated with the cells for 9 hr in the absence or the presence of 0.1 μM calphostin C for 9 or 24 hr. The inhibitor was added 30 min before TPA. Other conditions as in Fig. 1A. To light-activate calphostin C, the cells were incubated with the inhibitor under ordinary fluorescent light for 60 min [30]. (B) Dose dependence of the effect of calphostin C on the activity of PKC of membrane (●) and cytosolic (○) fractions from CHO cells. Calphostin C was incubated for 30 min at the indicated concentrations. (C) Effect of calphostin C on basal resistance to MTX. Calphostin C was added for 6 days at the indicated concentrations in the presence of 10^{-7} M (▲) or 2×10^{-7} M (■) MTX. MTX selection was applied until the formation of visible colonies (3 weeks). Results are the mean ± SE of three separate experiments.

that lead to the development of drug resistance, a first step in finding ways to decrease it. Given the results by Varshavsky [6] regarding the increase in resistance to MTX upon cell incubation with TPA and the fact that TPA activates PKC, we explored the possibility that PKC might be involved in the pathway leading to the production of MTX-resistant colonies. First, we confirm the observation made by Bojan [23], that TPA is able to generate resistance to MTX treatment in CHO cells. These authors also observed that TPA increased resistance to MTX, *N*-(phosphonacetyl)-L-aspartate and cadmium no more than three-fold in hamster cells. K1 cells are less prone to present resistance than 3T6 cells which might be inherent in the different cell type. We also report that the main mechanism for the observed resistance in CHO cells is gene duplication because of the increased copy number of the gene encoding for dhfr in the resistant colonies. In addition, we document that agents which cause calcium mobilization, such as ionophore A23187 and angiotensin II, and which activate PKC are able to increase the frequency of appearance of resistant colonies to MTX.

One interesting finding is that drug resistance can be demonstrated without maintaining the presence of the phorbol ester in the incubating medium throughout the whole selection with MTX. In fact, we show that the appearance of resistant colonies is readily observed if cells are incubated with TPA for 15 min. According to our results on PKC activity in CHO cells, this enzyme is maximally translocated and activated after 15 min of incubation with the phorbol ester (Fig. 1D). Therefore, considering both time courses, it is conceivable that the generation of resistant colonies to MTX could be triggered by the translocation and activation of PKC. The generation of resistant colonies by TPA might not be related to the proteolysis of this enzyme, given that the PKC inhibitor calphostin C cannot prevent PKC translocation by TPA whereas it is very effective in reducing the frequency of resistance to MTX produced by the phorbol ester to basal levels. PKC is present in CHO cells in different isoforms [24], mainly α and δ, with different responsiveness towards effectors. PKC might then constitute a sensor protein which further transmits the signal that triggers development of resistance to MTX. Our results with ionophore A23187, angiotensin II and TPA plus EGTA suggest that this resistance could be brought about, at least in part, by calcium-dependent isoenzymes. Since the four PKC inhibitors tested (H-7, glycyrrhetinic acid, staurosporine and calphostin C), used at concentrations that would not cause cell death, are able to counteract MTX resistance increased by TPA, it is suggested that PKC is part of the pathway that leads to generation of resistant colonies.

It is interesting to consider the dual effect, as shown in the Results section, exerted by staurosporine. This behaviour could be explained by the fact that staurosporine translocates PKC to membranes as shown in inside-out vesicles from erythrocyte membranes [19], hepatocytes [20] and in CHO cells (this report). The resulting membrane PKC activity after cell incubation with staurosporine depends on the concentration of this particular inhibitor. At low concentrations (5–10 nM), the translocated enzyme shows increased activity over the control, whereas at a higher concentration of staurosporine the translocated PKC is completely inhibited (Fig. 5D). These results are in agreement

Table 2. *In vitro* effect of PKC inhibitors

	PKC activity in membrane fraction (pmols/min × mg protein)	% of inhibition of translocated activity
Control	156 ± 14	
TPA (0.3 μg/mL, 15 min) + Inhibitor	711 ± 55	
H-7 (20 μM)	257 ± 18	81
H-7 (50 μM)	0	100
Staurosporine (5 nM)	595 ± 49	21
Staurosporine (10 nM)	535 ± 40	32
Staurosporine (100 nM)	0	100
Calphostin C (0.1 μM)	618 ± 43	17
Calphostin C (0.3 μM)	576 ± 45	24
Calphostin C (1 μM)	458 ± 35	45

CHO cells were incubated with TPA (0.3 μg/mL) for 15 min and then subjected to subcellular fractionation. PKC activity was determined as described in the Materials and Methods section, in the absence or in the presence of PKC inhibitors at the indicated concentrations. The inhibitors were added to the reaction mixture and incubated with membrane aliquots obtained from TPA-treated cells. Results are the mean ± SE of three separate experiments.

with those of Wolf and Baggiolini [19], who found that staurosporine translocated PKC and that the membrane-associated PKC was inhibited by staurosporine at concentrations of 20 nM and above, either in the absence or in the presence of phorbol ester. Therefore, at the concentration of 5 nM staurosporine used in the cell incubation for reasons of viability, the inhibitor could be acting as an agonist when incubated alone, but yet be able to counteract the action of TPA. In fact *in vitro*, staurosporine is able to inhibit in a dose-dependent manner PKC activity translocated to the membrane fraction by the action of TPA (Table 2). The dual effect shown by staurosporine could be interpreted as a further indication of the involvement of PKC in the generation of resistance towards MTX. In addition, calphostin C is able to abolish the effect on the resistance produced by staurosporine alone (Fig. 5C).

These results are in keeping with the behaviour showed by TNR9 cells, a cell line variant derived from 3T3, which do not develop drug resistance by amplification upon TPA incubation [25]. The analysis of the characteristics of these cells revealed that they show only 10–15% of the normal content of phosphorylated 80 kDa PKC substrate [26].

Clearly, PKC is involved in the cellular response to drugs that develop resistance through different mechanisms. It has been shown that the MDR phenotype, which induces resistance to various cytotoxic natural products such as anthracyclines, epipodophyllotoxins, antibiotics and *Vinca* alkaloids, is accompanied by changes in the activity of PKC [27, 28]. Activation of PKC *in vivo* results in phosphorylation of P-glycoprotein and a decrease in drug accumulation [29] while inhibition of PKC can partially reverse the MDR phenotype [30]. We have shown that the presence of PKC inhibitors counteracts the effect of TPA, which increases the number of colonies surviving MTX exposure mainly by gene amplification.

According to the results presented herein, we conclude that the process of formation of colonies resistant to MTX can take place through a mechanism involving PKC. Inhibition of PKC activity leads to a reduction in the number of resistant colonies increased by TPA. Thus mechanistically, basal resistance to MTX could also be dependent on PKC activity. To evaluate the possibility of clinical use of PKC inhibitors in chemotherapy treatment with MTX, we are studying conditions under which these inhibitors could decrease basal resistance generated by MTX alone. To this end, by using lower concentrations of MTX ($1–2 \times 10^{-7}$ M) and calphostin C for prolonged periods of time, we have been able to demonstrate that this PKC inhibitor is also effective in reducing basal resistance to MTX.

Acknowledgements—This work was supported by grants 92/0775 from FISss and SAF94–0177 from CICYT. The authors thank Dr Lawrence A. Chasin (Biol. Sci., Columbia Univ) and Dr J. C. Laguna (Pharmacology, University of Barcelona) for helpful discussions; Mr Robin Rycroft from the Language Advisory Department in revising the English manuscript; and Ms Cristina Alemany for technical assistance. C. J. C. was a recipient of a fellowship from CIRIT of Catalonia. V. N. is a recipient of a predoctoral fellowship from the Spanish Ministry of Education.

REFERENCES

1. Rogan AM, Hamilton TC, Young RC, Klecker RW and Ozols RF, Reversal of adriamycin resistance by verapamil in human ovarian cancer. *Science* **224:** 994–996, 1984.
2. Sirotnak FM, Moccio DM, Kelleher LE and Goutas LJ, Relative frequency and kinetic properties of transport-defective phenotypes among methotrexate-resistant L1210 clonal cell lines derived *in vivo*. *Cancer Res* **41:** 4447–4452, 1981.
3. Flintoff WF, Davidson SV and Siminovitch, Isolation and partial characterization of three methotrexate-resistant phenotypes from Chinese hamster ovary cells. *Somat Cell Genet* **2:** 245–261, 1976.
4. Haber DA, Beverley SM, Kiely ML and Schimke RT, Properties of an altered dihydrofolate reductase encoded by amplified genes in cultured mouse fibroblasts. *J Biol Chem* **256:** 9501–9510, 1981.
5. Alt FW, Kellems RE, Bertino JR and Schimke RT, Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells. *J Biol Chem* **253:** 1357–1370, 1978.
6. Varshavsky A, Phorbol ester dramatically increases incidence of methotrexate-resistance mouse cells: Possible mechanisms and relevance to tumor promotion. *Cell* **25:** 561–572, 1981.
7. Ferguson PJ, and Cheng Y-C, Transient protection of cultured human cells against antitumor agents by 12-O-Tetradecanoylphorbol-13-acetate. *Cancer Res* **47:** 433–441.
8. Barsoum J and Varshavsky A, Mitogenic hormones and tumor promoters greatly increase the incidence of colony-forming cells bearing amplified dihydrofolate reductase genes. *Proc Natl Acad Sci USA* **80:** 5330–5334, 1983.
9. Ciudad CJ, Urlaub G and Chasin LA, Deletion analysis

of the Chinese hamster dihydrofolate reductase gene promoter. *J Biol Chem* **263:** 16274–16282, 1988.
10. Noé V, Alemany C and Ciudad CJ, Determination of dihydrofolate reductase gene amplification from single cell colonies by quantitative polymerase chain reaction. *Anal Biochem* **224:** 600–603, 1995.
11. Sambrook J, Fritsch EF and Maniatis T, Gel electrophoresis. In: *Molecular cloning: A laboratory manual* (2nd edn, pp 149–185. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.
12. Bradford M, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
13. Hug H and Sarre TF, Protein kinase C isoenzymes: divergence in signal transduction?. *Biochem J* **291:** 329–343, 1993.
14. Hidaka H, Inagaki M, Kawamoto S and Sasaki Y, Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry* **23:** 5036–5041, 1984.
15. O'Brian CA, Fan D, Ward NE, Seid C and Fidler IJ, Level of protein kinase C activity correlates directly with resistance to adriamycin in murine fibrosarcoma cells. *FEBS Letts* **246:** 78–82, 1989.
16. Zheleznyak A and Brown E, Immunoglobulin-mediated phagocytosis by human monocytes requires protein kinase C activation: evidence for protein kinase C translocation to phagosomes. *J Biol Chem* **267:** 12042–12048, 1992.
17. O'Brian CA, Ward NE and Vogel VG, Inhibition of protein kinase C by the 12-*O*-tetradecanoyl phorbol-13-acetate antagonist glycyrrhetic acid. *Cancer Letts* **49:** 9–12, 1990.
18. Falcieri E, Martelli AM, Bareggi R, Cataldi A and Cocco L, The protein kinase inhibitor staurosporine induces morphological changes typical of apoptosis in MOLT-4 cells without concomitant DNA fragmentation. *Biochem Biophys Res Commun* **193:** 19–25
19. Wolf M and Baggiolini M, The protein kinase inhibitor staurosporine, like phorbol esters, induced the association of protein kinase C with membranes. *Biochem Biophys Res Commun* **154:** 1273–1279, 1988.
20. Díaz-Guerra MJ, Junco M and Boscà L, Oleic acid promotes changes in the subcellular distribution of protein kinase C in isolated hepatocytes. *J Biol Chem* **266:** 23568–23576, 1991.
21. Kobayashi E, Nakano H, Morimoto M and Tamaoki T, Calphostin C (UCN-1028C), a novel microbial compound is a highly potent and specific inhibitor of protein kinase C. *Biochem. Biophys Res Commun* **159:** 548–553, 1989.
22. Bruns RF, Miller FD, Merriman RL, Howbert JJ, Heath WF, Kobayashi E, Takahashi I, Tamaoki T and Nakano H, Inhibition of protein kinase C by calphostin C is light-dependent. *Biochem Biophys Res Commun* **176:** 288–293, 1991.
23. Bojan F, Kinsella AR and Fox M, Effect of tumor promoter 12-*O*-tetradecanoyl phorbol-13-acetate on recovery of methotrexate-, *N*-(phosphonacetyl)-L-aspartate-, and cadmium-resistance colony-forming mouse and hamster cells. *Cancer Res* **43:** 5217–5221, 1983.
24. Watanabe T, Ono Y, Taniyama Y, Hazama K, Igarashi K, Ogita K, Kikkawa U and Nishizuka Y, Cell division arrest induced by phorbol ester in CHO cells overexpressing protein kinase C-δ subspecies *Proc Natl Acad Sci USA* **89:** 10159–10163, 1992.
25. Herschman HR, A 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-nonproliferative variant of 3T3 cells is resistant to TPA-enhanced gene amplification. *Molec Cell Biol* **5:** 1130–1135, 1985.
26. Biemann HPN and Erikson RL, Abnormal protein kinase C down regulation and reduced substrate levels in non-phorbol ester-responsive 3T3-TNR9 cells. *Molec Cell Biol* **10:** 2122–2132, 1990.
27. Fine RL, Patel J and Chabner BA, Phorbol esters induce multidrug resistance in human breast cancer cells. *Proc Natl Acad Sci USA* **85:** 582–586, 1988.
28. Lee SA, Karaszkiewicz JW and Anderson WB, Elevated level of nuclear protein kinase C in multidrug-resistant MCF-7 human breast carcinoma cells. *Cancer Res* **52:** 3750–3759, 1992.
29. Hamada H, Hagiwara K-I, Nakajima T and Tsuruo T, Phosphorylation of the M_r 170,000 to 180,000 glycoprotein specific to multidrug-resistant tumor cells: Effects of verapamil, trifluoperazine, and phorbol esters. *Cancer Res* **47:** 2860–2865, 1987.
30. Ma L, Marquardt D, Takemoto L and Center MS, Analysis of P-glycoprotein phosphorylation in HL60 cells isolated for resistance to vincristine. *J Biol Chem* **266:** 5593–5599, 1991.