CELLULAR IMMUNOLOGY 153, 28–38 (1994)

Inhibition of T Cell Activation by Protein Kinase C Pseudosubstrates

P. BARJA,*,[1] A. ALAVI-NASSAB,* C. W. TURCK,† AND J. FREIRE-MOAR*

Department of Molecular Immunology, Syntex Discovery Research (S3-6), 3401 Hillview Avenue, Palo Alto, California 94304; and †Howard Hughes Medical Institute and Department of Medicine, University of California at San Francisco, San Francisco, California 94143

Received April 30, 1993; accepted August 18, 1993

PKC pseudosubstrate consensus sequences can be found in the amino terminal region of all the different PKC isoenzymes characterized to date. Here we have used four peptides corresponding to the putative pseudosubstrate sequences from the PKC isoenzymes α, γ, δ, and ϵ. These peptides showed PKC inhibitory activity when tested in a PKC-specific enzyme assay at concentrations of 25 to 100 μM, similar to what has been reported for the myristylated peptide KRTLR. Although the presence of a myristyl group at the amino terminal end of any of these peptides is not essential for their inhibitory activity, myristylation increased the inhibitory activity significantly. By contrast, the myristylated control peptide (GALRQQKNVHEVKN) was not active even at a 100 μM concentration. All of the PKC inhibitory peptides were also able to block PKC activity in a cell assay as demonstrated by their ability to inhibit the induction of IL-2R and TNF-β expression in Jurkat cells. Finally, we confirmed a previous report of the inhibitory activity of the myristylated peptide KRTLR and showed that other related peptides (N-m-RLTRK, N-m-RRLKT) are also active in these assays.

INTRODUCTION

Protein kinase C represents a family of more than seven isoenzymes involved in the signal transduction mechanism leading to the activation of T cells (1–4). Although a high degree of sequence homology is seen among all the members of the PKC family at their catalitic site (5, 6), there is an important difference in the regulatory domain that results in differential response to a variety of activators (7–9). This together with other lines of evidence (5, 10) indicates that these isoenzymes may have heterogenous functions in the signal transduction cascade.

In resting cells the enzyme is kept in the inactive state by the interaction of the regulatory domain with the catalytic site (5, 11–13). This interaction is disrupted by phospholipids and Ca^{2+}, which leads to the proteolytic removal of the inhibitory regulatory domain (11, 13–15). Several lines of evidence indicate that this inhibition occurs through the binding of a putative pseudosubstrate sequence to the substrate binding site in the catalytic domain of the enzyme (16–19).

This concept has opened a new approach to the generation of specific PKC inhibitors. In this regard House and Kemp (16) synthesized an octadecapeptide that is a potent

[1] Present address: Dept. de Bioquimica, Facultad de Biologia, Universidad de Santiago de Compostela, Spain.

0008-8749/94 $6.00

inhibitor of PKC. More recently O'Brian *et al.* (20) converted a PKC substrate peptide to an inhibitor by addition of a myristyl group at the amino terminal end of the peptide. The same myristylated pentapeptide was able to inhibit the expression of the IL-2R and IL-2 secretion in Jurkat cells activated by PHA or OKT3 mAb in the presence of TPA (21). We attempted to take these observations one step further, to try to develop isoenzyme-specific inhibitors, by using peptide pseudosubstrates corresponding to the regulatory sequences of different members of the PKC family. Here we report the PKC inhibitory activity in an enzyme assay of several myristylated and nonmyristylated peptide pseudosubstrates, and their capacity to block the expression of IL-2R and TNF-β in activated Jurkat cells.

MATERIALS AND METHODS

Reagents. Phycoerythrin-conjugated anti-IL2 receptor (anti-Tac) antibody was obtained from Becton–Dickinson (Mt. View, CA); IOT3 (anti-CD3) monoclonal antibody was from AMAC (Westbrook, ME); PKC peptide inhibitor (19–36) and phorbol 12-myristate 13-acetate were from Gibco/BRL (Gaithersburg, MD), H7 kinase inhibitor was purchased from Seikagaku Kogyoco (Tokyo, Japan); aprotinin, benzamidin, and leupeptine (protease inhibitors) were from Sigma (St. Louis, MO); AmpliTaq DNA polymerase was obtained from Perkin Elmer (Norwalk, CT); oligonucleotide primers TNF-β 5′ primer ATGACACCACCTGAACGTCTCTTC, TNF-β 3′ primer CGAAGGCTCCAAAGAAGACAGTACT, IL-2R 5′ primer GAATTTATCATTTCGTGGTGGGGCA, and IL-2R 3′ primer TCTTCTACTCTTCCTCTGTCTCCG were purchased from Clontech Laboratories (Palo Alto, CA); PKC assay system kit was purchased from Amersham (Arlington Heights, IL); [γ-^{32}P]ATP was obtained from DuPont NEN (Boston, MA).

Cell culture and activation. Jurkat cells were grown in RPMI 1640 medium with 10% FCS at 37°C and 5% CO_2 to reach log phase, at which time they were transferred to fresh medium at a density of 10^6 cell/ml. Aliquots of 10-ml cultures were incubated for 16 hr in the presence or absence of PMA (5 ng/ml), anti-CD3 (2 μg/ml), or both. PKC inhibitors were added at different concentrations (where indicated) together with the stimuli to culture. At the end of the incubation period, cells were harvested by centrifugation.

Electroporation. Jurkat cells in log phase were recovered by centrifugation and resuspended in Hanks' balanced saline solution plus 20% sucrose at 5×10^6 cell/ml. Peptides were added to the cell suspension to give final concentrations of 20, 50, 100, and 200 μM. Aliquots (400 μl) of cell suspensions were exposed to an electric field in a gene pulser cuvette (0.2-cm electrode gap) with a gene pulser system from Bio-Rad (Bio-Rad, Richmond, CA). A pulse of 750 V/cm was delivered for 0.3 msec. After 10 min at 4°C, the cells were removed from the chamber, centrifuged, and resuspended in fresh medium for analysis.

Cell surface immunofluorescence. Induction of IL-2 receptor expression in Jurkat cells was determined by immunofluorescence using an antibody against the p55 chain of the IL-2R molecule (CD 25). Briefly, cells were harvested by centrifugation washed free of medium and resuspended in Hanks' balanced solution containing 0.1% NaN. Cell suspensions were incubated with anti-TAC-PE for 20 min at 4°C and then washed to remove the free antibody before analysis. Cell fluorescence was analyzed by flow cytometry using a FACSTAR plus (Becton–Dickinson).

Message amplification phenotyping (MAPPing). Expression of TNF-β and IL-2R mRNA was determined by the MAPPing technique (22). Total RNA was extracted from cell pellets using RNazol (Biotecx Laboratories, Inc., Houston, TX). First-strand cDNA synthesis was performed as described earlier (23). Polymerase chain reaction amplification was carried out on a Perkin–Elmer Thermal Cycler set for 33 cycles using specific primers for human TNF-β and IL-2R from Clontech. The conditions for the PCR were as follows: denature 94°C for 1 min, primer anneal 55°C for 2 min, and primer extension 72°C for 3 min. PCR products were analyzed by electrophoresis on 1.5% agarose gel.

Peptide synthesis. Based on previous reports three pentapeptides were synthesized around the PKC pseudosubstrate sequences (KRTLR), as well as longer peptide sequences which were derived from the regulatory sequences of the PKC isoenzymes α, γ, δ, and ϵ (Table 1). The syntheses of the different peptides were carried out on a Model 350 Multiple Peptide Synthesizer (Advanced Chemtech, Louisville, KY) using diisopropylcarbodiimide/1-hydroxybenzotriazole-activated fluorenylmethyloxycarbonyl (FMOC)-protected amino acids (Bachem, Torrance, CA). Myristylation of the peptides was carried out as previously described (20). Fluorescein-labeled peptides were generated by the reaction of FITC with resin-immobilized protected peptides. After completion of the synthesis the resin-bound protected peptides were dried and then treated with 500 μl of trifluoroacetic acid/anisole/dimethylsulfide/ethanedithiol (50:3:3:1) for 3 hr at 0°C. Upon deprotection, 10 ml of anhydrous ether was added to the filtered mixture and the precipitated peptides were collected by centrifugation and extensively washed with ether. Dried peptides were purified by RP-HPLC on a C_{18}-Vydac (Hesperia, CA) column (4.6 mm × 25 cm) in 0.1% trifluoroacetic acid (TFA), using a linear gradient of acetonitrile from 0 to 65% over 90 min.

Protein kinase C activity. PKC activity from rat brain extracts was assayed using a commercially available kit from Amersham. Briefly, rat brain tissue was homogenated at 4°C in 3 vol of lysis buffer (50 m*M* Tris–ClH, pH 7.5; 5 m*M* EDTA; 10 m*M* EGTA; 1 m*M* PMSF; 10 m*M* benzamidine; 10 μg/ml leupeptin; 100 μg/ml aprotinin; 0.3% (w/v) 2-mercaptoethanol; 5 m*M* DTT; 1% DTT; 1% NP-40) using a Polytron. The homogenate was incubated for 30 min in ice and then centrifuged at 12,000*g* for

TABLE 1

Peptide Sequences

Peptide	Origin	Sequence
1		N-m-KRTLR
2		N-m-RLTRK
3		N-m-RRLKT
4	PKC-α	RFARKGALRQKNVHEVKN
5	PKC-γ	FCRKGALRQKVVHEV
6	PKC-ϵ	RKRQGAVRRRVHQV
7	PKC-δ	NRRGAIKQAKIHYIK
8	PKC-α	N-m-GALRQKNVHEVKN
9	PKC-γ	N-m-FCRKGALRQKVVHEV
10	PKC-ϵ	N-m-RKRQGAVRRRVHQV
11	PKC-δ	N-m-NRRGAIKQAKIHYIK

15 min; the supernatant was used immediately as a source of PKC activity in the enzyme assay.

RESULTS

Inhibition of PKC activity by pseudosubstrates. Synthetic peptides, containing putative PKC pseudosubstrate sequences, were tested for their ability to inhibit PKC in an "*in vitro*" enzyme assay using a crude extract from rat brain as the source of PKC activity.

Two types of peptides were tested (Table 1): one group was composed of myristilated pentapeptides based on several combinations of the amino acids KRTLR; and a second group was composed of peptides containing between 14 and 18 residues with sequences based on the regulatory sequences present in the different PKC isoenzymes. All of these peptides were active in the range of 10 to 100 μM concentrations (Fig. 1), although differences in their potency can be seen. The long peptides do not require myristilation for their inhibitory activity although the presence of the myristyl group at the amino terminal end of the peptide may slightly enhance the inhibitory effect. A control peptide (GALRQKNVHEVKN) lacking the consensus sequences for a PKC pseudosubstrate was inactive in this assay at a 100 μM concentration even after myristylation.

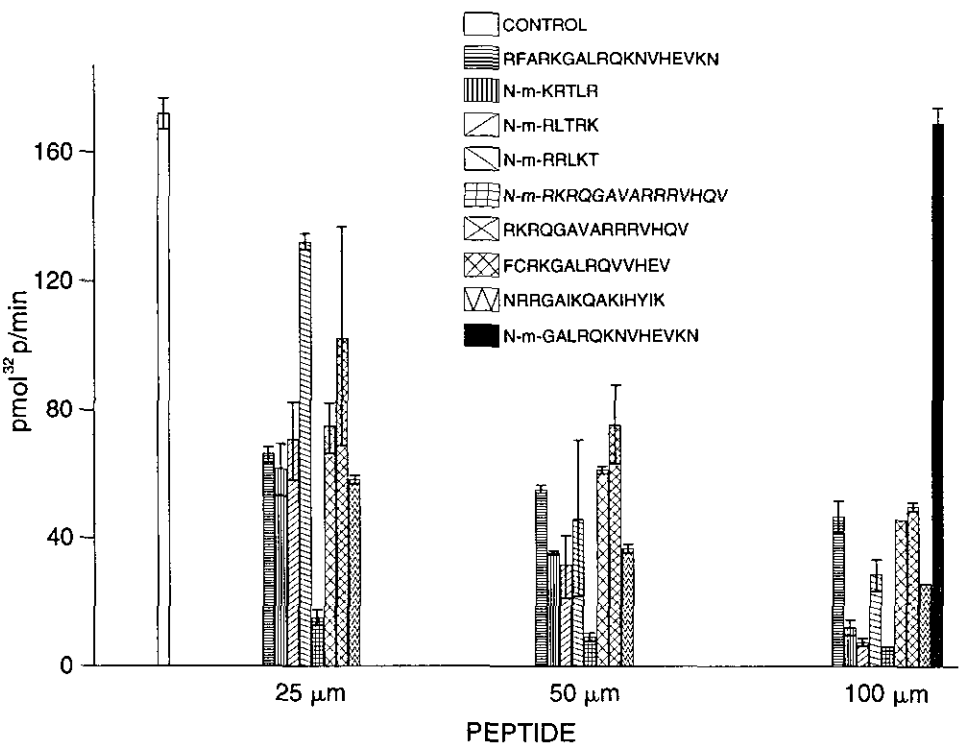

FIG. 1. Effect of peptides on PKC enzyme activity. Tissue extracts containing PKC activity were prepared from rat brain as indicated under Materials and Methods. PKC activity in the crude extract was determined by measuring the incorporation of ^{32}P in the PKC-specific substrate peptide. Different concentrations of each peptide were added to the reaction mixture to determine their PKC-inhibitory activity. Each experiment was done in triplicate. Values represent the means ± SD.

Incorporation of synthetic peptides in Jurkat cells. To analyze the incorporation of nonmyristylated peptides by Jurkat cells, peptides were fluorescently tagged at the amino terminal end with a FITC molecule. These FITC-labeled peptides were used to study the efficiency of peptide incorporation in Jurkat cells by direct uptake from the medium or electroporation. Fluorescence microscopy of these samples showed diffuse and uniform staining, indicating that the peptides can get into the cytoplasm of the Jurkat cells without electroporation (data not shown). The relative amount of peptide in the cell was monitored by flow cytometric analysis. Figure 2 shows the distribution of the cell fluorescense intensity from electroporated and nonelectroporated cells in the presence of 20 μM FITC-labeled peptide 5. No significant differences were seen in the uptake of the peptide under these conditions. At the same time the amount of peptide incorporated into the cells was dependent on the amount of peptide added to the media as can be seen in Fig. 3. Therefore, since Jurkat cells can absorb small peptides from the media without the need to create any artificial pores in the cell membrane, in all the subsequent experiments described below peptides were added directly to the medium.

Inhibition of IL-2R expression in Jurkat cells by PKC pseudosubstrates. To study the effects of PKC pseudosubstrates on the induction of IL-2R expression, Jurkat cells were incubated for 18 hr with PMA, anti-CD3, or the combination of PMA and anti-CD3. Figure 4 shows one representative experiment. Incubation of cells in the presence of anti-CD3 (2 μg/ml) or PMA (5 ng/ml) resulted in a small but significant induction of IL-2R expression, while the combination of PMA and anti-CD3 gives a strong induction, with more than 60% of the cells staining for IL-2R.

Incubation of Jurkat cells with anti-CD3 antibodies in the presence of 75 μM PKC inhibitory peptide (N-m-KRTLR) or the peptide corresponding to the reverse sequence

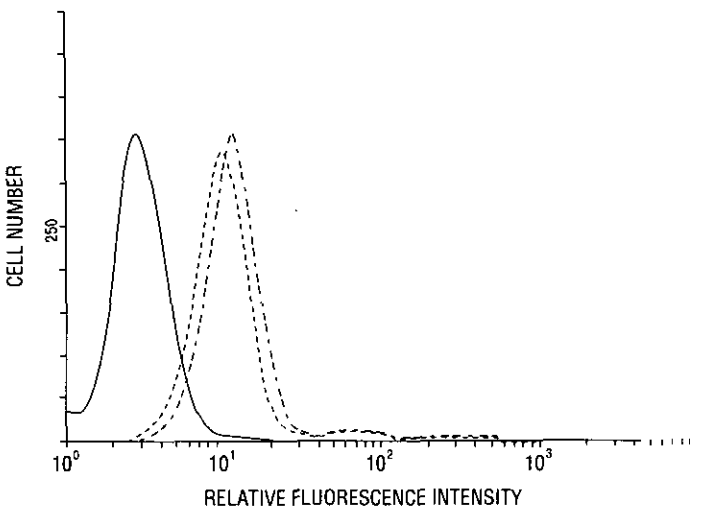

FIG. 2. Incorporation of FITC-labeled peptides into Jurkat cells. Jurkat cells from a log-phase culture were recovered by centrifugation and resuspended in medium containing 20 μM FITC-labeled peptide; an aliquot of the cells was electroporated as indicated under Materials and Methods. Electroporated and non-electroporated cells were incubated at 37°C for 2 hr and then analyzed by FACS. (– – –) Electroporated cells, (- - - -) nonelectroporated. Cells incubated at 4°C in the presence of the FITC peptide were used as negative control (——).

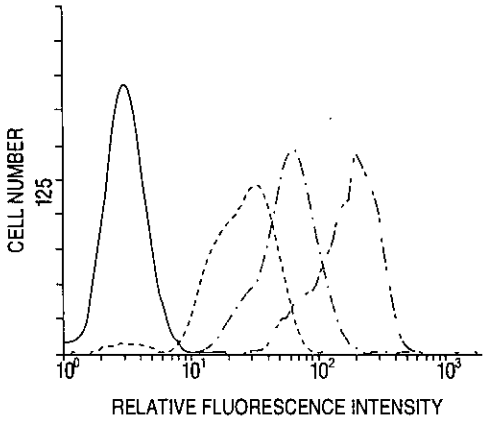

FIG. 3. Dose-dependent incorporation of peptide into Jurkat cells. Cells were incubated as indicated in the legend to Fig. 2 in the presence of different concentrations of FITC peptide, and the amount of peptide incorporated by the cells was monitored by FACS analysis as indicated under Materials and Methods. (- - -) 50 μM, (--·--) 100 μM, and (- — -) 200 μM. Control cells (——) were incubated at 4°C in the presence of 200 μM peptide.

(N-m-RLTRK) resulted in an inhibition of the induction of IL-2R expression (Fig. 5). Similar effects were found when other PKC-inhibitory peptides were included in the media at the time of the induction (Table 2). Although the inhibitory activity of these peptides was not total, the effects were reproducible in five independent exper-

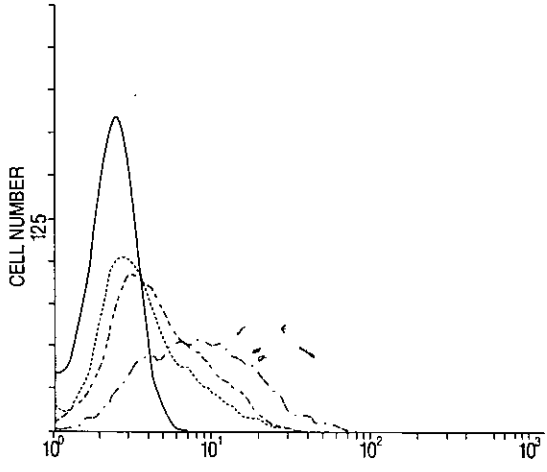

FIG. 4. Induction of IL-2R expression in Jurkat cells. Jurkat cells at a concentration of 10^6/ml were incubated for 16 hr at 37°C in the presence of PMA (5 ng/ml) (- - -), anti-CD 3 (2 μg/ml) (---), or the combination of both (- - - -) as indicated under Materials and Methods. IL-2R expression was determined by FACS analysis using anti-CD25-phycoerythrin-conjugated antibody as described under Materials and Methods. Control (——) represent unstimulated cells stained with anti-CD25-phycoerythrin-conjugated antibody.

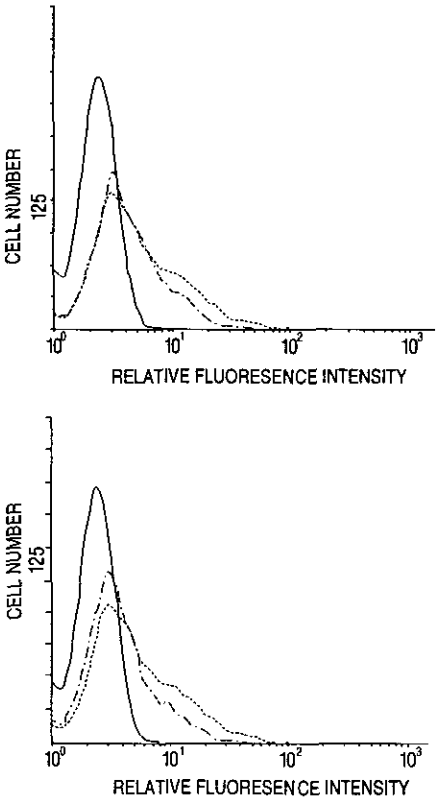

FIG. 5. Effect of PKC inhibitory peptides in the induction of IL-2R expression. Jurkat cells were stimulated with anti-CD3 antibodies as indicated in the legend to Fig. 4 in the presence (- - - -) or absence (– – –) of 75 μM peptide, and IL-2R expression was analyzed by FACS as previously indicated. Control (——) represents unstimulated cells stained with anti-CD25-phycoerythrin-conjugated antibody. Top, peptide N-m-KRTLR; bottom, peptide N-m-RLTRK.

iments. At the same time, the control peptide (N-m-GALRQKNVHEVKN), that showed no inhibitory activity in the PKC enzyme assay, had no effect in the induction of IL-2R expression by the different stimuli (Table 2).

Induction of IL-2R expression was also analyzed at the mRNA level by reverse transcriptase-PCR (Fig. 6). Treatment of Jurkat cells for 18 hr with PMA (5 ng/ml) or anti-CD3 (2 μg/ml) resulted in the induction of the expression of the mRNA coding for the IL-2R p55 chain. As previously shown (21) the induction of IL-2R was blocked by the PKC inhibitor H-7. In a similar way, the PKC peptide inhibitors (peptides 1 and 2) were also able to block the induction of the IL-2R mRNA by PMA and anti-CD3, while the control peptide had no effect.

Inhibition of TNF-β mRNA expression in Jurkat cells by PKC pseudosubstrates. To extend the previous described effects of these pseudosubstrates, we looked at the effect of several of these peptides on the induction of TNF-β expression in Jurkat cells by a PKC-mediated event. Jurkat cells were stimulated with PMA and anti-CD3 in the presence or absence of PKC pseudosubtrates for 18 hr, and induction of TNF-β mRNA expression was determined, as indicated previously for IL-2R, by the MAPPing technique, using specific primers for TNF-β. The results of these analyses are presented

TABLE 2

Inhibition by PKC Pseudosubstrates of IL-2R Induction in Jurkat Cells Treated with PMA and Anti-CD3

Peptide	Concentration (μM)	% Inhibition[a]		
		PMA	Anti-CD3	PMA + anti-CD3
N-m-KRTLR	50	4	25	15
	100	17	50	22
N-m-RLTRK	50	25	55	33
	100	53	70	45
N-m-RRLKT	50	10	ND	23
	100	12	ND	44
N-m-RKRQGAVARRRVHQV	50	20	11	19
	100	ND	42	38
RFARKGALRQKNVHEVKN	50	ND	ND	17
	100	17	31	52
N-m-GALRQKNVHEVKN	100	−8.0	−8.8	−8.7

[a] Percentage of inhibition was calculated by taking the percentage of cells expressing detectable CD25 in the sample that received only the respective inducer as 100% induction.

in Fig. 6. As previously reported (22) unstimulated Jurkat cells did not express detectable levels of TNF-β mRNA; however, incubation for 18 hr with PMA (5 ng/ml) or anti-CD3 (2 μg/ml) resulted in the induction of TNF-β mRNA. Addition of the PKC inhibitor H-7 at the time of the stimulation blocked the induction of TNF-β expression. In a similar manner, the presence of the myristylated peptides N-m-KRTLR and N-m-RLTRK at the time of addition of PMA or anti-CD3 to the cultures resulted in inhibition of TNF-β mRNA induction, while the myristylated control peptide (N-m-GALRQKNVHEV) had no effect.

DISCUSSION

The use of peptide pseudosubtrates as specific PKC inhibitors could offer great opportunities to analyze the signal transduction events involving the activation of PKC.

In this report we show that different peptides (Table 1) with sequences corresponding to the putative regulatory site of several PKC isoenzymes were able to inhibit the activity of PKC in an enzyme assay, with only small differences in their potency, while a truncated peptide (GALRQKNVHEVKN) containing only part of the pseudosubstrate sequence had no effect. Although PKC-α and -β/β' represent the majority of the PKC activity present in rat brain, and PKC-γ expression appears to be restricted to Purkinje cells (5, 10), we found that the PKC-γ peptide was as effective PKC inhibitor as the PKC-α peptide. At the same time the PKC-ϵ peptide give a higher degree of PKC inhibition than was seen with PKC-α peptide. These results indicate that all of these peptides are capable of inhibiting the major isoenzymes (PKC-α and -β) present in the rat brain, and they lack isoenzyme specificity in their inhibitory activity at the concentrations tested. However, more detailed studies using pure isolated PKC isoenzymes, and lower concentrations of the inhibitors, would be required to get a good estimation of their affinity for each isoenzyme.

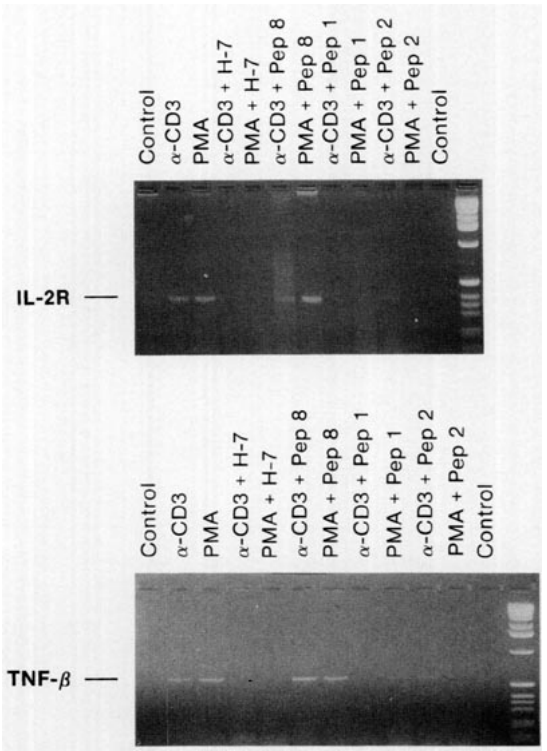

FIG. 6. Inhibition by PKC pseudosubstrates of IL-2R and TNF-β mRNA induction. Jurkat cells were treated with PMA (5 ng/ml) or anti-CD3 (2 μg/ml) for 16 hr at 37°C in the presence of different myristylated peptides (75 μM) or with H-7 (10 μM). Isolated total RNA from each sample was reverse transcribed and the cDNA pools were amplified by 33 cycles of PCR using specific primers for IL-2R (top) and for TNF-β (bottom) as indicated under Materials and Methods. Aliquots of the PCR products were analyzed by electrophoresis on a 2% agarose gel in TBE + ethidium bromide.

In a previous report (20) it was shown that myristilation of the pentapeptide KRTLR converts it from a PKC substrate to an inhibitor. This effect is thought to be due to an increase in the binding of the peptide through the myristyl group to the enzyme and, in that way, keeps the peptide in the catalytic site. When we compared the inhibitory activity of myristylated and unmyristylated peptide (RKRQGAVARRRVHQV) we found that the addition of a myristyl group does increase the inhibitory activity of the peptide.

We have also compared the inhibitory activity of three myristylated pentapeptides based on the sequence KRTLR. Our results showed that the peptide with the reverse sequence inhibited PKC activity in a manner similar to that of the normal peptide. However, the peptide N-m-RRLKT, where the two arginine residues are located side by side was less effective in inhibiting PKC activity. Although this peptide does not have the consensus sequence (RKXXXRXK) for a PKC pseudosubstrate (13), it is still able to inhibit PKC activity but at higher concentrations than that seen for the other peptides.

The inhibition of PKC activity by these pseudosubstrates was also shown in a cell assay, when we studied their effect in the induction of IL-2R and TNF-β expression

on Jurkat cells by PKC-mediated events. We found that all the pseudosubstrates inhibit IL-2R and TNF-β expression at concentrations of 50 to 100 μM. While we did not see an effect on cell viability for any of the peptide tested at these concentrations, we found some cytotoxic effects of myristylated peptides at concentrations higher than 150 μM.

A previous report (21) has shown that the peptide N-m-KRTLR inhibited IL-2 secretion and IL-2R induction by TPA plus either PHA or OKT3 mAb in Jurkat cells. Here we present evidence that the reverse peptide (N-m-RLTRK) and a related peptide (N-m-RRLKT) can inhibit the induction of IL-2R expression by PMA, anti-CD3, and PMA + anti-CD3 in Jurkat cells, when added to the medium at 50 or 100 μM (Table 2). Inhibition of PKC activity at a cellular level by these pseudosubstrates was further characterized by analyzing their effects on TNF-β mRNA expression after PMA or anti-CD3 treatment. The PKC inhibitor H-7 was used here to compare its inhibitory activity against that of the PKC pseudosubstrates. By looking at the inhibition of the induction of IL-2R and TNF-β expression by PMA and anti-CD3, we focused on the PKC pathway, avoiding any synergistic effects between PMA and anti-CD3. PMA treatment induces a direct activation of cellular PKC bypassing any receptor-mediated signal-transduction event, while treatment with anti-CD3 antibodies represents a more physiological mechanism of PKC activation. Based on this, the analysis of the effect of the pseudosubstrates on cells treated with PMA or anti-CD3 allows us to conclude that these peptides are able to inhibit intracellular PKC activity, resulting in interference with cellular events mediated by PKC activation.

Even though the peptide N-m-RRLKT was less effective as a PKC inhibitor in the enzyme assay at low concentrations, at higher concentrations it was as effective as the other two peptides. Given these facts, we found that the inhibition of IL-2R expression was also inhibited by the peptide N-m-RKRQGAVARRRVHQV, as well as the myristylated pseudosubstrates corresponding to the isoenzymes α, γ, and δ (data not shown). These results would indicate either that these pseudosubstrates have no isoenzyme specificity or that all the isoenzymes play an intricate part in IL-2R expression. The latter conclusion is less likely given the fact that normal thymocytes lacking PKC-δ readily show IL-2R expression (2), and even more significantly, Jurkat cells as normal T lymphocytes do not express detectable levels of PKC-γ (3, 24).

Myristylation of these peptides changes a hydrophilic molecule to an amphiphilic molecule, facilitating their incorporation inside the cell. However, the interaction of the myristylated peptide with the lipid bilayer of the cell membrane could also result in some nonspecific effects due to the alteration of the permeability of the cell membrane. This can be the cause for some of the cytotoxic effect of these peptides that were seen when they were added to the media in concentrations over 150 μM (data not shown).

A myristylated peptide (N-m-GALRQKNVHEV) corresponding to the truncated pseudosubstrate from PKC-α was used as a control because as predicted it had no effect on PKC activity measured in an enzyme assay. Consistent with this, we found that a 100 μM concentration of this peptide inhibited neither the induction of IL-2R (as determined by FACS or by RT-PCR) nor the induction of TNF-β mRNA expression (as determined by RT-PCR). These results demonstrate that the myristyl group had no effect by itself on the inhibition of IL-2R and TNF-β induction, and that the effects observed with the pseudosubstrates are really due to the inhibition of PKC activity. This was also confirmed when we used the pseudosubstrate RFARK-

GALRQKNVHEVKN to inhibit the induction of IL-2R expression by PMA and/or anti-CD3. In this assay the unmyristylated peptide was as active as the myristylated form of the same peptide (data not shown) or any of the other myristylated peptides, supporting also the concept that Jurkat cells are able to incorporate peptides from the media in an efficient manner.

ACKNOWLEDGMENTS

We thank Dr. J. Dunne and L. Chiu for their assistance and expertise with the flow cytometer analysis, and Dr. J. T. Ransom, D. Webb, B. H. Devens, and Dr. G. Peltz for their review of this manuscript. Dr. Barja was supported by a fellowship from the Spanish Ministry of Science and Education.

REFERENCES

1. Koretzky, G. A., Wahi, M., Newton, M. E., and Weiss, A., *J. Immunol.* **143,** 1692, 1989.
2. Freire-Moar, J., Cherwinski, H., Hwang, F., Ransom, J., and Webb, D., *J. Immunol.* **147,** 405, 1991.
3. Mischak, H., Kolch, W., Goodnight, J., Davidson, W. F., Rapp, U., Rose-John, S., and Mushinski, J. F., *J. Immunol.* **147,** 3981, 1991.
4. Terajima, J., Tsutsumi, A., Freire-Moar, J., Cherwinski, H., and Ransom, J. T., *Cell. Immunol.* **142,** 197, 1992.
5. Nishizuka, Y., *Nature* **334,** 661, 1988.
6. Parker, P. J., Kour, G., Marais, R. M., Mitchell, F., Pears, C., Schaap, D., Stabel, S., and Webster, C., *Mol. Cell. Endocrinol.* **65,** 1, 1989.
7. Ohno, S., Akita, Y., Konno, Y., Imajoh, S., and Suxuki, K., *Cell* **53,** 731, 1988.
8. Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K., and Nishizuka, Y., *J. Biol. Chem.* **263,** 6927, 1988.
9. Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K., and Nishizuka, Y., *Proc. Natl. Acad. Sci. USA* **86,** 3099, 1989.
10. Brandt, S. J., Niedel, J. E., Bell, R. M., and Young, W. S., *Cell* **49,** 57, 1987.
11. Bell, R. M., and Burns, D. J., *J. Biol. Chem.* **266,** 4661, 1991.
12. Nishizuka, Y., *Science* **233,** 305, 1986.
13. Bell, R. M., *Cell* **45,** 631, 1986.
14. Kisimoto, A., Kajikawa, N., Shiota, M., and Nishizuka, Y., *J. Biol. Chem.* **258,** 1156, 1983.
15. Melloni, E., Pontremoli, S., Michetti, M., Sacco, O., Sparatore, B., Salamino, F., and Horecker, B. L., *Proc. Natl. Acad. Sci. USA* **82,** 6435, 1985.
16. House, C., and Kemp, B. E., *Science* **238,** 1726, 1987.
17. Pearson, R. B., Wettenhall, R. E. H., Means, A. R., Hartshorne, D. J., and Kemp, B. E., *Science* **241,** 970, 1988.
18. Hardie, G., *Nature* **335,** 592, 1988.
19. Schaap, D., Parker, P. J., Bristol, A., Kriz, R., and Knopf, J., *FEBS Lett.* **243,** 351, 1989.
20. O'Brian, C. A., Ward, N. E., Liskamp, R. M., De Bont, D. B., and Van Boom, J. H., *Biochem. Pharmacol.* **39,** 49, 1990.
21. Ioannides, C. G., Freedman, R. S., Liskamp, R. M., Ward, N. E., O'Brian, C. A., *Cell. Immunol.* **131,** 242, 1990.
22. Brenner, C. A., Tam, A. W., Nelson, P. A., Engleman, E. G., Suzuki, N., Fry, K. E., and Larrick, J. W., *Biothechniques* **7,** 1097, 1989.
23. Frohman, M. A., Dush, M. K., and Martin, G. R., *Proc. Natl. Acad. Sci. USA* **85,** 8998, 1988.