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Divergent Effects of Protein Kinase C (PKC) Inhibitors Staurosporine and Bisindolylmaleimide I (GF109203X) on Bone Resorption

Suk Kyeong Lee and Paula H. Stern†‡*

*Catholic Research Institutes of Medical Science, The Catholic University, Seoul, Korea and †Department of Molecular Pharmacology and Biological Chemistry, Northwestern University Medical School, Chicago, IL 60611, U.S.A.

ABSTRACT. Activation of protein kinase C (PKC) has been suggested to play a role in bone resorption. However, phorbol esters, which activate PKC, have been reported to have both stimulatory and inhibitory effects on bone resorption. To study the role of PKC in bone resorption further, we have measured calcium release elicited by bone-resorbing hormones from isolated bones incubated with the PKC inhibitors staurosporine (ST) and the more PKC-selective ST analog bisindolylmaleimide I (GF109203X; GF). In fetal rat limb bone organ cultures, ST (1 μM) or GF (1 μM) significantly reduced the bone resorption induced by maximal concentrations of parathyroid hormone (PTH). However, when submaximal concentrations of PTH were used, lower concentrations of the two antagonists had divergent effects. GF (20–300 nM) acted solely as an antagonist, whereas ST (10–100 nM) significantly enhanced resorptive responses to PTH. ST also enhanced the bone resorption elicited by α-thrombin, tumor necrosis factor-α (TNF-α), and thyroxin (T4). ST alone had small stimulatory effects in some experiments. GF prevented the stimulatory effects of ST alone as well as the enhancing effect of ST on PTH-stimulated resorption. The divergent effects of ST and GF on the responses of bone to low concentrations of PTH and the ability of GF to antagonize the stimulatory effects of ST suggest that PKC isozymes have complex and even antagonistic effects on bone resorption. BIOCHEM PHARMACOL **60**;7: 923–926, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. protein kinase C; bone resorption; parathyroid hormone; staurosporine; GF109203X

An important role of PKC§ in bone resorption is indicated by several previous studies. In fetal mouse calvarial cultures, inhibition of PKC by AMG reduces the bone resorption induced by PTH, 1,25-D, or PGE [1]. Also, 1,25-D-stimulated bone resorption in fetal mouse limb bones and calvariae is inhibited by both AMG and ST [2]. The phorbol esters TPA and PDD stimulate resorption in neonatal mouse calvarial organ culture by a mechanism involving the local production of PGE [3]. In fetal rat limb bone cultures, Lorenzo and Sousa [4] found that the same phorbol esters (TPA and PDD) stimulate resorption through mechanisms independent of prostaglandin synthesis. Abraham *et al*. [5] also have shown that phorbol ester treatment of fetal rat limb bones causes resorption. All these data provide evidence that activation of PKC is one of the pathways through which hormones stimulate bone resorption. On the other hand, Ransjö and Lerner [6] observed that TPA inhibits bone resorption induced by PTH and PTH-related peptide of malignancy in neonatal mouse calvarial organ cultures. In the same study, they confirmed that TPA by itself stimulates bone resorption. Since both stimulatory and inhibitory effects of phorbol esters on bone resorption were observed under the same conditions in long-term cultures, it seems unlikely that the effects were due to activation in one case and down-regulation of PKC in the other. To clarify the role of PKC in bone resorption, we have determined the effects of the PKC inhibitor ST and the more PKC-specific ST analog bisindolylmaleimide 1 (GF109203X; GF) [7] on the resorption elicited by several hormones on isolated fetal rat limb bones.

MATERIALS AND METHODS

ST and GF were purchased from Calbiochem, PTH was obtained from Bachem, TNF-α was purchased from Collaborative Research, T4 was obtained from the Sigma Chemical Co., and α-thrombin was the gift of Dr. John Fenton. Fetal (19-day) rat (Harlan) limb bones were cultured by previously published techniques [8]. Briefly,

‡ Corresponding author: Paula H. Stern, Ph.D., Department of Molecular Pharmacology and Biological Chemistry, Northwestern University Medical School, 303 Chicago Ave., Chicago, IL 60611. Tel. (312) 503-8290; FAX (312) 503-5349; E-mail: p-stern@northwestern.edu

§ *Abbreviations*: PKC, protein kinase C; ST, staurosporine; GF, bisindolylmaleimide I (GF109203X);TNF-α, tumor necrosis factor-α; T4, thyroxin; AMG, 1-*O*-alkyl-2-*O*-methylglycerol; PTH, parathyroid hormone; 1,25D, 1,25$(OH)_2$-vitamin D_3; DMEM, Dulbecco's modified Eagle medium; PGE, prostaglandin E_2;TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; and PDD, phorbol-12,13-didecanoate.

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TABLE 1. Inhibitory effects of 20 nM–1μM GF on resorption stimulated with 1 or 0.5 nM PTH

Treatment	N	% Bone ^{45}Ca released	% Inhibition
Experiment #1			
Control	4	23.3 ± 3.2	
GF, 1 μM	4	18.0 ± 2.2	
PTH, 1 nM	4	46.5 ± 2.4*	
PTH + GF	4	18.6 ± 1.5†	100
Experiment #2			
Control	4	26.8 ± 0.6	
GF, 300 nM	4	22.7 ± 0.2	
PTH, 1 nM	4	61.1 ± 8.6*	
PTH + GF	4	42.6 ± 9.3‡	42
Experiment #3			
Control	4	27.4 ± 1.3	
GF, 20 nM	4	26.7 ± 2.2	
PTH, 1 nM	4	72.9 ± 5.2§	
PTH + GF	4	63.7 ± 3.5§†	18.7
Experiment #4			
Control	4	24.3 ± 2.6	
GF, 100 nM	4	28.2 ± 1.2	
PTH, 0.5 nM	4	34.1 ± 4.8‖	
PTH + GF	4	21.6 ± 1.5	100
Experiment #5			
Control	4	22.3 ± 1.0	
GF, 60 nM	4	21.1 ± 8.7	
PTH, 0.5 nM	4	39.8 ± 5.6‖	
PTH + GF	4	30.2 ± 8.7	48

Fetal rat limb bones pre-labeled with $^{45}CaCl_2$ were cultured in DMEM + 15% heat-inactivated horse serum containing reagents shown above. After 72 hr, resorption was quantified by measurement of ^{45}Ca released into the medium and retained in the bones. Data are expressed as means and standard errors of the responses of 4 bones per treatment. Percent inhibition was calculated from the differences between control and PTH-treated bones in the presence and absence of the inhibitor.

*$P = 0.01$ vs control.
†$P = 0.001$ vs PTH.
‡$P < 0.05$ vs PTH.
§$P < 0.001$ vs control.
‖$P < 0.05$ vs control.

bones were prelabeled by injection of 18-day pregnant rats with 200 μCi $^{45}CaCl_2$. Bones were cultured for 72 hr in DMEM + 15% heat-inactivated horse serum and the indicated treatments. Resorption was quantified by measurement of the ^{45}Ca released into the medium and that retained in the bones, and the results were expressed as the percentage of the bone ^{45}Ca released. Data are presented as means and SEM of the responses of at least 4 bones per treatment. Statistical significance was determined by analysis of variance followed by Fisher's Least Significant Difference Test.

RESULTS

PTH (0.5 or 1 nM) stimulated bone resorption 1.4- to 2.7-fold, compared with the basal response, as shown in Table 1. To test whether the bone resorption induced by PTH was mediated by PKC in our system, a PKC-selective inhibitor, GF109203X, was used. Co-treatment with 1 μM GF completely inhibited bone resorption (Table 1, Experiment #1) induced by 1 nM PTH. In addition, 300 and 20 nM GF also (42 and 18%) inhibited the resorption induced by 1 nM PTH significantly (Table 1, Experiments #2 and #3). When a lower concentration (0.5 nM) of PTH was used to induce bone resorption, 100 nM GF completely inhibited the resorption, and 60 nM GF inhibited the resorption by 48% (Table 1, Experiments #4 and #5).

TABLE 2. Inhibitory effect of 1 μM ST on bone resorption stimulated by PTH

Treatment	N	% Bone ^{45}Ca released
Experiment #1		
Control	4	19.1 ± 2.4
ST, 1 μM	4	22.5 ± 0.5
PTH, 0.6 nM	4	55.3 ± 1.9*
PTH + ST	4	17.6 ± 0.9

Fetal rat limb bone pre-labeled with $^{45}CaCl_2$ were cultured in DMEM + 15% heat-inactivated horse serum containing reagents shown above. After 72 hr, resorption was quantified by measurement of ^{45}Ca released into the medium and retained in the bones. Data are expressed as means and standard errors of the responses of 4 bones per treatment.

*$P < 0.001$ vs control.
†$P < 0.001$ vs PTH.

Similar experiments were performed using ST. PTH (0.6 nM) stimulated bone resorption over 2.9-fold, compared with the basal response (Table 2). Co-treatment with 1 μM ST inhibited the PTH-stimulated resorption completely. Unexpectedly, opposite effects were observed when bone resorption was stimulated with PTH in the presence of lower concentrations of ST (Fig. 1). The resorptive response to 0.25 or 0.5 nM PTH was enhanced by 100 nM ST, rather than inhibited (Figs. 1A, 2). Although there was variability in the response to a given concentration of PTH between experiments, the inhibitory effect of 1 μM ST and the potentiating effect of 10–100 nM ST were observed consistently, regardless of the magnitude of the PTH-induced bone resorption. Similar potentiating effects of ST also were seen in bones treated with α-thrombin (α-TH) (Fig. 1B), T4 (Fig. 1B), or TNF-α (Fig. 1C) when these bone-resorbing agents were used at submaximal concentrations. ST alone elicited slight stimulatory effects on bone resorption at a concentration of 100 nM, which sometimes (Fig. 2) but not always (Fig. 1, panels A–C) achieved statistical significance.

Interestingly, when both ST and GF were added together to the cultures, GF antagonized the stimulatory effects of ST on PTH-induced bone resorption, as well as inhibiting the ability of ST to enhance bone resorption by itself (Fig. 2).

DISCUSSION

Our present data showing inhibition of PTH-stimulated resorption by high concentrations of ST (Table 2) and the

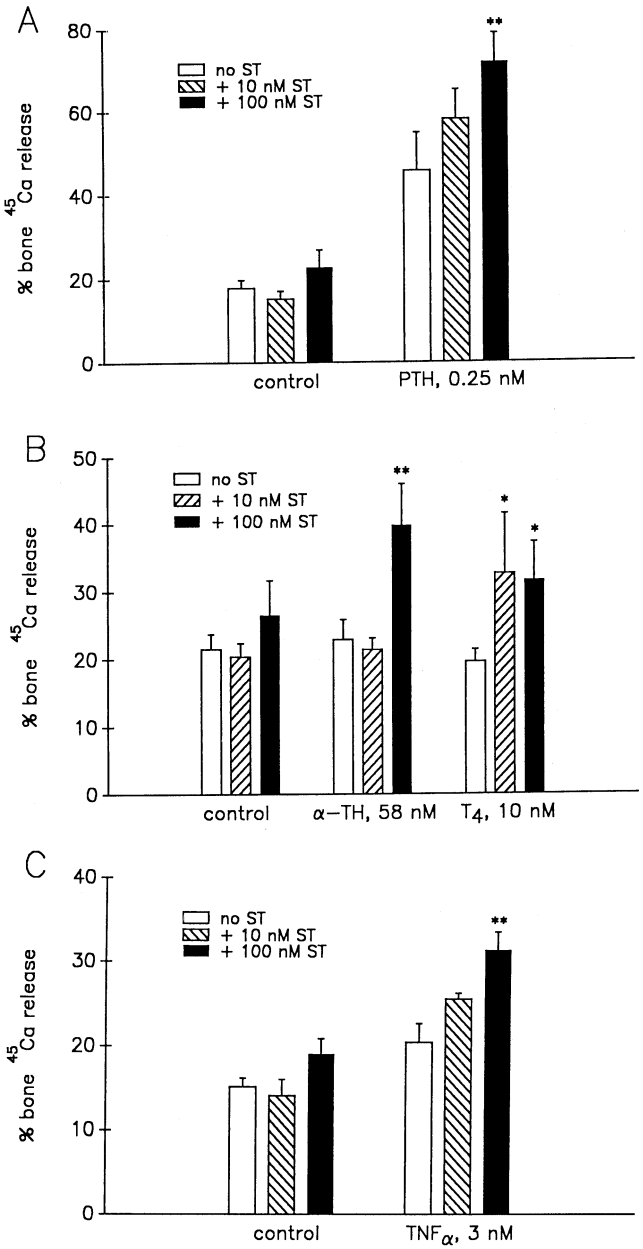

FIG. 1. Enhancement of stimulated bone resorption by 10–100 nM ST. Fetal rat limb bones prelabeled with $^{45}CaCl_2$ were cultured in DMEM + 15% heat-inactivated horse serum. Ten or one hundred nanomolar ST was added together with each stimulator of bone resorption. Stimulators were (A) 0.25 nM PTH, (B) 58 nM α-thrombin (α-TH) or 10 nM T4, and (C) 3 nM TNF-α. After 72 hr, resorption was quantified by measurement of ^{45}Ca released into the medium and retained in the bones. Data are expressed as means and standard errors of the responses of at least 4 bones per treatment. Key: (*) $P < 0.05$ and (**)$P < 0.01$, effect of ST plus stimulator compared with stimulator alone.

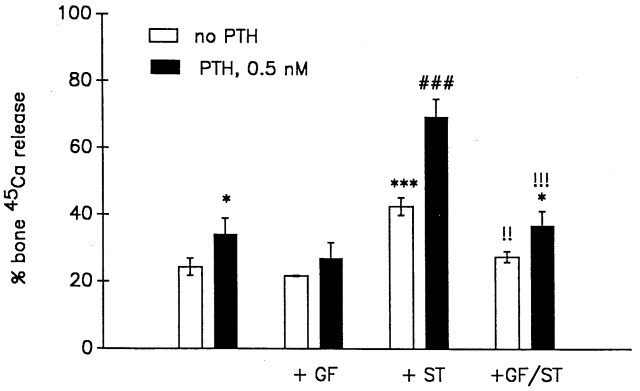

FIG. 2. Antagonism by GF of the stimulatory effects of ST on bone resorption. The experimental design was similar to that in Fig. 1. Treatments were 0.5 nM PTH, 300 nM GF, and 100 nM ST. Data are expressed as means and standard errors of the responses. Key: (*) $P < 0.05$ compared with control; (***) $P < 0.001$ compared with control; (###)$P < 0.001$ compared with control, PTH alone, or ST alone; (!!) $P < 0.01$ compared with ST only; (!!!) $P < 0.001$ compared with ST + PTH.

more PKC-selective ST analog GF (Table 1) are consistent with the conclusion indicated by earlier studies that PKC is critical for bone resorption [1, 2]. In contrast to these inhibitory effects of the high concentrations of the PKC inhibitors, the current results showed that when lower concentrations of the inhibitors were used with submaximal concentrations of PTH, GF continued to inhibit resorption, whereas ST effects were found to be biphasic. Lower concentrations of ST, but not of GF, enhanced the resorptive responses to submaximal concentrations of PTH, α-TH, T4, and TNF-α, and in some experiments elicited a small stimulatory effect even in the absence of agonists, possibly by potentiating the basal resorption occurring in the unstimulated cultures. In contrast, GF, over a 20 nM-1000 μM concentration range, inhibited effects of both maximal and submaximal concentrations of PTH.

ST is a relatively nonselective protein kinase inhibitor; at concentrations close to those that inhibit PKC, ST can inhibit protein kinase A [9]. This raises the possibility that either the inhibitory or stimulatory effects of ST could be mediated through a non-PKC pathway. In thymocytes, micromolar concentrations of ST caused apoptosis [10]. In calvarial organ cultures we have found that micromolar ST inhibits both thymidine and proline incorporation, whereas 1 μM GF does not inhibit macromolecular synthesis (data not shown). It seems likely that the inhibition of resorption by GF is a consequence of its inhibition of PKC, since GF is a more specific PKC antagonist [7]. In addition, earlier studies showed that another PKC-specific inhibitor, AMG, reduced the bone resorption induced by PTH, 1,25-D, or PGE [1, 2]. The mechanism by which inhibition of PKC can lead to inhibition of resorption remains to be determined.

The stimulatory/enhancing effects on resorption of the lower concentrations of ST were a somewhat unexpected finding. The inhibition of these stimulatory effects of ST by the selective PKC inhibitor GF suggests that they may be mediated by a ST-stimulated increase in PKC. In keratinocytes, ST has effects on cell morphology that suggest that it stimulates PKC in those cells [11]. In previous studies we found that in UMR-106 clonal osteoblastic cells, PTH elicits calcium transients that are enhanced by ST [12, 13].

It is conceivable that this increased intracellular calcium could lead to an activation of calcium-sensitive PKC isozymes in osteoblasts. We have reported that the calcium-sensitive α and β PKC isozymes, as well as the calcium-insensitive δ, ϵ, ζ, and ι isozymes, are present in osteoblasts [14, 15].

Biphasic effects of ST have been reported previously. In studies of thymocyte apoptosis, concentrations of ST in the nanomolar range antagonized thapsigargin-induced apoptosis [10], in contrast to the apoptotic effects of the higher ST concentrations. The biphasic effects of ST we observed in the bone organ cultures could be related to responses and sensitivities of different cell types. Osteoclasts, the cells mediating resorption, are inhibited directly by PKC activation [16]. Inhibition of this inhibitory PKC-dependent process by ST could lead to enhanced resorption, although this would not explain why other PKC inhibitors have not shown the biphasic effects that were observed with ST.

Although GF generally is considered to be a highly selective PKC inhibitor [7], in *in vitro* kinase assays it can inhibit MAPKAP kinase-1β and p70 S6 kinase [17]. It is not known whether these kinases are present in bone, are involved in resorption, or are affected by PTH. Thus, the possible roles of these other kinases in the observed responses are unclear.

The findings in the present study may be relevant for the previous seemingly disparate effects of phorbol ester on bone resorption, for which both stimulatory and inhibitory effects have been shown. The observations could also have importance for other tissues in which there are potentially antagonistic effects resulting from the activation or inhibition of different PKC isozymes.

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