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Staurosporine Enhances Parathyroid Hormone-Induced Calcium Signal in UMR-106 Osteoblastic Cells

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Parathyroid hormone (PTH) treatment of bone and kidney-derived cells not only activates adenylyl cyclase but also increases intracellular free calcium, and translocates protein kinase C (PKC) from cytosol to plasma membranes. We have found that acute phorbol ester pretreatment significantly decreases PTH-induced calcium transients and the effect of phorbol ester was antagonized by staurosporine (ST). Although the major effect of ST in that study was the reversal of the action of phorbol ester, it appeared that ST may also have promoted the effect of PTH directly. To further investigate the observation, we examined the effect of ST on the intracellular calcium transients induced by PTH and α-thrombin (α-TH). For calcium transient experiments, UMR-106 cells were loaded with 2 mM fluo-acetoxymethylester for 30 min at room temperature. The cells were then washed and suspended in buffer containing 1 mM calcium. Fluorescence was detected at 530 nm, with excitation at 505 nm. ST alone did not cause calcium transients, but enhanced the transients elicited by PTH when added 5 min before the hormone. Another protein kinase inhibitor H-7 likewise enhanced the calcium responses elicited by PTH, while genistein did not affect PTH response. Calcium transients elicited by α-TH were also enhanced by ST. The results suggest that there might be tonically activated endogenous protein kinase(s) which inhibit calcium signaling of some calcemic agents.

Key words : Staurosporine, PTH, α-thrombin, Calcium signal, Osteoblast, H-7

INTRODUCTION

PTH is an 84 amino acid single chain polypeptide that is secreted by the parathyroid gland under hypocalcemic conditions. PTH is the major short-term regulator of calcium homeostasis, increasing calcium release from bone and stimulates renal tubular calcium reabsorption (Potts *et al.*, 1982). It also increases calcium absorption from the gut indirectly by promoting the synthesis of 1,25-dihydroxyvitamin D in the proximal tubules of the kidney (Potts *et al.*, 1982).

The PTH receptor is a 80~85 kDa glycosylated protein located in the plasma membrane of clonal rat osteosarcoma cells (Shigeno *et al.*, 1988). cDNA clones that encode PTH receptors were isolated from opossum kidney and rat osteosarcoma cell cDNA libraries (Juppner *et al.*, 1991; Abou Samra *et al.*, 1992). The DNA sequences encoding the receptors predict that these are members of the large family of receptors that span the plasma membrane seven times and work by activating guanyl nucleotide-binding proteins (G proteins) on the inner surface of the membrane (Juppner *et al.*, 1991). Later, identical cDNAs that encode a 593-amino acid human PTH receptor were isolated from cDNA libraries constructed from human kidney and human osteoblast-like osteosarcoma cells (Schipani *et al.*, 1993), suggesting that the same PTH receptor is present in two major target organs for PTH.

Stimulation of adenylyl cyclase by coupling to a G protein is recognized as a major signal transduction pathway for PTH receptor. PTH receptor coupling to phospholipase C (PLC) has been identified as an alternate pathway of PTH signal transduction in bone and kidney, based upon recent experiments (Civitelli *et al.*, 1988; Abou Samra *et al.*, 1992). Consistent with this, PTH causes translocation of protein kinase C (PKC) from the cytosolic to the membrane fractions in osteoblastic cells (Abou Samra *et al.*, 1989).

The involvement of PKC, cAMP-dependent protein kinase (PKA), and other phosphorylation mechanisms in the rapid desensitization of the calcium response to PTH stimulation was investigated in osteoblast-like

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UMR-106 cells (Lee and Stern, 1994). In that study, we found that phorbol 12, 13-dibutyrate (PDB) pretreatment reduces subsequent calcium signaling by PTH, implying PKC in PTH response desensitization. We also observed that the PDB-induced decrease of the PTH response was blocked when ST was added to the cell suspension prior to the preincubation with PDB (Lee and Stern, 1994). Although the major effect of ST in the studies was the reversal of the action of PDB, it appeared that ST may also have promoted the effect of PTH directly. The current studies were undertaken to examine this more thoroughly with regard to calcium signaling of PTH. In addition, the effect of ST on another calcemic agent, α-thrombin (α-TH) was studied.

MATERIALS AND METHODS

Materials

Fluo-3 acetoxymethylester (fluo-3) was purchased from Molecular Probes, Inc. (Eugene, OR, USA) stored at -20°C in dark, and dissolved in dimethylsulfoxide (DMSO) just before use. PTH was obtained from Bachem, Inc. (Torrance, CA, USA), dissolved in 1 mM HCl, aliquoted, and kept frozen at -70°C. All other agents and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

UMR-106 cells

UMR-106 cells were grown in 75 cm^2 plastic flasks at 37°C in a humidified 5% CO_2 atmosphere in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 2.8 mM L-glutamine, 15% heat-inactivated horse serum, 100 U/ml Na-penicillin G. Medium was changed every three days. Cells between 7th through 20th passage were used for experiments.

Intracellular calcium measurement

UMR-106 cells were harvested from culture dishes by rapid (<3 min) treatment with 0.01% trypsin and 0.02% EDTA in phosphate-buffered saline. After washing, the cells (approx. 10^7 cells/ml) were loaded with 2 mM fluo-3-acetoxymethylester for 30 min at room temperature in a loading buffer containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 10 mM Hepes, 1% bovine serum albumin, and 2.5 mM probenecid. The buffer was adjusted to pH 7.4. After loading, cells were washed twice and resuspended in the loading buffer. 3×10^6 cells/2 ml were used for each measurement. The cell suspension in the cuvette was stirred continuously. A Perkin-Elmer LS-5B Luminescence Spectrometer was used for the fluorometric assays (excitation at 505 nm, emission at 530 nm).

After baseline fluorescence had been recorded for approximately 1 min, treatments were added in separate runs. Cells were preincubated for 5 min with vehicle or ST, and then stimulated with either 25 nM PTH or 29 nM α-TH.

Maximal fluorescence (F_{max}) was measured by the addition of digitonin (40 mM) or Triton X-100 (0.2%). There was no obvious difference between the F_{max} values obtained from digitonin or Triton X-100 treatment. F_{min} ($=F_{auto}$) is the fluorescence of the cell suspension without fluo-3 loading. Calcium concentration [nM] was calculated according to the formula (Lee and Stern, 1994): $[Ca^{2+}]=K_d\times(F-F_{min})/(F_{max}-F)$, where K_d is the dissociation constant for fluo-3 (400 nM) and F is the measured fluorescence of sample in the cuvette.

Unless otherwise specified, the results shown in figures are representative of at least four similar experiments. Data on graphs are expressed as means and standard errors of the responses of at least four experiments. Statistical significance was determined by analysis of variance followed by Fisher's least significant difference test.

RESULTS

The effect of staurosporine (ST) on the calcium response of PTH in UMR-106 cells

The effect of ST (an inhibitor of protein kinase) pretreatment on the response to PTH was examined (Fig. 1 and 2). A continuously stirred cell suspension was preincubated in a cuvette with vehicle (DMSO) or increasing concentration of ST for 5 min before being stimulated with 25 nM PTH. As shown in Fig. 1, 25 nM PTH induced an immediate increase in intracellular calcium concentration in the UMR-106 cells. ST alone did not cause calcium transients, but enhanced the calcium transients elicited by PTH. Preincubation of cells with 100 nM or 1 mM ST increased the PTH response to 147% or 118%, respectively, of the response obtained with PTH alone ($p<0.05$) (Fig. 2). Preincubation of the cells with another protein kinase inhibitor H-7 (1-(5-isoquinolinylsulfonyl)-2-methylpiperazine) also enhanced PTH response to 115% (25 μM) to 140% (50 μM) (data not shown). Each concentration of ST was prepared so that 1 μl of the DMSO solution added to the 2 ml cell suspension produced the indicated final concentration. DMSO itself was without any effect on the PTH response.

The effect of genistein on the calcium response of PTH in UMR-106 cells

To assess the effect of tyrosine kinase on the PTH-induced calcium responses, genistein (an inhibitor of protein tyrosine kinase) was used. Pretreatment of cells

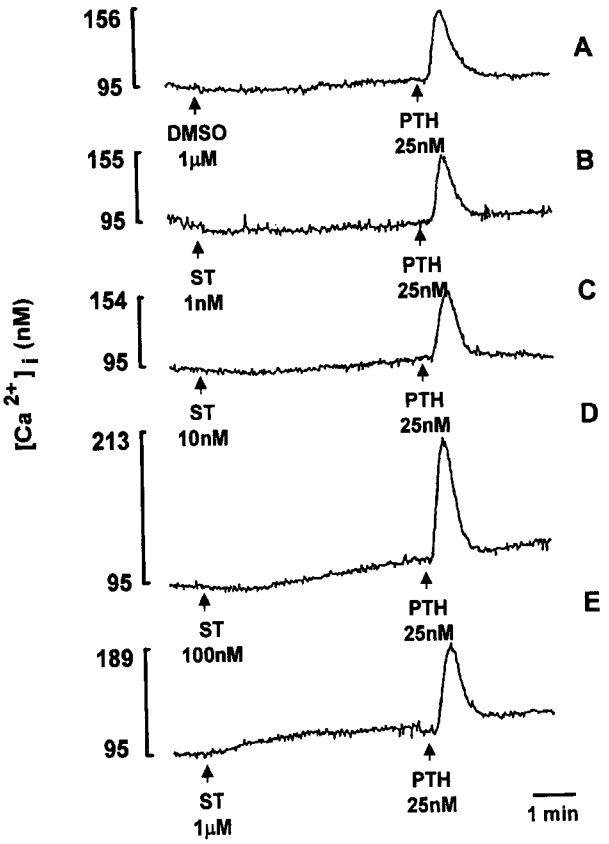

Fig. 1. The effect of staurosporine (ST) on the calcium response of PTH in UMR-106 cells. Each fluo-3-loaded UMR-106 cell suspension was preincubated for 5 min with 1 μl DMSO (A), 1 nM ST (B), 10 nM ST (C), 100 nM ST (D), or 1 μM ST (E) prior to the stimulation with 25 nM PTH.

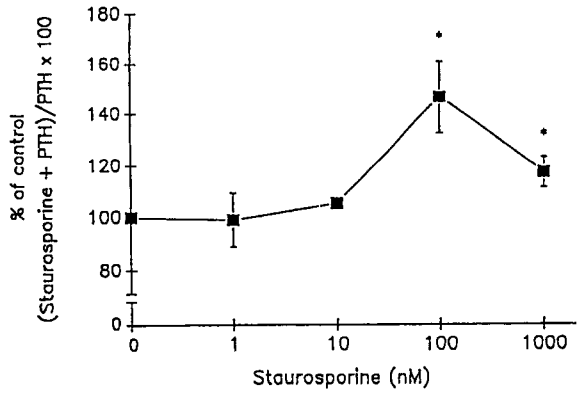

Fig. 2. Concentration-dependent effect of ST on the PTH-stimulated calcium signal in UMR-106 cells. The cell suspension was preincubated for 5 min with the indicated concentrations of ST, after which the cells were stimulated with 25 nM PTH. The results are the mean±SEM of at least four experiments and are expressed as the percentage of the response from untreated cells to the same concentration of PTH. *p<0.05 compared to PTH alone.

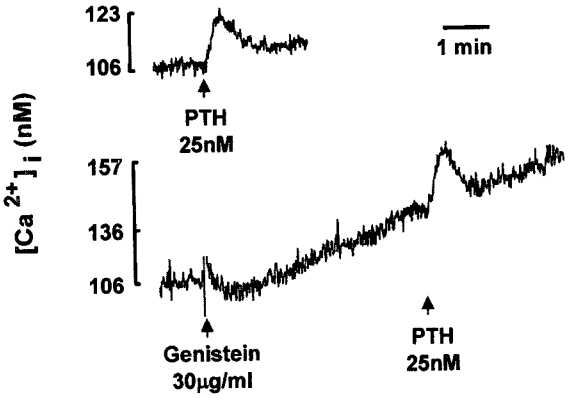

Fig. 3. Lack of effect of genistein pretreatment on the calcium response of PTH in UMR-106 cells. An aliquot of cells was preincubated for 5 min with 30 μg/ml genistein before the stimulation with 25 nM PTH. For comparison, a control PTH response was obtained from the cells unexposed to genistein.

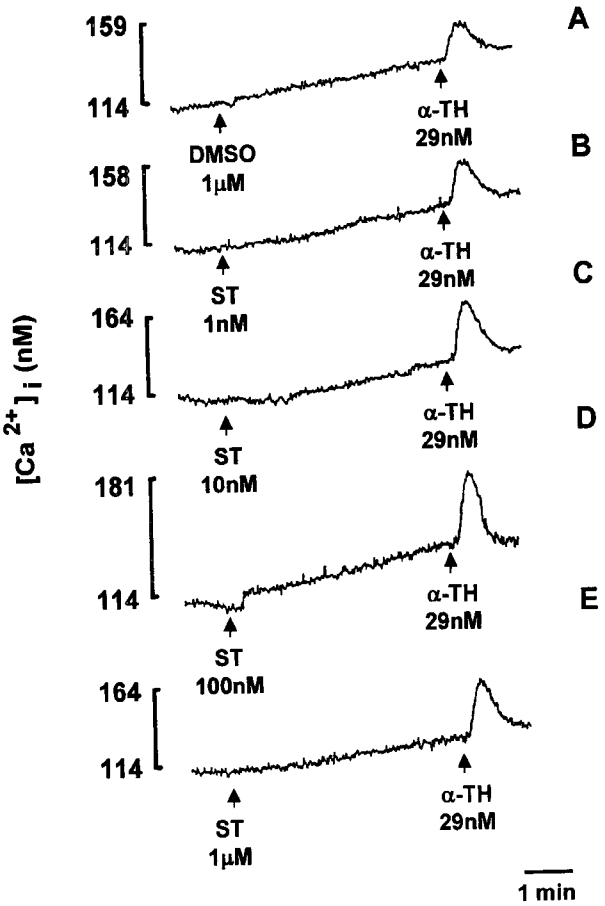

Fig. 4. The effect of ST on the calcium response of α-TH in UMR-106 cells. Each fluo-3-loaded UMR-106 cell suspension was preincubated for 5 min with 1 μl DMSO (A), 1 nM ST (B), 10 nM ST (C), 100 nM ST (D), or 1 μM ST (E) prior to the stimulation with 29 nM α-TH.

with 30 μg/ml genistein for 5 min had no effect on PTH response (Fig. 3). This concentration of genistein inhibited tyrosine kinase activity in UMR-106 cells (data not shown).

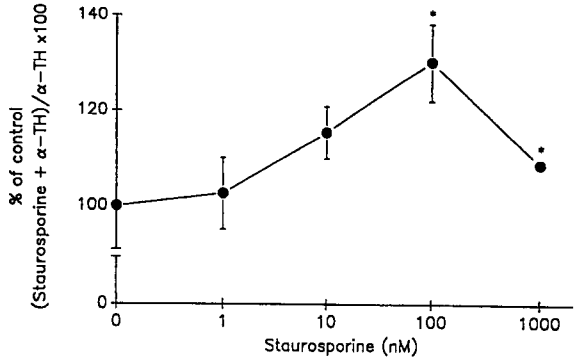

Fig. 5. Concentration-dependent effect of ST on the α-TH-stimulated calcium signal in UMR-106 cells. The cell suspension was preincubated for 5 min with the indicated concentrations of ST, after which the cells were stimulated with 29 nM α-TH. The results are the mean±SEM of at least four experiments and are expressed as the percentage of the response from untreated cells to the same concentration of 29 nM α-TH. *$p<0.05$ compared to α-TH alone.

The effect of staurosporine on the calcium response of α-TH in UMR-106 cells

To verify whether the ST effect is specific to PTH, the effect of ST on the calcium response to α-TH was tested. ST pretreatment for 5 min also increased the calcium transients elicited by α-TH stimulation (Fig. 4). At 10 nM, ST slightly increased α-TH response, and at concentrations higher than 100 nM it significantly ($p<0.05$) enhanced calcium transient to α-TH stimulation (Fig. 5).

DISCUSSION

Desensitization is defined as a decreased cellular response to a long-lasting or repeated stimulus. Increasing numbers of studies on receptor desensitization argue that receptor phosphorylation is closely related to the functional uncoupling of receptors from G proteins in many different receptors. The receptor phosphorylation leading to desensitization can be mediated by two distinct types of protein kinases; the second messenger-activated kinases and G protein coupled receptor kinases (Inglese *et al.*, 1993).

Single species of PTH/PTH-rp (PTH-related peptide) receptor can couple to both adenylyl cyclase and PLC, causing activation of PKA and PKC (Abou Samra *et al.*, 1989; Abou Samra *et al.*, 1992). In addition, PTH has been reported to cause tyrosine phosphorylation of pp60 c-src protein in MG-63 osteoblastic cells through yet unknown mechanism (Izbicka *et al.*, 1994). Thus, it is possible for PKC, PKA, and/or protein tyrosine kinase to play a role in the desensitization of PTH response as the receptor phosphorylation leading to desensitization can be mediated by the second messenger-activated kinases as well as G protein coupled receptor kinases (Inglese *et al.*, 1993).

Interestingly, the predicted PTH/PTH-rp receptor sequences from the opossum kidney (Juppner *et al.*, 1991) or rat osteosarcoma (Abou Samra *et al.*, 1992) cell lines contain consensus sequences for phosphorylation by PKC, suggesting the possibility of modification by PKC. In agreement with this, PKC was suggested to have a role in PTH receptor down-regulation and reduced binding affinity (Fukayama *et al.*, 1992). In addition, we also found that PKC activation by phorbol ester treatment inhibited calcium response of PTH (Lee and Stern, 1994) and that several PKC isozymes are present including PKC-α, β1, β2, δ, ε, ζ, η, and ι/λ in UMR-106 cells (Sanders and Stern, 1996).

Cells can have autocrine/paracrine stimulation by variety of factors secreted by themselves. PTH-related peptide (PTHrp), which reproduces the major biological actions of PTH *in vivo* and *in vitro* (Nissenson *et al.*, 1988), is produced in osteoblastic cells and its expression is upregulated by serum stimulation through transcriptional and posttranscriptional mechanisms (Falzon, 1996). Other regulators such as cytokines and growth factors as well as their receptors were found in osteoblastic cells implying their possible role as autocrine factors for bone cells (Bilbe *et al.*, 1996). These support the notion that osteoblastic cells could be stimulated by autocrine factors under ordinary cell culture condition and that some protein kinases could be stimulated at certain level resulting in partial receptor desensitization.

In this study, ST as well as H-7 potentiated the PTH-induced calcium responses. ST was initially described as a highly specific inhibitor of PKC, but further work has demonstrated that it inhibits several other kinases as well, including PKA (Ruegg and Burgess, 1989). Pretreatment with forskolin to activate PKA (Lee and Stern, 1994) as well as genistein to inhibit protein tyrosine kinase (Fig. 3) failed to affect the PTH-induced calcium transients in UMR-106 cells. Additionally, enhanced calcium response to α-TH was observed in ST-pretreated cells. In relation to our observation, PKC-β was shown to desensitize the α-TH-induced calcium response in endothelial cells (Vuong *et al.*, 1998).

The results we observed suggest that endogenous protein kinase(s) other than PKA or protein tyrosine kinase could be inhibited by ST and H-7 treatment resulting enhanced calcium responses to PTH and α-TH in our experimental condition. Generally, we regard cells as unstimulated in the absence of exogenous stimulation. The possibility that the normal state, even in an isolated cell culture system, involves autocrine stimulation is a very intriguing one. The current results with ST which has no effects of its own but enhances the responses to bone-resorbing factors, should

be considered with that possibility in mind.

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