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Studies on the Role of Protein Kinases in the TNF-Mediated Enhancement of Murine Tumor Cell-Endothelial Cell Interactions

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Abstract We have previously demonstrated that the exposure of mouse microvascular endothelium (MME) to tumor necrosis factor-α (TNF) led to the increased binding of mouse mastocytoma cells (P815) to endothelial monolayers (Bereta et al., in press). In the current study we examined the possible involvement of protein kinases in TNF signal transduction in the endothelial cells. PKA does not appear to play a role in the potentiation of binding by TNF. We found that the TNF-generated signal is inhibited by H-7 and sangivamycin, but not by staurosporine. TNF did not cause translocation of PKC to the cell membrane and its effect could not be completely mimicked by PMA nor by PMA in the presence of calcium-raising agents. Thus, we concluded that the "classical" PKC pathway is not completely responsible for TNF signalling in this system. We also found that staurosporine itself strongly enhanced adhesion of tumor cells to endothelium, utilizing a mechanism distinct from that of TNF. Although the data provide evidence for the role of kinases in the effect of TNF on binding of tumor cells to MME, this role appears to be a complex one.

Key words: tumor necrosis factor-α, tumor cell adherence, PKC, PKA, calcium

Tumor necrosis factor-α (TNF), a protein secreted mainly by activated macrophages, was originally defined by its antitumor activity. However, TNF is now recognized as a cytokine affecting many types of cells and displaying multiple biological activities involved in inflammatory and immunological processes (reviewed in Kunkel et al., 1989). The broad spectrum of TNF activities includes, among others, stimulation of fibroblast growth, activation of granulocytes, inhibition of synthesis and activity of lipoprotein lipase, stimulation of synthesis of cytokines, and stimulation of collagenase activity.

One target for TNF action is endothelium. Together with other cytokines, TNF mediates endothelial responses during injury or inflammation. For example, TNF causes endothelium to express procoagulant activity, increases the expression of Class I MHC antigens, and stimulates the production of chemotactic factors, other cytokines, and platelet-derived growth factor (PDGF) by endothelium (reviewed in Pober and Cotran, 1990). TNF has also been shown to stimulate or induce the expression of cell adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1) and endothelial-leukocyte adhesion molecule 1 (ELAM-1) on the endothelial cell surface (Pober and Cotran, 1990). These molecules are responsible for the binding of inflammatory cells to endothelium, a process which is required for their migration to sites of inflammation.

We have previously reported that TNF can also influence tumor cell adherence to endothelium (Bereta et al., in press). We demonstrated that pretreatment of mouse microvascular endothelial cells (MME) with TNF augmented the binding of P815 mouse mastocytoma cells. Cytokine-enhanced adhesiveness of endothelium was observed between 4–48 hours after addition of TNF. A similar time dependent increase in P815 binding to MME was observed when endothelium was treated with interleukin 1 (IL-1), or, to a lesser extent, with phorbol myristate

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acetate (PMA), an activator of protein kinase C (PKC).

To date, little is known about intracellular pathways involved in either the regulation of cell adhesion molecule expression or the mechanism of TNF action. The involvement of PKC was proposed for IFN-γ-mediated stimulation of ICAM-1 on human umbilical vein endothelial cells (HUVEC) (Renkonen et al., 1990). In addition, Lane et al. (1989, 1990) found that activators and inhibitors of PKC could affect ICAM-1 expression, which suggested that PKC was involved in this process. Several studies have indicated that PKC also plays a role in TNF signal transduction in a number of cell types. In lymphoid cell lines, TNF caused a transient activation and translocation of PKC (Schütze et al., 1990). TNF-mediated gene expression in melanoma cells was found to be regulated in a positive manner by PKC and in a negative manner by protein kinase A (PKA) (Johnson and Baglioni, 1990). Other studies have indicated that cAMP (Baud et al., 1988; Zhang et al., 1988) and tyrosine phosphorylation (Kohno et al., 1990) can play a positive role in modulating the TNF signal. The recent discovery of multiple TNF receptors has added another layer of complexity (reviewed in Sprang, 1990). Here, we examine the possible role of various protein kinases in TNF signal transduction leading to increased binding of P815 mastocytoma cells to endothelium. We provide evidence for the involvement of kinases in this process, but find that their role is a complex one.

MATERIALS AND METHODS

Maintenance of Cells

P815 mastocytoma cells were maintained in DBA/2J mice (Jackson Laboratory, Bar Harbor, ME). The cells were passaged every 6 to 8 days by intraperitoneal transfer of 0.1 ml ascites fluid. The exudates contained approximately 95% tumor cells in suspension with minimal contamination by inflammatory cells.

Murine microvascular endothelial cell cultures (MME), obtained initially from brain endothelium, were a gift from Dr. Robert Auerbach. They were grown in medium consisting of DMEM (Hazleton, Lenexa, KS), 20% FCS (HyClone, Logan, UT), 20% Sarcoma 180-conditioned medium, 5 mM L-glutamine, and antibiotics. The presence of significant amounts of angiotensin-converting enzyme activity was used to verify the endothelial nature of the cultures. The cobblestone appearance of confluent cultures provided additional ongoing confirmation of endothelial identity.

Adhesion Assay

P815 mastocytoma cells were labelled with ^{51}Cr (New England Nuclear, Boston, MA) as described previously (Antonia et al., 1989). Endothelial cells were grown to complete monolayers in 24-well tissue culture plates (Falcon, Lincoln Park, NJ). In each case, the monolayers were visually examined to confirm confluency. MME cells were incubated with specified reagents for the time period indicated in each experiment. After the incubation, the medium was aspirated, the monolayers were washed once with fresh medium, and 0.5 ml of RPMI 1640 supplemented with 2% FCS containing 3.5×10^5 radiolabelled cells were added to each well. The plates were incubated at 37°C in 5% CO_2-95% air for 1 hour. Upon completion of the incubation the monolayers were washed two times with RPMI 1640 medium to remove nonadherent cells. The content of each well was solubilized by adding 0.5 ml of 0.5 N NaOH and the level of radioactivity was determined in a Packard gamma counter (Downers Grove, IL). The percent of tumor cells that adhered was calculated as follows:

$$\% \text{ binding} = \frac{\text{labelled cells bound to monolayer}}{\text{labelled cells added to monolayer}} \times 100.$$

Determination of ^{35}S-Methionine Incorporation Into Cellular Proteins

When the cells reached confluency, the medium was replaced and sangivamycin or cycloheximide was added simultaneously with ^{35}S-methionine (1.6 μCi) to groups of 4 wells. After 4 hours of incubation, the medium was aspirated and the monolayers were washed three times with ice cold PBS. Cells were lysed by exposure to 0.5% Triton X-100 for 1 hour at 4°C. After collection of the content of each well, the protein was precipitated with 10% TCA (1 hour at 4°C). Precipitates were washed three times with PBS containing an excess of L-methionine to remove unincorporated ^{35}S-methionine. After the final centrifugation, the precipitates were dissolved in CytoScint (ICN Biomedicals, Irvine CA) and

transferred to vials, and the level of radioactivity was determined in a Beckman scintillation counter (Beckman Instruments Inc., Fullerton CA).

Measurement of ^{3}H-PDBu Binding to MME Monolayers

The binding of radiolabelled phorbol-12,13-dibutyrate (^{3}H-PDBu) (New England Nuclear, Boston, MA) to endothelial cells was performed according to the method of Jaken (1987). Briefly, monolayers of endothelial cells cultured in 12-well dishes were left untreated or treated with TNF (100 U/ml) at 37°C for various periods of time. The cells were then washed thoroughly and incubated for 15 minutes at 37°C in RPMI 1640 medium containing BSA (1 mg/ml) and ^{3}H-PDBu (100 nM). For estimation of nonspecific binding, cells were incubated with ^{3}H-PDBu in the presence of an excess of unlabelled PMA (10 μM). After incubation, the monolayers were washed three times with ice-cold medium containing BSA and the cells were removed from the wells using trypsin-EDTA. The contents of the wells were transferred to vials and the level of radioactivity was determined in a Beckman scintillation counter.

Measurement of PKC Activity

PKC activity was measured according to the method of Kikkawa et al. (1983) with some modifications. Briefly, monolayers of MME cells were pretreated for 5 or 60 minutes with TNF or PMA. Cells were harvested from plates with an extraction buffer containing calcium chelators, protease inhibitors, and 2-ME, followed by lysis of the cells by sonification. Cytosolic and membrane fractions were separated by centrifugation. Proteins were released from the membranes with detergent and supernatants obtained by additional centrifugation were designated as the membrane fractions. PKC in all fractions was partially purified on DE-52 ion-exchange chromatography columns. PKC activity was measured by enzymatic transfer of radiolabelled phosphate from ATP to histone. The reaction was started by the addition of samples containing 20 μg of protein to the reaction mixture (total volume of 250 μl containing 20 mM Tris (pH 7.6), 10 mM $MgCl_2$, 2 mM $CaCl_2$, 65 μg histone IIIS, 40 μM ATP, 45 μg phosphatidylserine, 40 nM PMA, and 1 μCi [γ^{32}P] ATP (6,000 Ci/mmol, New England Nuclear)). In control reactions, to determine the level of calcium- and phospholipid-independent kinase activity, calcium, phosphatidylserine, and PMA were replaced by 5 mM EGTA. After incubation for 10 minutes at 30°C, the reaction was stopped by addition of 750 μl of 20% TCA and 250 μl of 0.4% BSA. Precipitates were collected on Millipore filters (0.45 μm), washed with 10% TCA, and dried. Radioactivity on the filters was measured in liquid scintillation cocktail (CytoScint, ICN Biomedicals, Irvine, CA) by using a Beckman scintillation counter (Beckman Instruments Inc., Fullerton, CA). Net PKC activities were determined from the differences between mean triplicate reactions performed in the presence of PKC activators and duplicate control reactions.

Determination of Cyclic AMP Levels in MME

Endothelial cells were grown in 6-well tissue culture plates (Falcon) as described above. When the cells reached confluency, they were preincubated for 30 minutes in RPMI 1640 containing 2% FCS, 2 mM glutamine, antibiotics, and 3-isobutyl-1-methyl-xanthine (IBMX), an inhibitor of cAMP phosphodiesterase which prevents cleavage of cAMP. Factors to be studied were then added to the incubation medium for various times at 37°C at concentrations optimal for their activity in the adhesion assay. Following completion of the incubation, the medium was aspirated and the monolayers were washed twice with ice-cold PBS. To release cAMP from the cytoplasm, the cells were incubated overnight with 5% TCA at 4°C. After collection of the contents of each well, TCA was removed from the samples by extraction 5 times with 4 ml of water-saturated ether. Samples were dried under an air stream at 70°C and the level of cAMP was determined using a ^{125}I-cAMP radioimmunoassay kit (New England Nuclear, Boston, MA). After removal of TCA from the wells, the remaining cells were washed with PBS and dissolved in 1 M NaOH, and the protein concentration was determined. The amount of cAMP was calculated per 1 mg of cellular protein.

Reagents

Human recombinant TNF was purchased from ICN Biochemicals (Cleveland, OH) and dissolved in RPMI 1640 medium containing 10% FCS. Cholera toxin (ChTx) and cycloheximide (Sigma, St. Louis, MO), H-7 and HA-1004 (Sei-

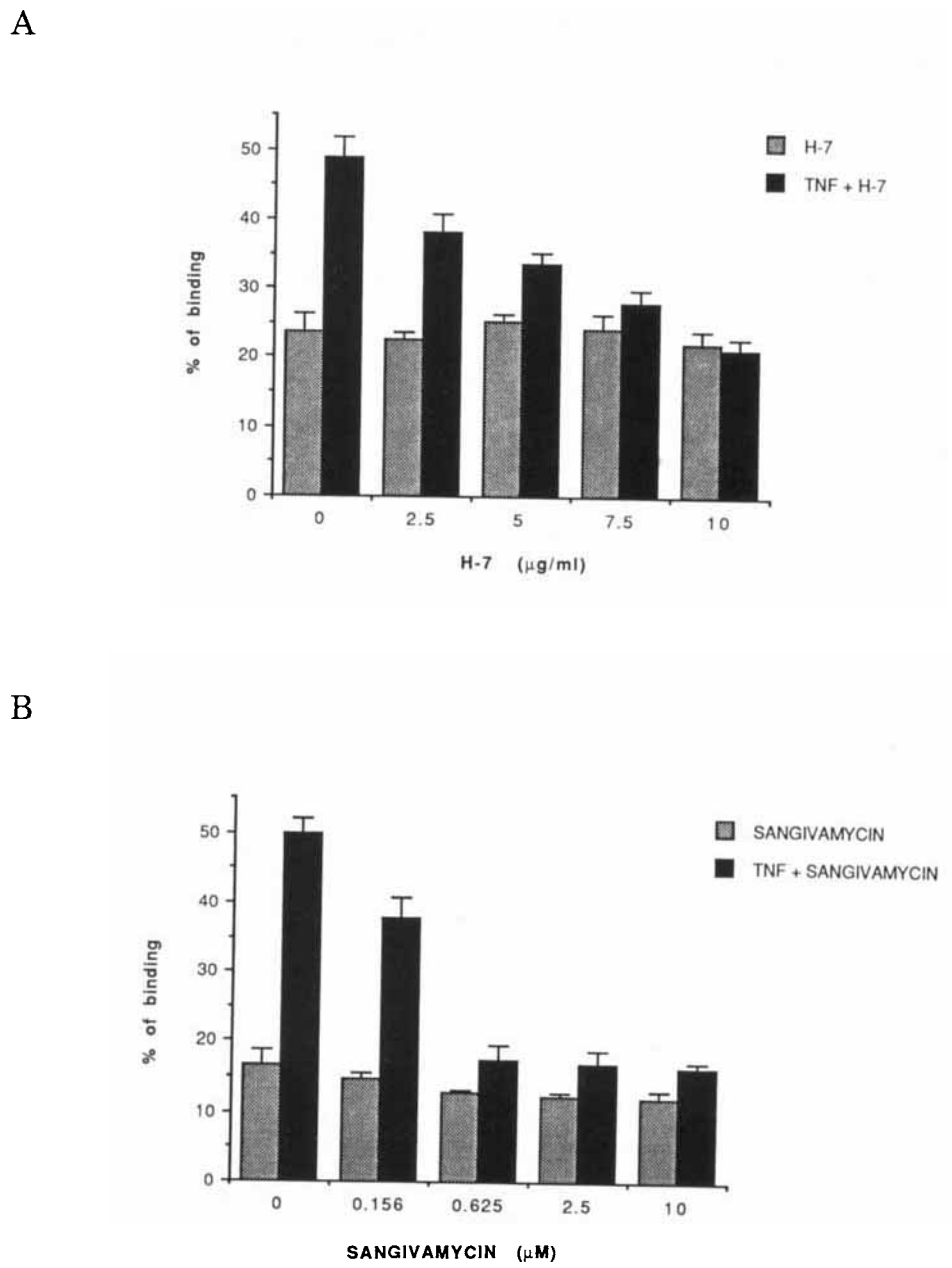

Fig. 1. Adherence of P815 cells to MME monolayer pretreated for 4 hours with various concentrations of H7 (**A**) or sangivamycin (**B**) and TNF (100 U/ml) in the presence of either inhibitor. Bars represent the mean adherence of 3 experiments ± SD. Each determination was done in quadruplicate. DMSO alone (sangivamycin solvent) at a concentration of 0.4% had no effect on binding. For sangivamycin at a concentration ≥ 0.625 μM and H-7 at a concentration ≥ 7.5 μg/ml, $P < 0.001$ compared with TNF alone.

kagaku America, Inc., St. Petersburg, FL) were dissolved in LPS-free water (Sigma, St. Louis, MO). PMA, forskolin, 1,9-dideoxyforskolin, IBMX, and dibutyryl-cAMP (db cAMP) (Sigma, St. Louis, MO), A23187 (Eli Lilly, Indianapolis, IN), and ionomycin (Calbiochem, San Diego, CA) were dissolved in ethanol. Staurosporine (Boehringer Mannheim, Indianapolis, IN), thapsigargin (Sigma), and sangivamycin (a gift from the National Cancer Institute, Bethesda, MD) were dissolved in DMSO. Stock solutions were prepared as follows: PMA, ionomycin, A23187, and db cAMP, 10 mM; forskolin, dideoxyforskolin, IBMX, thapsigargin, 5 mM; sangivamycin,

TABLE 1. Incorporation of ^{35}S-Methionine Into Cellular Proteins of MME Incubated With Sangivamycin or Cycloheximide[a]

	^{35}S-Methionine incorporation			
	cpm	% Incorporation[b]	% Inhibition Protein Synthesis[c]	% Inhibition of TNF effect[d]
Control	79,516 ± 5246[e]	12.51	—	—
Sangivamycin				
10.00 μM	56,870 ± 4788	8.95	28.23	100.0
0.156 μM	72,757 ± 3620	11.45	8.50	36.4
Cycloheximide				
10.000 μg/ml	4,662 ± 412	0.73	94.13	100.0

[a]MME cells were incubated with sangivamycin or cycloheximide for 4 hours in the presence of ^{35}S-methionine and incorporation of radioisotope was calculated as described in Materials and Methods.
[b]Total radioactivity added per well at the start of the experiment was 635,620 cpm.
[c]Compared with control (untreated) monolayers.
[d]Inhibition of TNF-mediated increase in P815 binding to MME.
[e]Each data point represents the mean ± SD of 4 wells from a single experiment. Similar data were obtained in other experiments.

2.5 mM; and staurosporine, 0.5 mM. All stock solutions of reagents were stored as recommended by the manufacturer.

Statistical analysis

Data were analyzed by Student's t-test.

RESULTS

The Effect of PKC Inhibitors on the Regulation of Tumor Cell Binding to Endothelium

We examined the effect of different protein kinase inhibitors including H-7, HA1004, sangivamycin, and staurosporine on the TNF-mediated increase in adherence of tumor cells to endothelium. H-7 and HA1004 are isoquinolinesulfonamide compounds which inhibit various protein kinases, including PKC and PKA, but differ in their binding affinities and resulting inhibition constants (K_i) with respect to particular enzymes. Within this group H-7 is the most potent inhibitor of PKC, while HA1004 is the weakest one and is commonly used as a control for H-7 (Hidaka et al., 1989).

Endothelial cells were incubated for 4 hours with TNF (100 U/ml), with varying concentrations of H-7, or with TNF in the presence of H-7. Following the incubation, the monolayers were washed and ^{51}Cr labelled P815 cells were added for 1 hour. Nonadherent tumor cells were removed. Pretreatment of endothelial cells with H-7 alone did not affect the binding of tumor cells to the monolayer. However, when H-7 was added to the endothelial cultures simultaneously with TNF (prior to incubation with tumor cells), the increase in adherence usually observed following cytokine treatment was inhibited in a dose-dependent fashion (Fig. 1A). As can be seen in Figure 1A, at 10 μg/ml (28 μM), H-7 was able to bring the TNF-mediated increase in adherence of P815 cells back to the level of binding in the absence of TNF (first bar). Pretreatment of endothelial monolayers with HA1004, even at a concentration of 10^{-4} M had no effect either on the basal level of tumor cell binding or on the TNF-mediated increase in P815 adherence to endothelium (data not shown).

Sangivamycin, a purine nucleoside analogue, is another potent inhibitor of PKC. Unlike H-7, which inhibits PKC and PKA to a similar extent, sangivamycin is a significantly weaker inhibitor of PKA than PKC (Loomis and Bell, 1988). We found that exposure of MME for 4 hours to TNF in the presence of sangivamycin led to the inhibition of the up-regulatory effect of TNF on tumor cell binding to endothelium (Fig. 1B). The effect of sangivamycin was dose dependent, but the concentration required for complete inhibition of the TNF effect on binding (0.625 μM) was at least 10 times less than the inhibition constant (K_i) which has been estimated for inhibition of native PKC by sangivamycin in in vitro studies (Loomis and Bell, 1988). We also observed a slight decrease in the basal level of tumor cell binding to endothelium after treatment of MME with sangivamycin alone. We have previously shown that cycloheximide slightly diminished the attachment of P815 cells to MME and completely abolished the effect of TNF (Bereta et al., in press). Because of the resemblance of the

sangivamycin effect to that of cycloheximide, together with the fact that the former has also been shown to inhibit protein synthesis to some extent (Cohen and Glazer, 1985), we wanted to rule out the possibility that the observed effects of sangivamycin were due to inhibition of protein synthesis. We found that high concentrations of sangivamycin (10 μM) were able to decrease the incorporation of ^{35}S-methionine into cellular proteins. However, at a low concentration (0.156 μM), sangivamycin did not significantly diminish protein synthesis, but did inhibit the effect of TNF (Table 1). Thus, inhibition of protein synthesis per se did not account completely for the inhibitory effect of sangivamycin on TNF action.

Staurosporine, a microbial alkaloid, is a uniquely potent inhibitor of PKC, since it is active at nanomolar concentrations. However, like other kinase inhibitors, staurosporine exhibits only limited selectivity among protein kinases (Tamaoki et al., 1986; Rüegg and Burgess, 1989). Surprisingly, we found that staurosporine did not behave like other PKC inhibitors with respect to tumor cell binding to endothelium. Pretreatment of endothelial monolayers with nanomolar concentrations of staurosporine led to a significant increase in tumor cell adhesion to endothelium. The effect of a 100 nanomolar solution of staurosporine was even greater than the maximal effect we observed with TNF alone. Moreover, when endothelium was pretreated with both compounds simultaneously, the number of tumor cells that bound to the monolayer was always more than when endothelial cells were treated with a single factor (Fig. 2A). This effect was most significant when MME monolayers were incubated with low concentrations of TNF (10 U/ml) and staurosporine (0.1 nM and 1 nM) (Fig. 2B). We have previously shown that a TNF-mediated increase in adherence is not observed for at least 4 hours after addition of the cytokine (Bereta et al., in press). In contrast, the effect of staurosporine was observed as early as 30 minutes after its addition to endothelial monolayers. Longer incubation of endothelium with staurosporine led to a further increase in tumor cell adherence (Fig. 2C). This effect was maintained for at least 24 hours (data not shown).

To examine whether or not inhibition of protein synthesis could prevent the stimulatory effect of staurosporine, we preincubated endothelial cells with staurosporine in the presence of cycloheximide. We found that cycloheximide was able to modify the effect of staurosporine to a limited degree (about 25%), indicating that protein synthesis did not account completely for the effect of staurosporine. In addition, unlike the other reagents that we studied (TNF, PMA, H-7, HA1004, and sangivamycin), staurosporine caused substantial changes to the morphology of endothelial cells in culture (Fig. 3).

Binding of PDBu to Endothelial Monolayers

For most cells, activation of PKC is accompanied by its translocation from the cytosol to the plasma membrane. Since PKC represents the cellular binding site for phorbol diesters, PKC translocation to the plasma membrane can be identified by the increase in specific PDBu binding to intact cells (Jaken, 1987). We examined the binding of PDBu to endothelial cells after incubation of the cells with TNF (100 U/ml) for various periods of time. We observed no significant changes in the specific binding of phorbol diester to MME, which indicated that PKC had not been translocated to the plasma membrane following treatment of endothelium with TNF (Table 2). These data are in agreement with the results of preliminary studies in which we measured the activity of Ca^{2+}- and phospholipid-dependent kinase (PKC) in cytosolic and membrane fractions of MME cells following their treatment with PMA or TNF. We found that exposure of MME to PMA for 5 minutes or 1 hour resulted in a marked translocation of the enzyme from the cytosol to the membrane, so that more than 90% of the total PKC activity was present in a membrane-bound form. In contrast, no such effect was observed after treatment of endothelium with TNF. One hour of exposure of MME to the cytokine led to a decrease in the activity of membrane-associated PKC, without any significant changes in the activity of the enzyme in the cytosol (data not shown).

The Effect of Adenylate Cyclase Activators on P815 Binding to Endothelial Monolayers

Because of the limited specificity of protein kinase inhibitors, it is difficult to draw unequivocal conclusions concerning their biological effects. We could not exclude the possibility of PKA participation in our model simply because HA1004 had no effect on TNF-mediated adherence. We therefore investigated the effect of ChTx and forskolin in this system. These agents

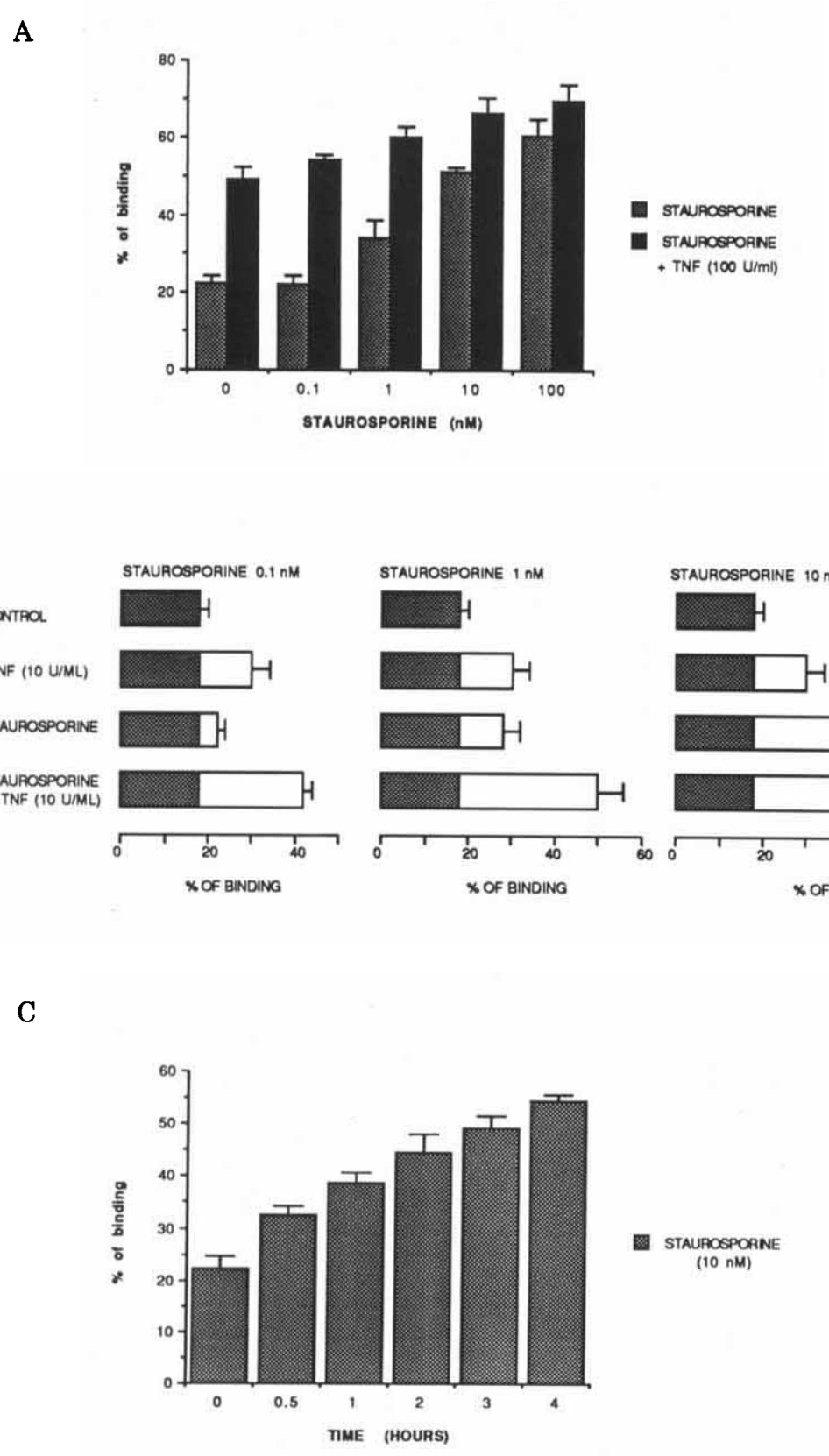
A
% of binding
STAUROSPORINE (nM)
STAUROSPORINE
STAUROSPORINE + TNF (100 U/ml)
B
STAUROSPORINE 0.1 nM
STAUROSPORINE 1 nM
STAUROSPORINE 10 nM
CONTROL
TNF (10 U/ML)
STAUROSPORINE
STAUROSPORINE + TNF (10 U/ML)
% OF BINDING
C
% of binding
TIME (HOURS)
STAUROSPORINE (10 nM)

Figure 2.

activate adenylate cyclase, which leads to an increase in cAMP levels in the cytoplasm and in turn to activation of PKA. Forskolin activates adenylate cyclase directly, whereas the effect of cholera toxin is mediated by stimulatory G protein (G_S). We found that both ChTx and forskolin increase tumor cell binding to endothelium in a dose-dependent manner after 24 hours of incubation (Fig. 4A,B). However, the increase in P815 adherence which followed incubation of endothelium with either ChTx or forskolin was significantly lower than that observed following incubation with TNF. It should also be noted that a longer period of incubation was required for the effects of ChTx and forskolin to become apparent when compared with the time required for an increase in adherence due to TNF (Fig. 5). While the rate of increase in tumor cell-endothelial cell binding was similar for forskolin or ChTx versus TNF during the period of 4 to 24 hours, TNF induced its major effect during the first 4 hours, the period in which the other two agents had a negligible effect. Similar results were obtained after treatment of MME with db cAMP, an analogue of cAMP, which is able to penetrate the cell membrane (data not shown). For this reason, PKA activity would not appear to play a dominant role in the enhancement of binding by TNF. This is not to imply that PKA does not play any role in binding. For example, we compared the effects of forskolin and its analogue, 1,9-dideoxyforskolin, on tumor cell binding to endothelium. Dideoxyforskolin shares most biological activities with forskolin but lacks the ability to activate adenylate cyclase (Laurenza et al., 1989). We found that unlike forskolin, dideoxyforskolin had no effect on P815 cell binding to endothelium, which suggests that adenylate cyclase is involved in the increase in tumor cell binding observed after forskolin treatment of MME (Fig. 4B).

Fig. 2. Adherence of P815 cells to MME monolayer: (A) endothelial cells pretreated for 4 hours with various concentrations of staurosporine (gray bars) or with TNF (100 U/ml) in the presence of staurosporine (black bars); (B) endothelial cells pretreated for 4 hours with different concentrations of staurosporine or staurosporine plus TNF (10 U/ml); (C) endothelial monolayers pretreated with staurosporine (10 μM) for various periods of time. Bars represent the mean adherence ± SD of 3 (A) or 2 (B and C) experiments and each determination was done in quadruplicate. In all experiments binding changes caused by staurosporine at a concentration ≥ 10 nM were statistically significant ($P < 0.001$).

It should be noted that incubation of endothelial monolayers with forskolin or db cAMP for 24 hours led to morphological changes in the cells (Fig. 3). These changes were also observed after incubation of MME with ChTx, but to a lesser extent.

The Effect of Stimulators of Adenylate Cyclase and TNF on Endothelial cAMP Levels

We studied cAMP concentration in endothelial cells after addition of ChTx, forskolin, or TNF. As shown in Figure 6, both ChTx and forskolin produced approximately a fivefold increase in cAMP levels in endothelial cells. The effect of forskolin was immediate, whereas changes in cAMP concentration following addition of ChTx were slower, reaching maximal levels in 30 minutes. TNF, unlike forskolin and ChTx, did not cause any changes in the cAMP concentration in MME cells (Fig. 6). More prolonged (up to 5 hours) incubation of endothelium with TNF also had no effect on cAMP levels. This, taken in conjunction with the quantitation and time course differences between TNF and forskolin or ChTx suggests that, unlike these latter agents, TNF does not exert its effect via PKA.

The Effect of Calcium Ions on the Regulation of Tumor Cell Binding to Endothelium

Calcium ions are regarded as an important intracellular transduction messenger in the regulation of cell growth and differentiation. Increase in cytosolic-free Ca^{2+} concentration is often accompanied by activation of PKC. In some systems these two secondary messengers act synergistically (Tyers and Harley, 1986; Berridge, 1987; Kumagai et al., 1987), whereas in others they can act independently (Farese et al., 1987; Rasmussen et al., 1990). Because our results indicated the possible involvement of PKC in up-regulation of tumor cell adhesion to endothelium, we studied the influence of increased calcium levels on this process.

Incubation of MME with the calcium ionophore ionomycin alone for 4 hours led to a slight decrease in tumor cell binding to endothelial monolayers. On the other hand, ionomycin inhibited the TNF-mediated increase in P815 adherence to endothelium in a dose-dependent manner. We also found that Ca^{2+} ionophores could inhibit not only the effect of TNF, but that of PMA as well (Fig. 7A). Similar results were obtained when ionomycin was replaced with the

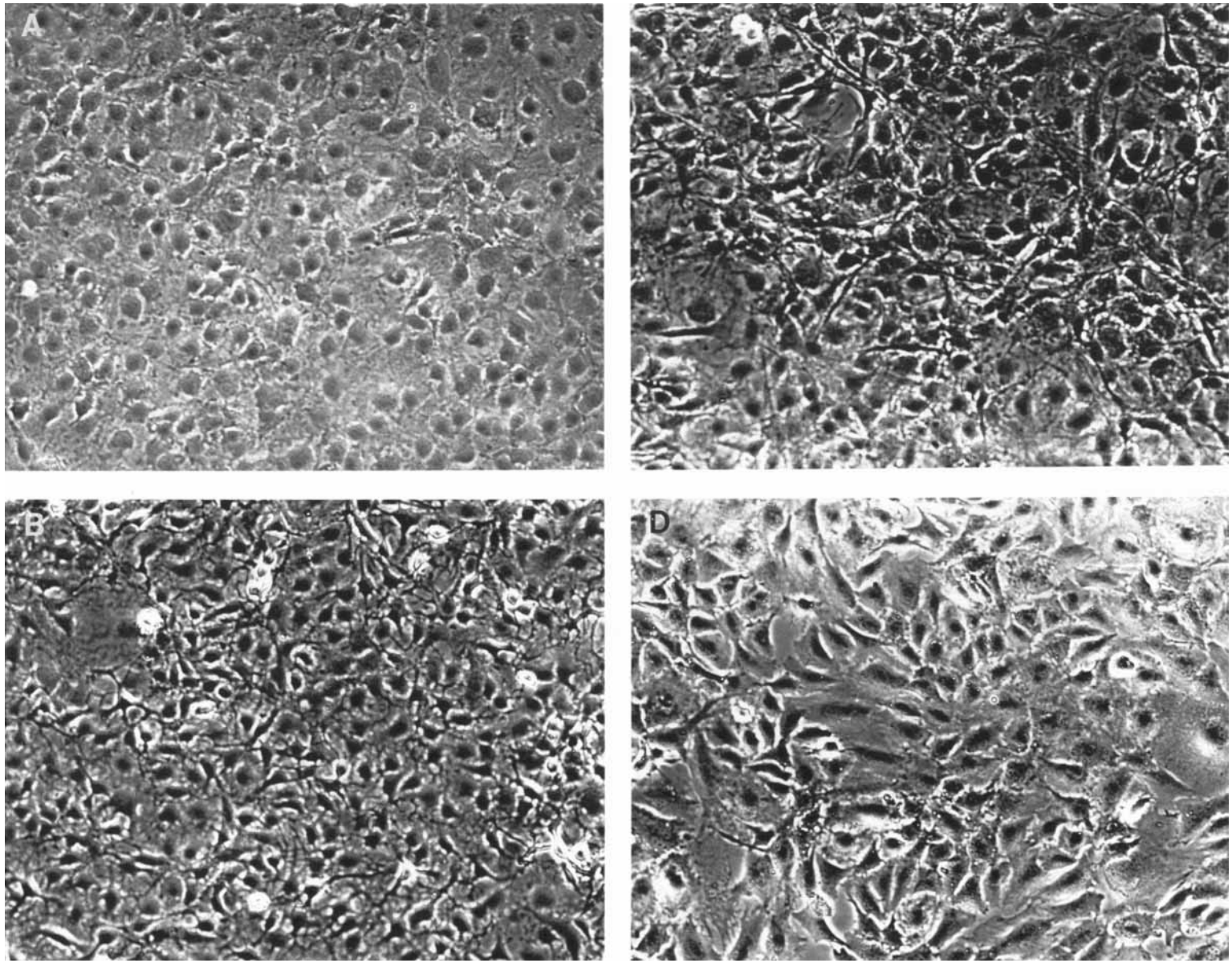

Fig. 3. Morphological changes in MME monolayers. (**A**) Nontreated cells, (**B**) cells treated for 4 hours with staurosporine (10 nM), (**C**) cells treated for 24 hours with forskolin (2 μM), and (**D**) cells treated for 24 hours with ionomycin (10 μM).

TABLE 2. Binding of ^{3}H-PDBu to Endothelial Monolayers

TNF treatment (min)	Experiment 1	Experiment 2
0.0	10.5 ± 1.8[a]	11.3 ± 0.3
2.5	9.3 ± 1.4	nd[b]
5.0	7.6 ± 1.9	9.8 ± 1.1
10.0	8.4 ± 1.6	11.6 ± 1.3
15.0	10.2 ± 2.1	11.2 ± 1.4
20.0	8.0 ± 1.5	13.9 ± 1.1
30.0	nd	11.3 ± 1.3

[a]Results are expressed as pmol of ^{3}H-PDBu bound per 10^7 endothelial cells. Nonspecific ^{3}H-PDBu binding in the presence of competitor PMA (10 μM) was subtracted from total values. Data points represent the mean of assays in duplicate (Exp. 1) or triplicate (Exp. 2) ± SD.
[b]Not determined.

Ca^{2+} ionophore A23187, which acted in the same concentration range.

The effect of ionophores on binding was also time dependent. A23187 was added 6, 4, or 2 hours prior to, or at the same time, or 2 hours following addition of TNF to endothelial monolayers. The strongest inhibition of the TNF effect was observed when addition of ionophore preceded the addition of TNF (Fig. 7B). However, partial inhibition of the TNF-mediated increase in tumor cell binding to endothelium was also observed when A23187 was added for the last 2 hours of a 4-hour incubation of MME with TNF. The inhibition by ionophores of the TNF-mediated increase in adherence of P815 to endothelium was observed up to 24 hours after addition of these factors and was accompanied by significant changes in cell morphology (Fig. 3).

To confirm whether or not the effects of ionomycin and A23187 resulted from an increase in the calcium ion level in the cytosol and were not simply due to toxic effects by ionophores on cell functions, we utilized thapsigargin, another calcium-raising agent, in some experiments. Thapsigargin has been shown to elevate the cytosolic Ca^{2+} concentration by a mechanism involving mobilization of calcium ions from intracellular stores, followed by the entry of extracellular Ca^{2+} ions. However, thapsigargin does not have the properties of a calcium ionophore (Takemura, 1989; Thastrup, 1989). We found that thapsigargin, like the ionophores, strongly inhibited the TNF- or PMA-mediated increase in P815 binding to MME. The complete abolition of the TNF or PMA effect by thapsigargin was observed when MME were incubated with the factors for 4 hours in the presence of 2.5 μM thapsigargin (Fig. 7C). This inhibition was still present, although to a lesser extent, after 24 hours of incubation of MME with the factors plus thapsigargin (data not shown).

DISCUSSION

TNF elicits a wide variety of responses in target cells; however, the molecular mechanism of its action is not completely understood. A number of second messenger systems (e.g., PKC and PKA) have been implicated in different TNF-induced responses (Zhang et al., 1988; Brenner et al., 1989; Schütze et al., 1990). For example, it has been shown that stimulation of IL-6 gene expression in human FS-4 fibroblasts by TNF is associated with increased cAMP levels leading to stimulation of PKA (Zhang et al., 1988). In contrast, we did not observe any changes in the level of cAMP following the treatment of MME with TNF, which argues against a role for PKA in the TNF effect. Although ChTx and forskolin treatment of MME resulted in small increases in the number of tumor cells attached to endothelial monolayers, they had essentially no effect during the 4-hour incubation period, which is crucial for TNF to exert its action. Thus, PKA is not involved in TNF-induced enhancement of binding. This is consistent with our failure to detect increases in endothelial cell cAMP levels after treatment with TNF. However, since both ChTx and forskolin did enhance binding, PKA may play a role in the binding phenomenon itself.

PKC, the most extensively studied intracellular messenger, has also been suggested as a TNF signal transducer in some cells. TNF was shown to enhance membrane-associated PKC activity in human AF-2 fibroblasts (Brenner et al., 1989) and to cause translocation and activation of PKC in human lymphocytic, monocytic, and erythroid cell lines (Schütze et al., 1990). However, the effect of TNF on IL-6 gene expression in human FS-4 fibroblasts (Zhang et al., 1988) and on the respiratory burst in human neutrophils (Laudanna et al., 1990) appeared to be independent of the PKC-controlled pathway.

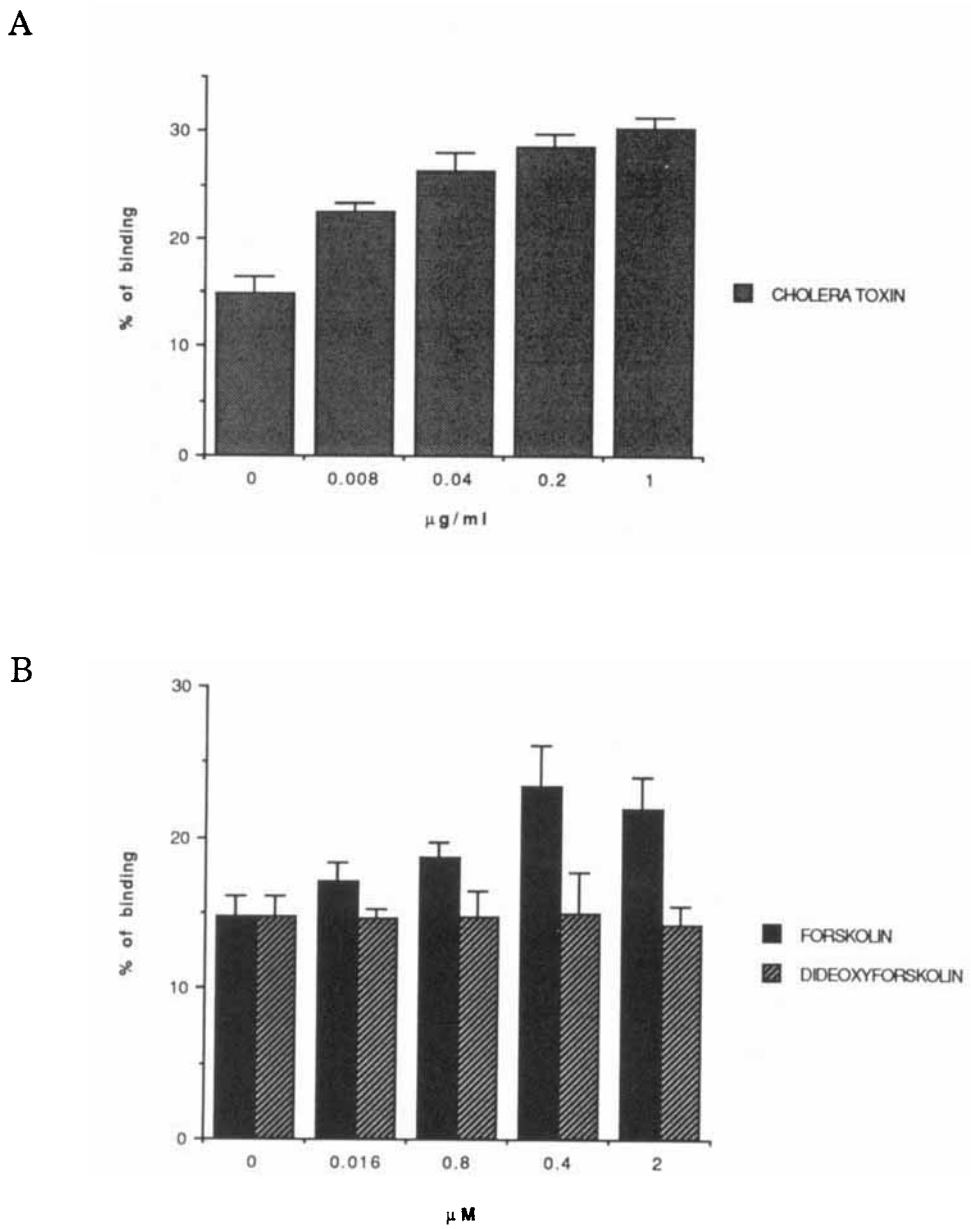

Fig. 4. Adherence of P815 cells to MME monolayer pretreated for 24 hours with various concentrations of (**A**) cholera toxin (gray bars), (**B**) forskolin (black bars), or dideoxyforskolin (hatched bars). Bars represent mean adherence ± SD of 3 experiments for ChTx and forskolin and 2 experiments for dideoxyforskolin. In addition, ethanol alone (forskolin solvent) at a concentration of 0.1% had no effect on binding. Changes observed after treatment of MME with ChTx (1 μg/ml) and forskolin (0.4 μM) were statistically significant ($P < 0.01$ and $P < 0.02$, respectively).

We have previously shown that with respect to stimulation of tumor cell adhesion to endothelium, PMA elicited changes with similar kinetics as TNF, although the effect of the phorbol diester was significantly weaker than that of the cytokine. During signalling through inositol phospholipid breakdown, two second messengers are generated: inositol triphosphate (IP_3), which releases Ca^{2+} from intracellular stores, and diacylglycerol (DG) (Kikkawa and Nishizuka, 1986). In most cells DG and calcium ions act synergistically to stimulate PKC (Wolf et al., 1985; Tyers and Harley, 1986; Kumagai et al., 1987; McCrady et al., 1988). Since PMA mimics the effect only of DG in PKC activation, achievement of full physiological responses often requires the exposure of cells to phorbol diester in the presence of calcium ionophore. Our experiments, however, showed that when ionomycin or thapsigargin accompanied the addition of

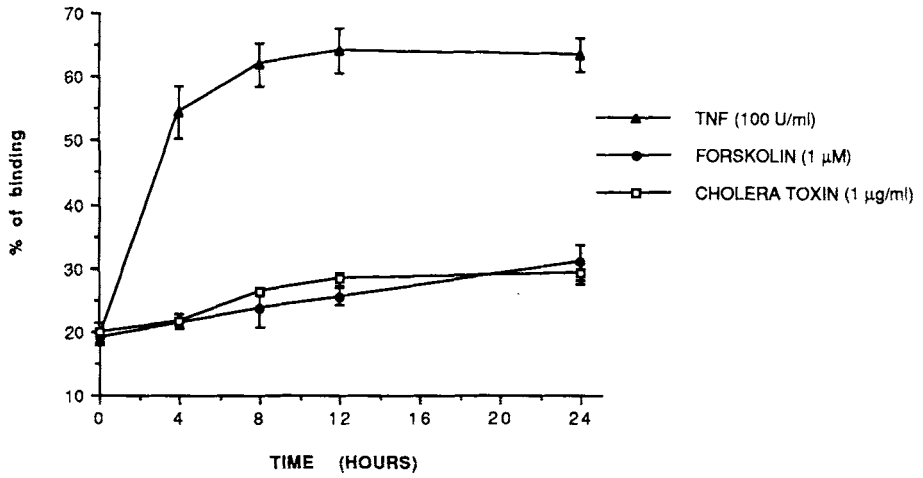

Fig. 5. Adherence of P815 cells to MME monolayer pretreated for various periods of time with: (▲) TNF (100 U/ml), (□) cholera toxin (1 μg/ml), (●) forskolin (1 μM). Data points represent the mean adherence ± SD of 3 experiments and each determination was done in quadruplicate.

PMA to endothelium, the effect of phorbol diester was not enhanced. An increase in intracellular calcium levels antagonized the effect of PMA on tumor cell binding to endothelium. Such antagonistic effects of PMA and ionomycin, although not a common mechanism, have been shown in the regulation of T cell receptor mRNA levels in human thymocytes (Martinez-Valdez et al., 1988). The failure of substitution for TNF by phorbol diester and calcium ionophore or thapsigargin suggested that signalling through inositol phospholipid breakdown can not be the major mechanism in TNF signal transduction pathway in our model.

We found that calcium-raising agents were able to diminish the TNF-mediated increase in tumor cell binding to endothelium. The strongest inhibition of the cytokine effect was observed when addition of ionophore preceded the addition of TNF, suggesting that calcium ionophores might influence the binding of the cytokine to its receptors. Indeed, it has been shown that exposure of human HeLa cells and murine L(S) cells (Johnson and Baglioni, 1988), as well as human neutrophils (Porteu and Nathan, 1990), to calcium ionophore A23187 decreased the binding of TNF to cell surface receptors. However, since the effect of TNF was partially inhibited even when A23187 was added 2 hours following the addition of the cytokine, and because we observed inhibition of the PMA effect by ionophores or thapsigargin, it is possible that

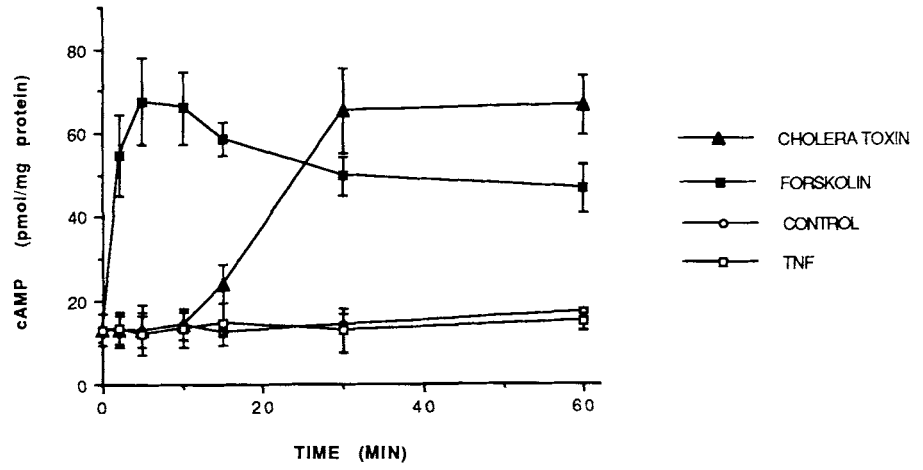

Fig. 6. Changes in cAMP levels in MME cells incubated with IBMX alone (100 μM) (○), or with forskolin (1 μM) (■), ChTx (1 μg/ml) (▲), or TNF (100 U/ml) (□) in the presence of IBMX (100 μM). Data represent the mean of 3 experiments ± SD and each determination was done in duplicate.

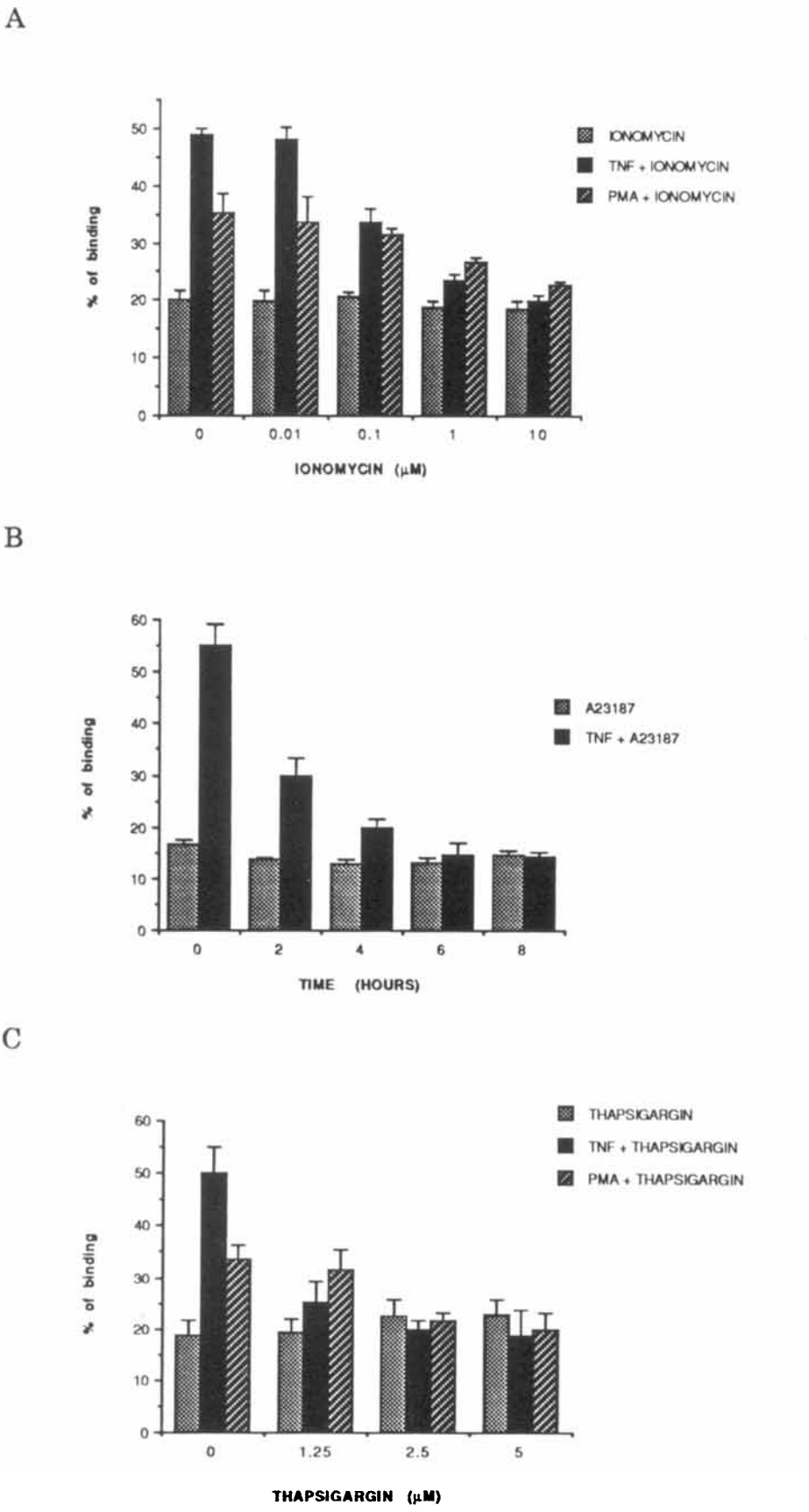
A
% of binding
IONOMYCIN
TNF + IONOMYCIN
PMA + IONOMYCIN
IONOMYCIN (μM)
B
% of binding
A23187
TNF + A23187
TIME (HOURS)
C
% of binding
THAPSIGARGIN
TNF + THAPSIGARGIN
PMA + THAPSIGARGIN
THAPSIGARGIN (μM)

Figure 7.

increased calcium levels may also interfere with intracellular mechanisms leading to enhanced binding of tumor cells to MME. It should be noted that in addition to modulating the activity of PKC, Ca^{2+} exerts its influence by activation of calmodulin-dependent protein kinases, and it is possible that this mechanism may be involved in the Ca^{2+} effect on binding that we observed.

The translocation of PKC from the cytosol to the cell membrane is regarded as evidence of its activation (Thomas et al., 1987). By examining PDBu binding to intact cells, Schütze et al. (1990) found that TNF caused translocation of PKC in several, although not all, human cell lines. The authors demonstrated that the increase in PDBu binding mediated by TNF was transient, reaching maximal level (2–3-fold increase) when the cells were treated with TNF for 4–6 minutes. In our system, however, incubation of endothelial monolayers with TNF up to 30 minutes did not result in any significant changes in PDBu binding to the cell surface. However, there is increasing evidence showing the lack of correlation between translocation and biological effects of PKC in different cells (Bosca et al., 1989). For example, Salari et al. (1990) showed that platelet activating factor, thrombin, and prostacyclin caused stimulation of both cytosolic and particulate-derived PKC in rabbit platelets. Moreover, IL-3 appeared to activate the preexisting membrane-associated PKC rather than elicit its translocation from the cytoplasm in a mast cell/megakaryocyte line R6-XE.4 (Pelech et al., 1990). It is unlikely that such mechanisms play a role in the effect of TNF in our model, since we did not observe an increase in PKC activity in both the cytosolic and membrane fractions of MME cells exposed to the cytokine.

We found that two inhibitors of PKC, H-7 and sangivamycin, were able to abolish the effect of TNF on tumor cell adherence to endothelial monolayers. Both of these inhibitors have been used to implicate the involvement of PKC in many biological effects (Clark et al., 1987; Steele and Brahmi, 1988; Burke et al., 1989). The inhibitory activities of H-7 and sangivamycin result from their competition with ATP at the ATP binding site of protein kinases (*Hidaka et* al., 1984; Loomis and Bell, 1988). Since the ATP binding site of PKC displays striking homology with other kinases (Hanks et al., 1988), these inhibitors cannot be very selective. For example, H-7 inhibits cAMP- and cGMP-dependent kinases, in addition to PKC (Hidaka et al., 1984). Sangivamycin has been shown to also inhibit nuclear protein kinases (Saffer and Glazer, 1981) and rhodopsin kinase (Lebioda et al., 1990). The list of enzymes sensitive to these inhibitors is certainly not complete. Thus, inhibition of the TNF effect by H-7 and sangivamycin, although suggesting the involvement of PKC in TNF signal transduction, cannot be regarded as conclusive proof.

We also found that neither quercetin (≤ 10 μM), an inhibitor of tyrosine kinase, nor W7 (≤ 30 μM), an antagonist of calmodulin, were able to significantly inhibit the effect of TNF (unpublished data). These observations suggest that tyrosine kinase and calmodulin-dependent protein kinase do not participate in TNF signal transduction leading to increased adherence of tumor cells.

In comparison with the effects of H-7 and sangivamycin, the effect of staurosporine on MME is intriguing. In spite of expectations, staurosporine, which is currently known to be the most potent inhibitor of PKC, did not inhibit TNF-mediated increases in tumor cell binding to endothelium. In contrast to the other protein kinase inhibitors, staurosporine alone was a potent enhancing agent for tumor cell binding to endothelium. Other investigators have also found paradoxical effects using staurosporine as an inhibitor. For example, Sako et al. (1988) demonstrated that in mouse epidermal cells staurosporine failed to block phorbol diester responses and induced by itself phorbol diester-

Fig. 7. (**A**) Adherence of P815 cells to MME monolayer pretreated for 4 hours with various concentrations of ionomycin (gray bars), TNF (100 U/ml) in the presence of ionomycin (black bars), or PMA (100 nM) in the presence of ionomycin (hatched bars). Data represent the mean adherence of 3 experiments and each determination was done in quadruplicate. (**B**) Adherence of P815 cells to MME monolayers incubated with TNF for 4 hours. A23187 was added 4 or 2 hours prior to addition of TNF, at the same time as addition of TNF, or 2 hours following the addition of TNF. Time (abscissa) represents periods of exposure of monolayers to A23187. Control represents levels of binding in the absence of A23187. (**C**) Adherence of P815 cells to MME monolayers pretreated for 4 hours with various concentrations of thapsigargin (gray bars), TNF (100 U/ml) in the presence of thapsigargin (black bars), or PMA (100 nM) in the presence of thapsigargin (hatched bars). Data represent the mean adherence of 2 experiments and each determination was done in quadruplicate. Inhibition of PMA or TNF by ionomycin at ≥ 1 μM or by thapsigargin at ≥ 2.5 μM were statistically significant ($P < 0.03$ for the inhibition of PMA and $P < 0.002$ for the inhibition of TNF by either agent).

like effects in cell differentiation. Recently, Dierks-Ventling et al. (1989) showed that staurosporine, like PMA, induced urokinase-type plasminogen activator in epithelial cells and that the effect of both agents was additive. Thus, in certain circumstances, staurosporine appears to mimic PKC-dependent effects rather than inhibit them.

In our model, exposure of endothelium to both TNF and staurosporine led to a greater increase in P815 binding than treatment of MME with either factor alone. This fact, along with observations that the effect of staurosporine appeared earlier than that of TNF and was not abolished by cycloheximide, led to the conclusion that the mechanisms by which TNF and staurosporine caused enhanced tumor cell binding to endothelium are distinct from one another. Since phosphorylation of cell surface receptors may lead to their down-regulation and desensitization (Sibley et al., 1987; Chatila and Geha, 1988), it is tempting to speculate that staurosporine might elicit its effect by the opposite mechanism, that is, inhibition of phosphorylation of certain surface proteins which could lead to their activation. It is, however, more likely that the effects of staurosporine may result from physical alterations of the cell membrane, a possibility consistent with the changes in the cell morphology we observed after treatment of MME with this inhibitor (Fig. 3).

Taken together, the data we have presented demonstrate that kinases are involved in the effect of TNF on tumor cell adherence to endothelium. Recognizing the poor specificity of the various inhibitors, a PKC-like enzyme still remains a possible candidate for a TNF-generated second messenger. Recent studies of PKC, have changed the general concepts concerning the enzyme's structure, activation, and regulation. The term "PKC" is now often replaced by "PKC family," since several subspecies of this enzyme have been found and characterized (Nishizuka, 1988). It has been shown that these subspecies differ with respect to their activation requirements (some of them appear to be Ca^{2+} independent), regional localization, and substrate specificity, implying that each subspecies plays a different role in signal transduction (Nishizuka, 1988; Berry and Nishizuka, 1990). Recently Bird and Saklatvala (1990), studying down-modulation of EGF binding to fibroblasts by IL-1 and TNF, showed that although the effect of cytokines is similar to that caused by PKC activators, their pattern of phosphorylation of the EGF receptor is quite different. Moreover, cytokine effects were not inhibited by staurosporine nor by a deficiency of PKC. These observations indicated that IL-1 and TNF elicited their effects on the EGF receptor by activation of a protein kinase distinct from "classical" PKC. A similar mechanism of TNF action on MME cells is suggested by our results. It is also possible that TNF may induce more than one second messenger pathway. A mechanism involving multiple transduction pathways has already been proposed for IL-1 (Mizel, 1990). The interaction between distinct pathways stimulated by TNF may result in full expression of the increase in the adhesive properties of endothelium, while activation of individual kinases by PMA, forskolin, or cholera toxin are able to mimic only part of the response. Further studies are necessary to determine possible differences in the pattern of TNF- and PMA-induced phosphorylation of membrane proteins and to better characterize kinase(s) involved in TNF signal transduction. This work is now in progress.

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