Pergamon

Biochemical Pharmacology, Vol. 47, No. 4, pp. 667–672, 1994.

0006-2952/94 $6.00 + 0.00

PROTEIN KINASE C ACTIVATION PROMOTES CELL SURVIVAL IN MATURE LYMPHOCYTES PRONE TO APOPTOSIS

MIGUEL LUCAS,* VÍCTOR SÁNCHEZ-MARGALET, AUREO SANZ and FRANCISCA SOLANO

Departmento de Bioquímica Médica y Biología Molecular, Hospital Universitario Virgen Macarena, Facultad de Medicina, Avenida Sánchez Pizjuán 4, 41009 Sevilla, Spain

(*Received* 15 *July* 1993; *accepted* 4 *October* 1993)

Abstract—The putative protein kinase C (PKC) inhibitors polymyxin B and staurosporine were used to test the influence of PKC activity on the viability of lymphocytes. The cytotoxic effect of polymyxin B was characterized and it was found to be both time and dose dependent, with an LD_{50} in micromolar range, and counteracted by phorbol myristate acetate (PMA). To explore further the possible mechanism of action involved in polymyxin B-induced cell death, PKC activity and intracellular calcium were measured in polymyxin B-challenged lymphocytes. Polymyxin B inhibited PKC activity in both resting (25% inhibition) and PMA-stimulated (50% inhibition) cells, and increased intracellular calcium without disruption of the plasma membrane, a signal which is known to trigger apoptosis. Additionally, a number of experiments were conducted to assess the effect of staurosporine on PKC activity, cell growth, cell death and survival of mature lymphocytes. Staurosporine inhibited PKC activity in a dose-dependent manner (K_i close to 1 μM) and this effect correlated to some extent with the inhibition of [^{3}H]thymidine incorporation and the breakdown of DNA into oligonucleosome-sized fragments. These results support the hypothesis that PKC is involved in the survival of mature lymphocytes undergoing apoptosis.

Key words: apoptosis; mature lymphocyte; cell survival; free calcium; polymyxin B; staurosporine

Apoptosis (programmed cell death) is characterized by chromatin condensation, fragmentation of DNA into oligonucleosome-sized fragments, shrinkage and progressive cell degradation [1, 2]. Apoptosis occurs during embryo morphogenesis, the development of immune tolerance and tissue degeneration [3, 4]. Moreover, IL-2 has been shown to block glucocorticoid-induced apoptotic death of IL-2-dependent T lymphocytes [5]. Possible intracellular signaling mechanisms involved in the initiation of apoptosis include influx of calcium [6]; an ATP-dependent Ca^{2+} uptake system in liver nuclei [7] and an increase in intranuclear free Ca^{2+}, all of which have been related to DNA fragmentation [8]. However, altered expression of oncogenes c-fos and c-myc [9] appears to be involved in apoptosis, whereas an inner mitochondrial membrane protein encoded by the oncogene bcl-2 has been shown to block programmed cell death [10], although mitochondrial DNA is not fragmented during apoptosis [11]. Recent articles [12, 13] have shown that apoptotic cell death induced by c-myc is inhibited by bcl-2 and a novel mechanism for oncogene cooperation of potential interest in carcinogenesis is indicated.

Agents that stimulate PKC†, namely the tumour-promoting phorbol ester PMA, have been shown to inhibit DNA fragmentation in thymocytes and isolated thymocyte nuclei [14], in chronic lymphocytic leukemia cells [15] and in radiation-produced apoptosis *in vitro* [16]. We have previously reported the induction of apoptosis in mature lymphocytes by the omission of fetal bovine serum or the addition of polymyxin B to the culture medium; this effect is inhibited by tumour-promoting phorbol ester (PMA) and the possible involvement of PKC was pointed out [17]. In addition, we have shown that decreased PKC activity is associated with apoptosis in freshly isolated rat hepatocytes [18]. We decided to test the possible involvement of PKC in programmed cell death by analysing apoptosis-related events in mature lymphocytes undergoing apoptosis following incubation with PKC inhibitors polymyxin B and staurosporine. Changes in $[Ca^{2+}]_i$, protein kinase C activity and internucleosomal breakdown, as well as cell survival and proliferation, determined by vital dye exclusion and [^{3}H]thymidine incorporation, respectively, were studied in mature lymphocytes challenged with the above-indicated agents under a number of experimental regimes.

MATERIALS AND METHODS

Chemicals. Agarose was from BRL; λ-DNA/*Hind*III-digested molecular mass markers, proteinase K, ribonuclease A and collagenase were from Boehringer-Mannheim (Germany); bleomycin was from Almirall (Spain); α-L-phosphatidylserine, 1-3-diolein, lysine-rich histone, PMA, polymyxin B, *N*-laurylsarcosine and ethidium bromide were from the Sigma Chemical Co. (Poole, U.K.); RPMI medium, foetal bovine serum, streptomycin and penicillin were from Flow Laboratories (Irvine, U.K.). [γ^{32}P]-ATP was from Amersham (U.K.).

DNA electrophoresis. DNA was extracted from peripheral blood lymphocytes as described previously

* Corresponding author. FAX (34) 5 455 7352.

† Abbreviations: PKC, protein kinase C; PMA, phorbol myristate acetate; HBSS, Hanks' balanced salt solution.

[17] with minor modifications. Lymphocytes, 2×10^6, were resuspended in 0.3 mL Tris–EDTA buffer (50 mM Tris, 10 mM EDTA, pH 8.0) and supplemented with 0.5% (w/v) *N*-laurylsulfate and 0.5 mg/mL proteinase-K. After 1 hr at 50° the incubation medium was supplemented with 0.5 mg/mL deoxyribo-nuclease-free ribonuclease-A, and further incubated at 50° for 1 hr. DNA was precipitated with ethanol at −60° and dissolved in Tris–EDTA buffer. Samples were heated at 65° and supplemented with loading buffer (10 mM EDTA, pH 8, containing 0.25% Bromophenol blue, 1% low-gelling-temperature agarose) at a 1:5 (v/v) ratio. Electrophoresis was carried out in 2% agarose gel, and the buffer used was 80 mM Tris/20 mM phosphate/2 mM EDTA, pH 8.

Protein kinase C assay. PKC activity was assayed as phospholipid-sensitive, Ca^{2+}-dependent phosphorylation of lysine-rich histone according to the procedure described in Ref. 18 with minor modifications. In brief, lymphocytes (4×10^6) were sonicated for 15 sec at 4° in calcium- and magnesium-free HBSS. A crude homogenate was prepared by centrifugation at 15,000 *g* for 10 min to remove debris, nuclei and mitochondria. PKC activity was assayed in a medium containing 25 mM Tris–HCl buffer pH 8, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.25 mg/mL α-L-phosphatidylserine, 0.6 mg/mL 1,3-diolein, 1 mg/mL lysine-rich histone and 10 μM [^{32}P]ATP. The reaction was started by addition of 20 μL of the homogenate to 180 μL of the reaction mixture, leading to a final concentration of 1 mg protein/mL. After 15 min incubation at 37°, the reaction was stopped by spotting 75 μL aliquots of the assay mixture onto Whatman 3MM filters. Thereafter the filters were washed three times with an excess of 10% trichloroacetic acid. Radioactivity was counted in a β-spectrometer. Specific radioactivity was calculated from an unwashed filter spotted with 10 μL of the incubation mixture.

Measurement of $[Ca^{2+}]_i$. Fluo-3, the calcium-sensitive fluorescein-derived chromophore [19] was used to determine $[Ca^{2+}]_i$ by flow cytometry [20], which allows analysis of simultaneous changes in cell shape by determination of forward-angle and side-angle light scattering of cells. Cells, 1–5 × 10^6 mL in HBSS, were loaded for 40 min with 4 μM Fluo-3, from a stock of 2 mM in DMSO. After washing out the medium, following centrifugation at 500 *g*, the cells were resuspended in HBSS. Aliquots of the cell suspension were diluted in HBSS just before starting the experiment. Analysis of the cells was performed using a FACScan flow cytometer (Becton Dickinson). Excitation was from an argon laser at 488 nm, emission at 525 nm was measured on a linear scale. In some experiments, the fluorescence units were converted into $[Ca^{2+}]_i$ as described [21]. The calibration procedure includes the determination of F_{max} and F_{min}, which were achieved with 4 μM Br-A23187 and 2 mM $MnCl_2$, respectively. Intracellular free calcium, $[Ca^{2+}]_i$, was calculated by the equation:

$$[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$$

where K_d, the dissociation constant for Ca^{2+}-bound Fluo-3, is 400 nM and F represents the experimental fluorescence.

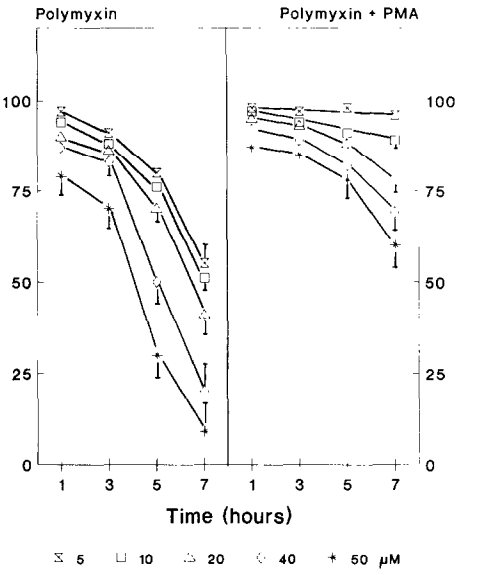

Fig. 1. Time- and dose-dependent effect of polymyxin B and PMA on cell viability. Lymphocytes, 2×10^6/mL, were incubated in PBS containing 1.2 mM $MgCl_2$ and 0.6 mM $CaCl_2$. The incubation medium was supplemented with polymyxin B at the indicated concentrations (see symbols under the *x*-axis), in the absence (left panel) and in the presence (right panel) of 200 nM PMA. Aliquots of the cell suspension were removed at the indicated time intervals, mixed with an equivalent volume of 0.4% Trypan blue in 0.7% saline and counted in a hemocytometer. Results are given as per cent of viable cells, i.e. control lymphocytes incubated without the above indicated compounds and processed at the same time intervals for Trypan blue staining. Data are the means and SE obtained from four separate experiments. Differences were analysed by the Student's *t*-test for paired values. The effect of PMA was statistically significant (P values inferior to 0.001) even after 3 hr incubation.

Nuclear staining and flow cytometry. For the purpose of nuclear staining as a measure of cell viability, lymphocytes suspended in PBS were incubated with 5 μM propidium iodide. Acquisition and analysis were performed with the FACScan and Chronys Research software in the flow cytometer by measuring fluorescence at 570–575 nm.

Flow cytometry measurements were performed on a FACScan (Becton Dickinson, San Jose, CA, U.S.A.). Forward and orthogonal light scattering and fluorescence signals were collected in list mode. Additional details are described in the legends to the figures.

RESULTS

We studied the survival of lymphocytes upon polymyxin B treatment and the effect of the tumour-promoting phorbol ester PMA. The experiments demonstrated a time- and dose-dependent cytotoxic effect of polymyxin B in the micromolar range, under conditions (see Fig. 1) which allowed survival of nearly 80% of control lymphocytes after 7 hr

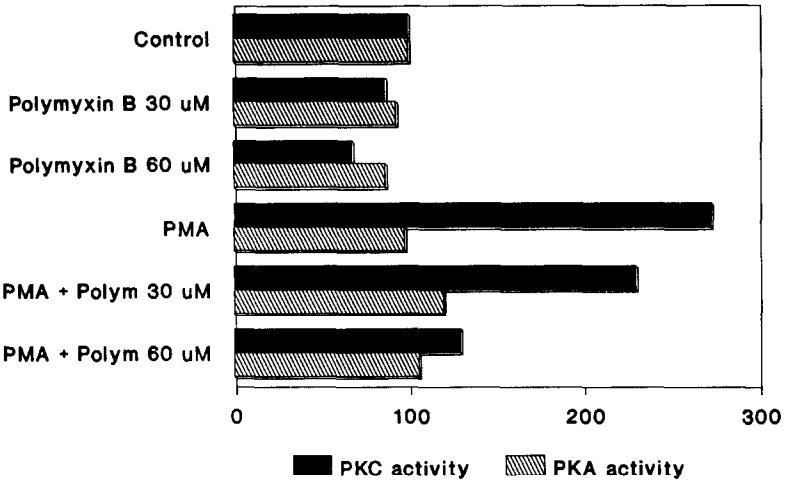

Fig. 2. *In vitro* effect of polymyxin B and PMA on protein kinase activity. Freshly isolated lymphocytes were cultured for 17 hr; the indicated compounds were added and 7 hr later the cells were harvested. The activities of PKC and cAMP-dependent protein kinase (PKA) were determined in the crude homogenate prepared by sonication of lymphocytes. PMA concentration was 200 nM. Results, given as per cent of controls, are the means of three experiments.

incubation. At 50 μM polymyxin B, the half-life approached 3.5 hr and the LD_{50} was close to 12 μM polymyxin after 7 hr incubation. PMA, at a concentration of 200 nM, counteracted the effect of polymyxin B; in fact the viability of lymphocytes within 3 hr incubation in the presence of both polymyxin B and PMA was clearly improved, as compared to lymphocytes incubated with polymyxin B, and the cytotoxic effect of polymyxin B was inhibited by PMA, even after 3 hr incubation, to a degree which depended on the concentration of polymyxin B.

In a set of experiments we assessed the effect of polymyxin B and PMA on protein kinase activity. The assays were performed in crude homogenates obtained from lymphocytes incubated under various conditions (see legend to Fig. 2) which demonstrated that polymyxin B produced minor inhibition of PKC activity in resting lymphocytes whereas it inhibited by nearly 50% PKC activity in PMA-stimulated lymphocytes. Cyclic AMP-dependent protein kinase (PKA) was almost unmodified under the described experimental conditions (see Fig. 2).

Polymyxin B produced an increase in $[Ca^{2+}]_i$ in lymphocytes reaching values close to 1000 nmol/L which were further increased upon the addition of calcium ionophore indicating the intactness of the plasma membrane. This was further studied in experiments designed to determine the disruption of the plasma membrane by analysing the entry of the nuclear staining propidium iodide. In fact, the addition of either polymyxin B or the calcium ionophore failed to increase the fluorescence of cells incubated in the presence of propidium iodide; this was in contrast to the sudden increase in fluorescence which follows the addition of the pore former detergent digitonin (see Fig. 3).

Another set of experiments was designed to

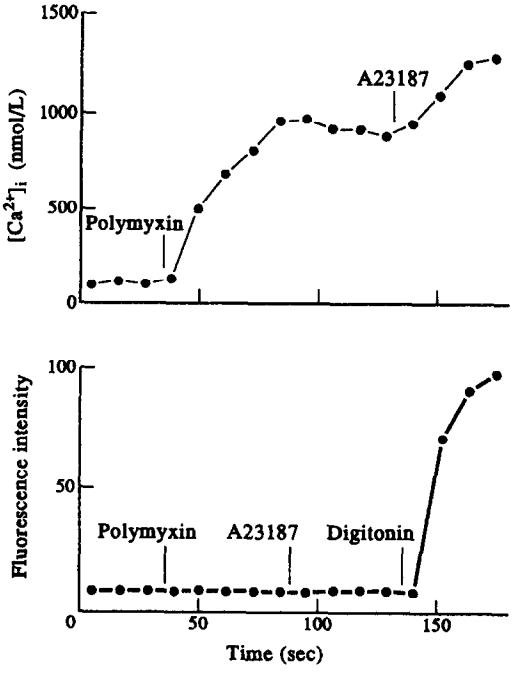

Fig. 3. Effect of polymyxin B on $[Ca^{2+}]_i$. Freshly isolated lymphocytes were resuspended in PBS containing 1.2 mM $MgCl_2$ and 0.6 mM $CaCl_2$ and supplemented with either 4 μM Fluo-3 (upper panel) or 5 μM propidium iodide (lower panel). Lymphocytes were acquired and analysed with the Chronys software in the flow cytometer and $[Ca^{2+}]_i$ was calculated as described in Materials and Methods. Monocytes were excluded from the mononuclear subpopulation according to size and granularity by forward and orthogonal light scattering. Arrows refer to the addition at the indicated time intervals of 50 μM polymyxin B, 8 μM Br-A23187, 20 μg/mL digitonin. Results are representative of experiments from four other cell batches.

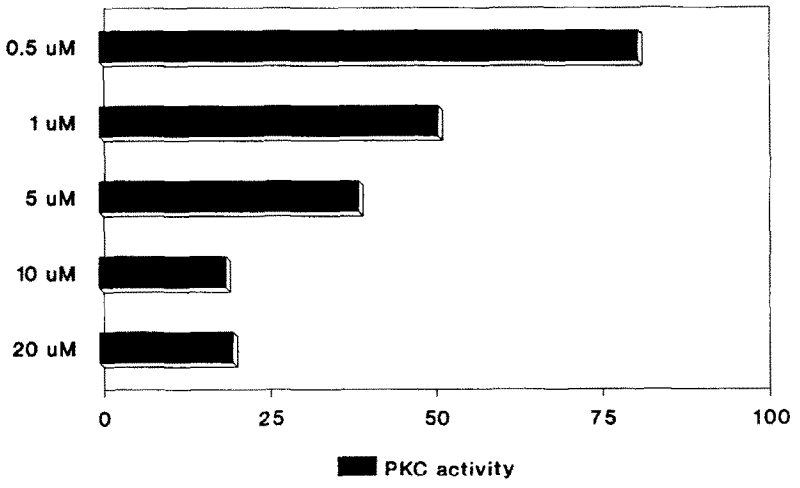

Fig. 4. Effect of staurosporine on PKC activity. Lymphocytes were incubated in RPMI supplemented with 10% foetal bovine serum in the absence (controls) and in the presence of staurosporine at the indicated concentrations. After 24 hr, the incubation medium was washed out and lymphocytes were sonicated to determine PKC activity. Results are given as per cent of activity in control cells, which was considered 100%. Further details were as described in Materials and Methods and in the legend to Fig. 2.

determine the effect of staurosporine and we found an inhibition of PKC activity in mature lymphocytes with a K_i close to 1 μM (see Fig. 4). The degree of inhibition of PKC correlated to some extent with the deleterious effect on cell viability (nuclear staining with propidium iodide), cell growth ([^{3}H]-thymidine incorporation) and the breakdown of DNA in oligonucleosome-sized fragments. This was observed in lymphocytes which were cultured for 48 hr in the presence of phytohaemagglutinin and further incubated for either 24 or 4 hr in the presence of 5×10^{-6}–5×10^{-13} M staurosporine. In fact, the time- and dose-dependent effect of staurosporine (see Figs 5 and 6) was characterized by the following: (a) after 24 hr incubation, the half-maximal effect on both [^{3}H]thymidine incorporation and cell death was reduced to 1–3 nM staurosporine; (b) shorter incubation of lymphocytes, 4 hr, required much higher staurosporine concentrations to obtain half-maximal effects which were reached at 0.02 and 1 μM staurosporine for [^{3}H]thymidine incorporation and cell death, respectively; (c) internucleosomal breakdown was triggered, after 4 hr incubation at staurosporine concentrations above 0.5 μM; (d) incubation for 24 hr showed optimum cleavage of DNA at 0.5–1 μM staurosporine; the cytotoxic effect of a higher dose of staurosporine (10 μM in Fig. 6) could hinder the triggering of events involved in the apoptosis of cells.

A striking finding concerning apoptosis in mature lymphocytes was that following 72 hr culture in phytohaemagglutinin-containing medium: a high proportion of cells underwent apoptosis, as judged by the ladder-like electrophoretic pattern of DNA, and it was clearly enhanced by 1 μM staurosporine. PMA counteracted the "spontaneous" DNA breakdown but failed to restrain the staurosporine-induced apoptosis. PMA together with forskolin produced a smear of highly degraded DNA which was hindered by staurosporine leading to the ladder-like pattern of DNA breakdown (Fig. 7).

DISCUSSION

The counteracting action of PMA on the apoptosis of mature lymphocytes previously reported [17] is extended in the present work by experiments showing the inhibition by PMA of polymyxin B-induced cell death in mature lymphocytes (Fig. 1) and the blocking effect of PMA on the breakdown of DNA in long-term culture of phytohemagglutinin-stimulated lymphocytes (Fig. 7). These results agree with data previously reported [14–16] involving PKC in the suppression of apoptosis, although its precise role has not been elucidated [22]. Cell death was studied in the present work with the vital dye Trypan blue and by nuclear staining with propidium iodide; the internucleosomal breakdown of DNA was analysed by electrophoresis. In spite of the fact that cell swelling and permeabilization of the plasma membrane is a characteristic of necrotic death, apoptotic death *in vitro*, in the absence of phagocytosis, is terminated by a phase where the apoptotic cell becomes permeable to vital dyes [23].

From the experiments describing an increase in $[Ca^{2+}]_i$ in polymyxin B-challenged lymphocytes (Fig. 3), it may be argued that calcium ions, by activating the endonuclease, are involved in polymyxin B-induced DNA breakdown. In addition, the minor effect of polymyxin B on PKC activity in resting lymphocytes could enhance the triggering of apoptosis induced by the increased in $[Ca^{2+}]_i$. This explanation is supported by the model proposed by McConkey *et al.* [2] on the triggering of apoptosis

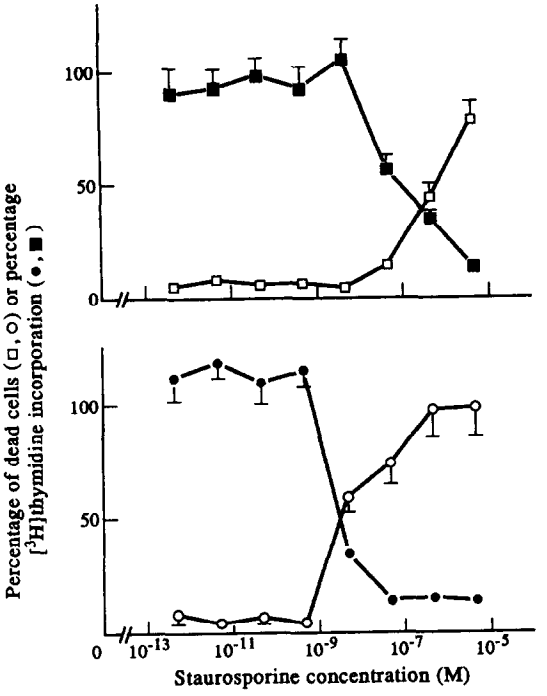

Fig. 5. Effect of staurosporine on [^{3}H]thymidine incorporation and cell viability. Lymphocytes were cultured in microtiter plates for 48 hr in the presence of 4 μg/mL phytohaemagglutinin and, thereafter, the culture medium was supplemented with the indicated staurosporine concentrations. After either 4 (upper panel) or 24 (lower panel) hr [^{3}H]thymidine was added and 5 hr later aliquots were removed to determine cell death by flow cytometry, and plates were processed in the harvester to measure the incorporation of [^{3}H]thymidine. The values of incorporated [^{3}H]thymidine (filled symbols) were referred to 100% value given to control cells. The percentage of dead cells (open symbols) at each staurosporine concentration was directly obtained from the FACScan software (Becton Dickinson) and further referred to the value obtained in the control cells which was below 5%. Results are the means ± SEM of four separate experiments.

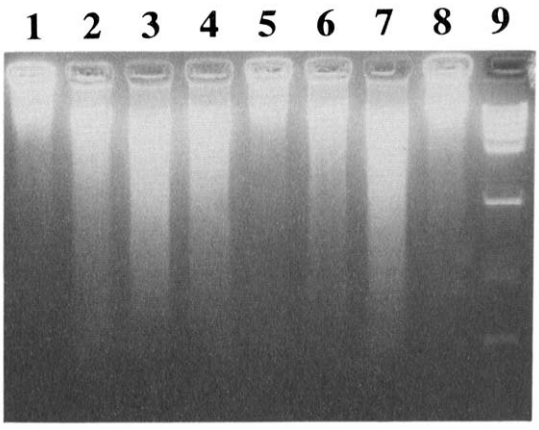

Fig. 6. Analysis of DNA breakdown. Effect of staurosporine. Lymphocytes, 2×10^6, were cultured in 1 mL RPMI supplemented with 10% foetal bovine serum and 4 μg/mL phytohaemagglutinin for 48 hr, staurosporine was added to the tubes and the culture was further continued for 24 hr. Lymphocytes were spun down and DNA extracted as described in Materials and Methods. The concentrations of staurosporine were as follows: 0, lanes 1 and 5; 0.1 μM, lanes 2 and 6; 1 μM, lanes 3 and 7; 10 μM, lanes 4 and 8. Lane 9 was loaded with λ-DNA digested *Hind*III. Electrophoresis included samples of DNA obtained from identical experiments with two separate cell batches.

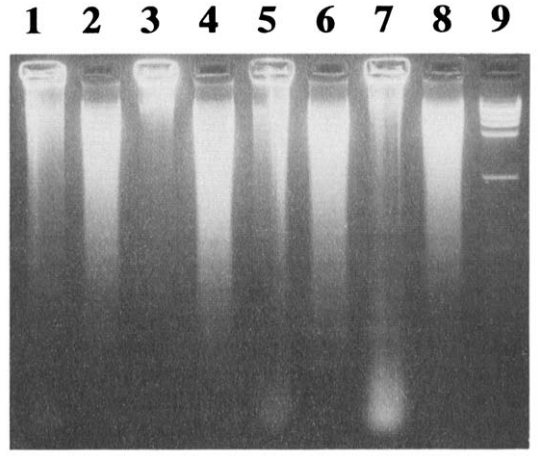

Fig. 7. Analysis of DNA breakdown. Effect of PMA, forskolin and staurosporine. Lymphocytes, 2×10^6, were cultured in 1 mL RPMI supplemented with 10% foetal bovine serum and 4 μg/mL phytohaemagglutinin for 72 hr. Afterwards, the compounds indicated below were added to the culture tubes and DNA was extracted 24 hr later. Additions were as follows: (a) none, lane 1; (b) 1 μM staurosporine, lane 2; (c) 50 nM PMA, lane 3; (d) 1 μM staurosporine plus 50 nM PMA, lane 4; (e) 0.1 μM forskolin, lane 5; (f) 0.1 μM forskolin plus 1 μM staurosporine, lane 6; (g) 0.1 μM forskolin, plus 50 nM PMA, lane 7; (h) 0.1 μM forskolin plus 1 μM staurosporine and 50 nM PMA, lane 8. Lane 9 is the molecular mass marker λ-DNA digested with *Hind*III. Single experiment representative of two others.

by cytosolic calcium in the absence of PKC activation. In this regard, the inhibition produced by PMA of polymyxin B-induced apoptosis could be explained too, since PMA produced a considerable activation of PKC activity even in the presence of polymyxin B (see Fig. 2).

Staurosporine is an inhibitor of PKC activity (see Fig. 4) and a potent trigger of DNA fragmentation in mature lymphocytes which was not counteracted by PMA (see Fig. 7). Therefore, staurosporine-challenged lymphocytes were analysed to test the possibility that PKC activity is required to promote both cell survival and the inhibition of apoptosis in mature lymphocytes by preserving the functional integrity of DNA. The rationale of these experiments relies on the dependency of cell survival upon the integrity of DNA which is required to transcribe and to pass the genetic information during mitotic division. With this in mind, we measured in cultured

lymphocytes the time- and dose-dependent effect of staurosporine on the rate of DNA synthesis, by [^{3}H]-thymidine incorporation, and the survival of lymphocytes, by propidium iodide staining. We observed a higher sensitivity to staurosporine of DNA synthesis, as compared to membrane permeability changes and cell death: after 4 hr culture half-maximal effects on inhibition of [^{3}H]-thymidine incorporation and triggering of lymphocyte death were obtained at 0.08 and 1 μM staurosporine, respectively. Following 24 hr culture the sensitivity of both phenomena could not be discriminated; in fact, both the inhibition of [^{3}H]thymidine incorporation and cell death were produced at much lower concentrations and half-maximal effects were obtained at concentrations close to 3 nM staurosporine (see also Figs 5 and 6). These results may be explained assuming that staurosporine, a potent inhibitor of PKC activity in mature lymphocytes, arrested the cell cycle and made lymphocytes prone to cleavage of DNA by endonucleases and to apoptotic events. On the other hand, the involvement of tyrosine-kinase can not be ruled out since staurosporine has been described to inhibit tyrosine phosphorylation [24].

From the data in the present and previous [17] work it is tempting to conclude that PKC is directly involved in the survival of mature lymphocytes which can be made prone to apoptosis by a number of conditions. In fact, lymphocytes under long-term culture or deprivation of fetal serum in the culture medium underwent apoptosis which was hindered by PMA.

Acknowledgement—This work has been financially supported by grant No. 92/0399 from the Fondo de Investigaciones Sanitarias de la Seguridad Social, Ministerio de Sanidad y Consumo.

REFERENCES

1. Wyllie AH, Kerr JFR and Currie AR, Cell death: the significance of apoptosis. *Int Rev Cytol* **68**: 251–356, 1980.
2. McConkey DJ, Orrenius S and Jondal M, Cellular signalling in programmed cell death (apoptosis). *Immunol Today* **11**: 120–121, 1990.
3. Arends MJ, Morris RG and Wyllie AH, Apoptosis. The role of endonuclease. *Am J Pathol* **136**: 593–608, 1990.
4. Wyllie AH, Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* **284**: 555–556, 1980.
5. Nieto MA and López-Rivas A, IL-2 protects T lymphocytes from glucocorticoid-induced DNA fragmentation and cell death. *J Immunol* **142**: 4166–4170, 1989.
6. McConkey DJ, Nicotera P, Hartzell P, Bolloma G, Wyllie AH and Orrenius S, Glucocorticoids activate a suicide proceed in thymocytes through an elevation of cytosolic Ca^{2+} concentration. *Arch Biochem Biophys* **269**: 365–370, 1989.
7. Nicotera P, McConkey DJ, Jones DP and Orrenius S, ATP stimulates Ca^{2+} uptake and increases the free Ca^{2+} concentration in isolated liver nuclei. *Proc Natl Acad Sci USA* **86**: 453–457, 1989.
8. Bellomo G, Perotti M, Taddei F, Mirabelli F, Finardi G, Nicotera P and Orrenius S, Tumor necrosis factor α induces apoptosis in mammary adenocarcinoma cells by an increase in intranuclear free Ca^{2+} concentration and DNA fragmentation. *Cancer Res* **52**: 1342–1346, 1992.
9. Buttyan R, Zakeri Z, Lockshin R and Wogelmuth D, Cascade induction of c-fos, c-myc and heat shock 70K transcripts during the regression of the ventral prostate gland. *Mol Endocrinol* **2**: 650–657, 1988.
10. Hockenberry D, Nuñez G, Milliman C, Schreiber RD and Korsmeyer SJ, Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* **348**: 334–336, 1990.
11. Murgia M, Pizzo P, Sandona D, Zanovello P, Rizzuto R and Di Virgilio F, Mitochondrial DNA is not fragmented during apoptosis. *J Biol Chem* **267**: 10939–10941, 1992.
12. Bissonnette RP, Echeverri F, Mahboubi A and Green R, Apoptotic cell death induced by C-myc is inhibited by bcl-2. *Nature* **359**: 552–554, 1992.
13. Fanidi A, Harrington EA and Evan GI, Cooperative interaction between C-myc and bcl-2 proto-oncogenes. *Nature* **359**: 554–556, 1992.
14. McConkey DJ, Harztell P, Jondal M and Orrenius S, Inhibition of DNA fragmentation in thymocytes and isolated thymocyte nuclei by agents that stimulate protein kinase C. *J Biol Chem* **264**: 13399–13402, 19089.
15. McConkey DJ, Aguilar-Santelises M, Hartzell P, Eriksson I, Mellstedt H, Orrenius S and Jondal M, Induction of DNA fragmentation in chronic B-lymphocytic leukemia cells. *J Immunol* **146**: 1072–1076, 1991.
16. Tomei DL, Kanter P and Wenner CE, Inhibition of radiation-produced apoptosis *in vitro* by tumor promoters. *Biochem Biophys Res Commun* **155**: 324–331, 1988.
17. Lucas M, Solano F and Sanz A, Induction of programmed cell death (apoptosis) in mature lymphocytes. *FEBS Lett* **1**: 19–20, 1991.
18. Sánchez V, Lucas M, Sanz A and Governa R, Decreased protein kinase C activity is associated with programmed cell death (apoptosis) in freshly isolated rat hepatocytes. *Biosci Rep* **12**: 199–206, 1992.
19. Minta A, Kao JPY and Tsien RY, Fluorescent indicators for cytosolic calcium based on Rhodamine and Fluorescein chromophores. *J Biol Chem* **264**: 8171–8178, 1989.
20. Vandenberghe PA and Ceuppens JL, Flow cytometric measurement of cytoplasmic free calcium in human peripheral blood T lymphocytes with fluo-3, a new fluorescent calcium indicator. *J Immunol Methods* **127**: 197–202, 1990.
21. Lucas M and Solano F, Opposite effect of cytochalasin B on agonist-induced respiratory burst in neutrophils and monocytes. *Biochem Pharmacol* **42**: 425–430, 1991.
22. Forbes IJ, Zalewski PD, Giannakis C and Cowled PA, Induction of apoptosis in chronic lymphocytic leukemia cells and its prevention by phorbol ester. *Exp Cell Res* **198**: 367–372, 1992.
23. Dive C, Gregory CD, Phipps DJ, Evans DL, Milner AE and Wyllie AH, Analysis and discrimination of necrosis and apoptosis (programmed cell death) by multiparameter flow cytometry. *Biochim Biophys Acta* **1133**: 275–285, 1992.
24. Berkow RL, Granulocyte-macrophage colony-stimulating factor induces a staurosporine inhibitable tyrosine phosphorylation of unique neutrophil proteins. *Blood* **79**: 2446–2454, 1992.