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Induction of the Collagenase Phorbol Ester Response Element by Staurosporine

Maria C. Shoshan and Stig Linder

Division of Experimental Oncology, Department of General Oncology, Radiumhemmet, Karolinska Institute and Hospital, S-171 76 Stockholm, Sweden

Abstract The indol alkaloid staurosporine is a potent inhibitor of protein kinase C, but has also been shown to have certain effects paradoxically similar to those of protein kinase C–activating phorbol esters. We show here that collagenase mRNA expression is stimulated by 10 nM staurosporine in normal and *ras*-oncogene–transformed rat fibroblasts. The kinetics of collagenase mRNA induction by staurosporine were slow compared to induction by phorbol ester. Staurosporine induction of the collagenase promoter appeared to be mediated via the TPA response element (TRE). Induction did not involve any increase in *jun* mRNA expression and did not require expression of c-Jun. Prolonged treatment with phorbol ester to deplete protein kinase C did not inhibit stimulation of the collagenase promoter by staurosporine. Instead, involvement of cAMP-dependent protein kinase (PKA) was indicated by inhibition of staurosporine induction by the PKA inhibitor H-89. In addition, raised levels of cAMP were observed during the first hour of staurosporine treatment. Altogether, our data indicate that staurosporine induces a PKA-dependent pathway leading to c-Jun–independent activation of the collagenase TRE element.

Key words: staurosporine, protein kinases, collagenase, AP-1, gene induction

The indol alkaloid staurosporine is widely used as a potent inhibitor of protein kinase C (PKC) activity [Tamaoki, 1991]. In the nanomolar range staurosporine will usually inhibit responses elicited by PKC-activating phorbol esters, for example, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA). Nevertheless, effects of staurosporine which are paradoxically similar to, or which potentiate, TPA effects have been described in the literature. These include induction of ornithine decarboxylase expression [reviewed in Nakadate, 1989], potentiation of NGF stimulation of neurotensin expression [Tischler et al., 1991], and induction of c-*fos* and of maturation in mouse keratinocytes [Sako et al., 1988; Dlugosz and Yuspa, 1991]. Dierks-Ventling et al. [1989] reported that staurosporine induces translocation of PKC to membranes in vitro and induction of urokinase-type plasminogen activator in vivo in a porcine epithelial cell line. The mechanism(s) underlying these findings is unknown.

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Address reprint requests to Stig Linder, Division of Experimental Oncology, Radiumhemmet, Karolinska Institute and Hospital, S-171 76 Stockholm, Sweden.

Phorbol esters activate protein kinase C (PKC) and cause a transient induction of the early response genes c-*jun* and c-*fos*. The AP-1 transcription factor consists of homodimers of Jun proteins or heterodimers between Jun and Fos proteins [Ransone and Verma, 1990; Angel and Karin, 1991]. Phorbol ester–induced binding of c-Jun protein to the phorbol ester responsive element (TRE) occurs rapidly and is paralleled by dephosphorylation of serine and threonine residues proximal to the DNA binding domain of c-Jun [Boyle et al., 1991]. Regulation of expression of several matrix metalloproteases has been shown to involve PKC [Aznavoorian et al., 1993], for example, phorbol ester treatment may induce the expression of interstitial collagenase (MMP-1), stromelysins (MMP-3 and MMP-10), and 92 kDa, type IV collagenase (MMP-9).

We here show that staurosporine as well as TPA may stimulate collagenase (interstitial collagenase, MMP-1) mRNA expression in rat fibroblasts and fibrosarcoma cells, albeit with different kinetics. Stimulation by staurosporine appears to be mediated by the TPA-responsive element in the collagenase promoter, but does not require expression of c-Jun.

MATERIALS AND METHODS

Materials

Reagents were provided as follows: staurosporine either from Upstate Biotechnology (Lake Placid, NY) or from Sigma (St. Louis, MO); H-89 from Calbiochem AG (Lucerne, Switzerland); 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) from Sigma; tissue culture reagents from Gibco (Paisley, Scotland).

Cell Cultures

Rat embryo fibroblasts (REF) and BRN-5 rat fibrosarcoma cells were propagated as monolayer cultures in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal calf serum, 100 U/ml penicillin, and 50 μg/ml streptomycin. MCF-7 and C8161 cultures were grown in Eagle's Medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. REF were isolated from rat embryos as described [Linder et al., 1990]. BRN-5 cells were derived from REF after cotransfection with polyoma large-T antigen and *ras* [Engel et al., 1993]. A14 cells were derived from *ras*-transfected REF by glucocorticoid hormone promotion, and have been shown to lack expression of c-*jun* mRNA [Marshall et al., 1993]. A14 cells lack both homologues of the c-*jun* gene as determined by Southern blot analysis (S. Linder, unpublished observations). C8161 is an established human melanoma cell line [Welch et al., 1991]. MCF-7 is an estrogen receptor–positive breast carcinoma cell line [Soule et al., 1973].

Plasmids and Transfections

Plasmids pcoll − 517/+63CAT and pcoll − 517/+63CATmTRE were obtained from M. Karin [Angel et al., 1988]. In plasmid 2 × TRE-CAT, a synthetic oligonucleotide consisting of a duplicated collagenase TRE element was inserted into plasmid pBLCAT [Marshall et al., 1993]. The DNA sequences of the relevant regions of the promoters were verified by the dideoxynucleotide method using a Pharmacia kit. Plasmid Δ9 encodes a dominant negative Jun protein (v-Jun devoid of its activation domain) previously shown to block AP-1–stimulated transcription [Lloyd et al., 1991]. Hybridization probes for rat collagenase and mouse c-*jun* were kindly provided by C. Quinn and R. Bravo, respectively [Quinn et al., 1990; Ryseck et al., 1988], whereas *jun*-B and *jun*-D probes were obtained from the American Type Culture Collection. Cells were transfected with the calcium phosphate technique [Graham and van der Eb, 1973] as described [Sambrook et al., 1989]. Cell extracts were prepared 40 hr after transfection. Measurement of CAT activity (conversion of [^{14}C] chloramphenicol to the monoacetate form) was made using standard procedures [Gorman et al., 1982]. Enzymatic activity was corrected for the amount of protein in the cell extract. Each experiment was performed at least twice and data from representative experiments are shown. Each value presented is the average of duplicate or triplicate determinations.

Northern Blot Analysis

Cellular RNA was extracted at 65°C with phenol equilibrated with 0.2 M Na acetate/10 mM EDTA, pH 5.5 [Edmonds and Caramela, 1969], and then precipitated and dissolved in water. The RNA was denatured in formaldehyde/formamide and loaded on 2.2 M formaldehyde-containing agarose gels. Blotting to nitrocellulose filters was performed as described [Thomas, 1980]. Hybridizations to DNA fragments labeled by random priming was carried out in 50% formamide/10% dextran sulphate for 16 hr at 42°C.

RESULTS

Induction of Collagenase mRNA by Staurosporine

Secondary or tertiary cultures of exponentially growing rat embryo fibroblasts (REF) were exposed to 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA; 50 ng/ml) or staurosporine (10 nM) for 4 to 24 hr. Total cellular RNA was isolated and the levels of interstitial collagenase mRNA were determined by Northern blot hybridization using a rat cDNA probe (Fig. 1A). Treatment with TPA resulted in some stimulation of collagenase mRNA expression at 4 and 7 hr, while at 24 hr collagenase expression was low. Staurosporine treatment also resulted in increased levels of collagenase mRNA. This increase was stronger than that induced by TPA, but was not detected before 24 hr of treatment (Fig. 1A).

Staurosporine stimulated the expression of collagenase mRNA also in a *plt* + T24-H-*ras* transformed REF cell line (BRN-5) (Fig. 1B). In these cells, collagenase mRNA was constitutively expressed, presumably due to stimulation

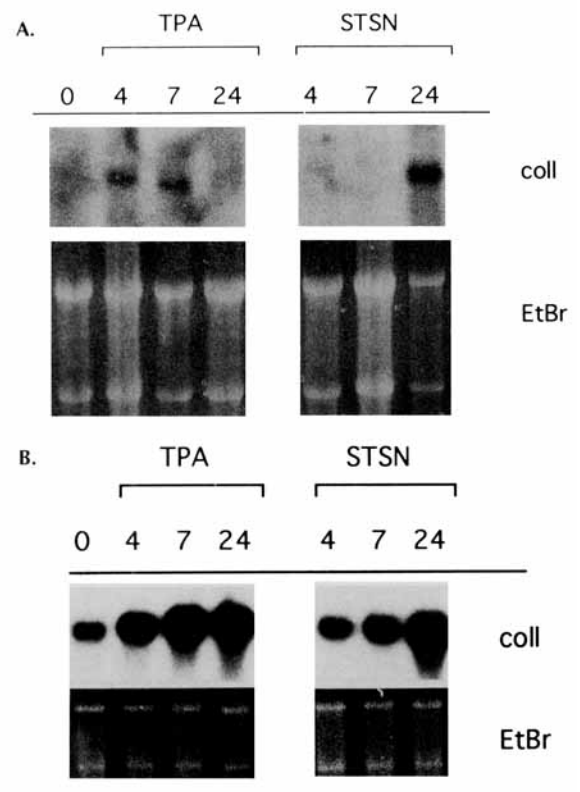

Fig. 1. Induction of collagenase mRNA by TPA and staurosporine. **A:** REF cells were treated with 50 ng/ml TPA or 10 nM staurosporine (STSN) for the indicated times (in hours). Total cellular RNA was isolated and hybridized to a rat collagenase probe. **B:** *plt* + *ras*–transformed cells (cell line BRN-5) were treated with 50 ng/ml TPA or 10 nM staurosporine (STSN) for the indicated times (in hours) and subjected to Northern blot analysis. Agarose gels were photographed before transfer to nitrocellulose membranes (EtBr).

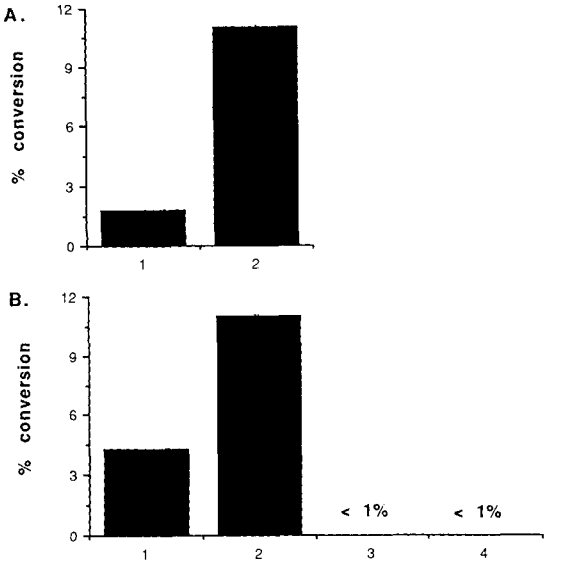

Fig. 2. Staurosporine induction of the collagenase TRE. REF cells were transfected with the indicated plasmids. After 16 hr the cells were washed and treated with staurosporine (10 nM; 24 hr). **A:** Transfection with pcoll − 517/+63CAT. **1,** Control; **2,** staurosporine. **B:** Transfection with 2 × TRE-CAT (**1** and **2**) or pcoll − 517/+63CATmTRE containing a mutated TRE site (**3** and **4**). 1, 3, Controls; 2, 4, staurosporine. The percentage of total [^{14}C] chloramphenicol converted to the monoacetate form during 45 min incubation was determined and the value corrected for the amount of cellular protein in the extract (average of duplicate petri dishes).

by the Ras oncoprotein. A small increase in collagenase mRNA was detected after 7 hr of treatment, and strong stimulation after 24 hr. TPA treatment resulted in a more rapid stimulation of collagenase mRNA in BRN-5 cells, but in contrast to REF cells this effect remained after 24 hr (Fig. 1B).

Staurosporine Induces Collagenase Expression Through the TRE Element

REF cells were transfected with a reporter plasmid in which the collagenase promoter is fused to the CAT gene (pcoll − 517/+63CAT). As shown in Figure 2A, staurosporine stimulated the activity of the collagenase promoter in REF cells. The level of stimulation varied between different experiments, probably because of cellular variations in growth/proliferation.

Calphostin C, another inhibitor of PKC, was tested in the same way for stimulation of the promoter activity, and was found to have no effect (not shown).

Stimulation of the collagenase promoter by phorbol esters is dependent on the TPA response element (TRE), shown to bind the AP-1 transcription factor [Angel and Karin, 1991]. The expression of a reporter in which the TRE element is mutated (pcoll − 517/+63CAT-mTRE) was undetectable in both staurosporine-treated and untreated REF cells (Fig. 2B). To examine whether staurosporine stimulates the activity of isolated TRE elements, REF cells were transfected with a plasmid containing tandem collagenase TRE elements (made through the insertion of a synthetic oligonucleotide) upstream of the CAT gene. As shown in Figure 2B, the expression of the duplicated TRE element was stimulated by staurosporine. Similar results were obtained using transformed BRN-5 cells (not shown).

Inhibition by a Dominant Negative *v-jun* Mutant and by E1A

Plasmid Δ9 encodes a v-Jun protein lacking the transactivation domain. Δ9 has been demon-

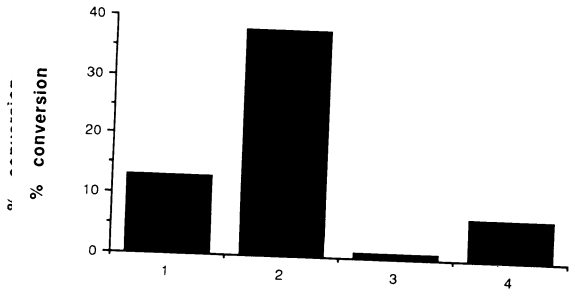

Fig. 3. Induction by staurosporine is repressed by the v-*jun* mutant Δ9 and by E1A. REF cells were transfected with pcoll – 517/+63CAT and either the Δ9 dominant, negative v-jun mutant or adenovirus E1A (12S cDNA) before staurosporine treatment for 24 hr as described in Figure 2. **1,** pcoll – 517/+63CAT; **2,** pcoll – 517/+63CAT with staurosporine (10 nM); **3,** pcoll – 517/+63CAT + Δ9 with staurosporine; **4,** pcoll – 517/+63CAT + E1A with staurosporine.

strated to inhibit the activity of the polyomavirus TRE [Lloyd et al., 1991]. REF cells were simultaneously transfected with pcoll – 517/+63CAT and Δ9. As shown in Figure 3, Δ9 abolished the activity of the collagenase promoter reporter in staurosporine-treated cells.

The 243R adenovirus E1A protein inhibits the induction of the collagenase TRE by TPA [Offringa et al., 1990]. Staurosporine induction of the pcoll – 517/+63CAT reporter was repressed by plasmid p00512S, a cDNA mutant encoding the adenovirus 243R E1A protein (Fig. 3).

Staurosporine Induction Does not Require c-Jun

Phorbol ester induction of collagenase is paralleled by stimulation of c-*jun* and *jun*B transcription and alterations in c-Jun phosphorylation [Angel and Karin, 1991]. Accordingly, c-*jun* mRNA levels in BRN-5 cells were transiently increased following TPA treatment, whereas no increase was observed following staurosporine treatment (Fig. 4A). Similarly, the expression of *jun*B or *jun*D mRNA was not stimulated by staurosporine in A14 or BRN-5 cells (not shown).

The absence of c-*jun* mRNA induction does not exclude involvement of c-Jun in the staurosporine response, as staurosporine might induce post-translational modifications of this protein. To further elucidate the potential role of c-Jun, an REF cell line defective in the c-*jun* gene (A14 cells) [Marshall et al., 1993] was treated with staurosporine. As shown in Figure 4B, collagenase mRNA was induced in A14 cells. Induction was observed already after 4 hr of staurosporine treatment, and was strongest after 24 hr. Induction of pcoll – 517/+63CAT activity was observed in A14 cells, an induction which was blocked by the dominant negative v-*jun* mutant (not shown).

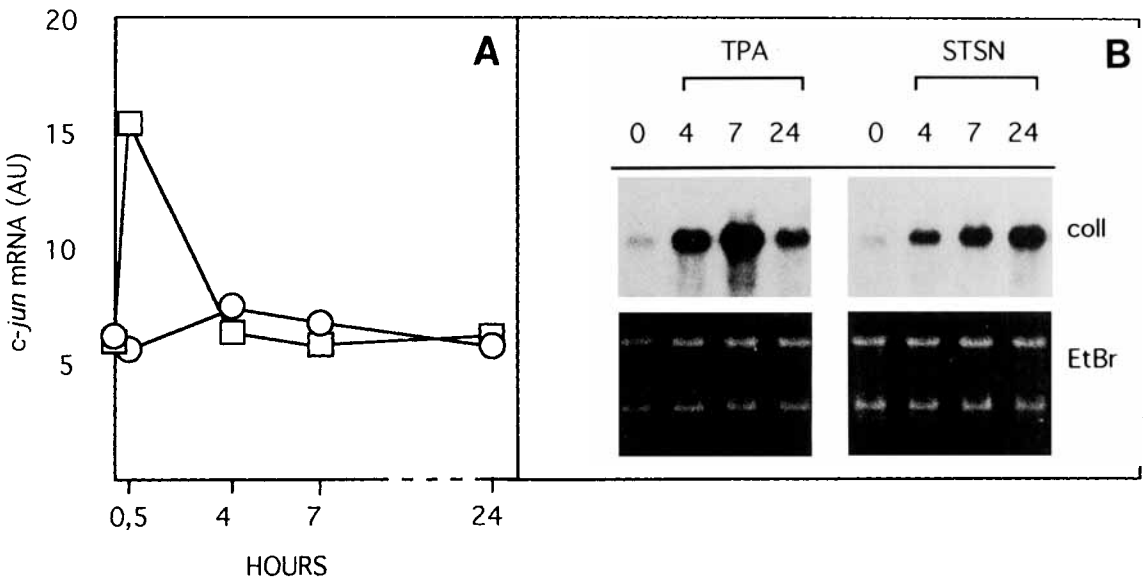

Fig. 4. Staurosporine does not induce c-*jun* mRNA in BRN-5 cells but induces collagenase mRNA in c-*jun*–defective A14 cells. **A:** The expression of c-*jun* mRNA was determined in BRN-5 cells by Northern blot analysis following TPA (squares) or staurosporine (circles) treatment. The levels of c-*jun* mRNA were quantitated by densitometric scanning of an autoradiographic film. **B:** The c-*jun*–defective A14 cell line was exposed to TPA or staurosporine (STSN) for the indicated times (in hours). Total cellular RNA was isolated and subjected to Northern blot analysis using a rat collagenase cDNA probe.

Stimulation of the Collagenase Promoter by Staurosporine Remains After Predepletion of PKC

In order to deplete PKC activity in REF cells, the cells were treated with TPA (100 ng/ml) for 24 hr prior to transfection with the pcoll − 517/+63CAT reporter plasmid. The continued presence of TPA after transfection inhibited only slightly the stimulation of reporter by staurosporine (Fig. 5A). Without staurosporine, the pcoll − 517/+63CAT activity in cells grown in the continued presence of TPA was similar to that in control cells (Fig. 5A).

Stimulation of Collagenase Promoter Is Abolished by a PKA Inhibitor

After transfection, REF and A14 cells were treated with PKA inhibitor H-89 (20 μM) for 30 min before addition of staurosporine. This treatment abolished stimulation of pcoll − 517/+63CAT by staurosporine in REF cells (Fig. 5B) and in A14 cells (not shown). In contrast, treatment with the PKA activator dibutyryl-cAMP did not affect pcoll − 517/+63CAT activity in A14 cells (conversion was 3–4% in both control and dibutyryl-cAMP–treated cells). Neither did dibutyryl-cAMP affect collagenase mRNA levels in REF cells (not shown).

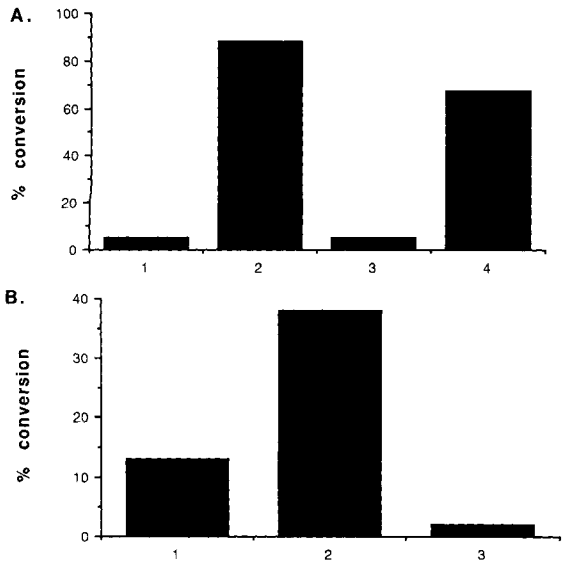

Fig. 5. Induction by staurosporine is not blocked by PKC depletion, but by the PKA inhibitor H-89. Staurosporine treatment was as described in Figure 2. **A:** REF cells transfected with pcoll − 517/+63CAT and treated with TPA (100 ng/ml for 24 hr before and after transfection) in order to deplete them of PKC activity. **1,** Control; **2,** staurosporine; **3,** PKC-depleted cells; **4,** staurosporine treatment of PKC-depleted cells. **B:** REF cells transfected with pcoll − 517/+63CAT plasmid. **1,** Control; **2,** staurosporine; **3,** staurosporine and H-89 (20 μM; added 30 min before staurosporine).

Staurosporine Induces Raised Levels of cAMP

The results obtained with H-89 prompted us to investigate the effects of staurosporine on cellular levels of cAMP. Staurosporine treatment of REF and A14 cells resulted in rapidly raised cAMP levels lasting for at least 45 min (Fig. 6).

The Effect of Staurosporine is Cell-Type Dependent

To determine whether staurosporine may stimulate the activity of the collagenase promoter in cell types other than rat fibroblasts, we studied human C8161 melanoma and human MCF-7 breast carcinoma cells. The results of the transient transfection analyses are presented in Figure 7. Staurosporine treatment induced collagenase promoter activity in C8161 cells but did not stimulate the reporter activity in MCF-7 cells.

DISCUSSION

Staurosporine is considered a highly potent inhibitor of PKC. There are, however, indications that staurosporine has other effects which, paradoxically, may even parallel effects of phorbol esters, including the ability to translocate cytosolic PKC to membranes [Dierks-Ventling et al., 1989; Dlugosz and Yuspa, 1991]. The

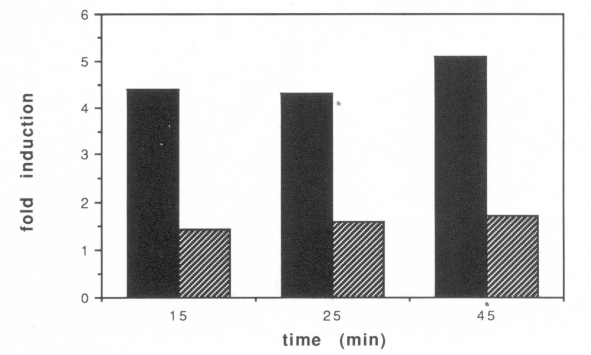

Fig. 6. Staurosporine induces raised cAMP levels. Cells were treated with staurosporine (10 nM) for the time periods indicated, whereupon they were lysed in distilled water containing 5 mM EDTA in order to inhibit phosphodiesterases. The supernatants were then processed according to the manufacturer's instructions for a cAMP [^{3}H] assay system kit (Amersham, Solna, Sweden). Similar results were obtained when the experiment was repeated. Solid columns, REF cells; hatched columns, A14 cells.

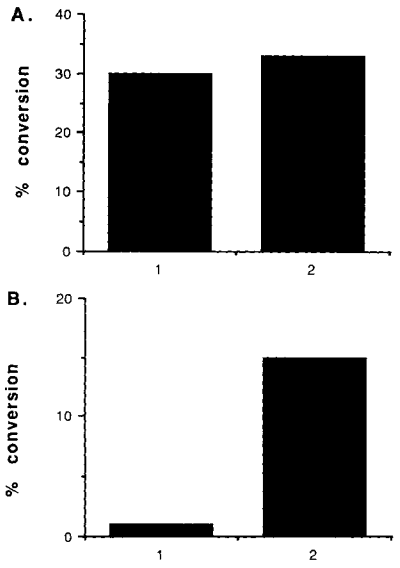

Fig. 7. Staurosporine effects on collagenase promoter activity in MCF-7 and C8161 cells. MCF-7 breast cancer (**A**) and C8161 melanoma (**B**) cells were transfected with pcoll − 517/+63CAT and treated with staurosporine as described in Figure 2. **1**, Controls; **2**, staurosporine.

present study reports another such effect of staurosporine, namely its ability to induce collagenase expression both in untransformed and *ras*-transformed REF cells. Although the stimulative effect of staurosporine did not appear to be restricted to fibroblasts, it is probably cell type–dependent: C8161 human melanoma cells, but not MCF-7 breast carcinoma cells, were also found to respond.

In previous work, involvement of active PKC as a mediator of the staurosporine response was indicated by the finding that the PKC inhibitor bryostatin partially blocked staurosporine-mediated differentiation responses in primary keratinocytes [Dlugosz and Yuspa, 1991]. We show here that staurosporine-mediated stimulation of collagenase reporter activity is not significantly affected by downregulation of PKC by long-term treatment with TPA. Two isozymes of PKC (η and ζ) are not affected by TPA [Nishizuka, 1992], but their sensitivity towards bryostatin has, to our knowledge, not been investigated.

Phorbol esters increase the expression of interstitial collagenase and a number of other proteinases [Aznavoorian et al., 1993]. Induction of collagenase gene transcription by TPA is dependent on the formation and binding of the AP-1 (Jun/Fos) complex to the TPA response element (TRE) in the collagenase promoter [Angel and Karin, 1991]. Again, the paradoxical nature of induction by staurosporine is reflected in the finding that it also acts via TRE. However, the kinetics of induction by staurosporine were much slower than those by TPA, and—unlike TPA-mediated induction—induction by staurosporine did not involve increased c-*jun* or *jun*B transcription. Since strong induction of collagenase mRNA was observed in the c-*jun*–defective A14 cell line, it is possible that c-Jun is not at all involved. Nevertheless, staurosporine induction of the collagenase promoter was blocked by a v-*jun* dominant negative mutant and also by E1A, which is known to inhibit AP-1 stimulation of the collagenase TRE [Offringa et al., 1990].

H-89, a selective inhibitor of protein kinase A (PKA), abolished staurosporine stimulation of the collagenase promoter. This, together with the observation that staurosporine induces raised levels of cAMP, suggests that staurosporine induction is dependent on PKA activity and might be mediated by PKA-dependent ATF/CREB transactivating proteins. It is interesting to note that it has been demonstrated that the AP-1 site in the c-*jun* promoter is activated by phosphorylated CREB protein [Lamph, 1991]. However, the mechanism of induction of the collagenase promoter by staurosporine is likely to be complex, since treatment with dibutyryl-cAMP did not give any significant increase in either collagenase promoter activity or in collagenase mRNA levels. We therefore conclude that PKA activity appears to be necessary, but not sufficient, for induction by staurosporine.

The findings presented here contribute to the growing insight that the use of staurosporine in cell and molecular biology may have unexpected effects. More importantly, the results also indicate that staurosporine triggers a largely uninvestigated pathway to gene induction via TPA-responsive promoter elements.

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