JOURNAL OF BONE AND MINERAL RESEARCH
Volume 17, Number 5, 2002

Prostaglandin E_2 Induces Interaction Between hSlo Potassium Channel and Syk Tyrosine Kinase in Osteosarcoma Cells

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ABSTRACT

Prostaglandins (PGs) are important mediators of bone response to growth factors, hormones, inflammation, or mechanical strains. In this study, we show that in MG63 osteosarcoma cells, prostaglandin E_2 (PGE_2) produces the opening of a large conductance Ca^{2+}-dependent K^+ channel (BK). This PGE_2-mediated channel opening induces the recruitment of various tyrosine-phosphorylated proteins on the hSlo α-subunit of BK. Because the C-terminal domain of hSlo encompasses an immunoreceptor tyrosine-based activation motif (ITAM), we show that the Syk nonreceptor tyrosine kinase, reported yet to be expressed mainly in hematopoietic cells, is expressed also in osteoblastic cells, and recruited on this ITAM after a PGE_2-induced docking/activation process. We show that Syk/hSlo association is dependent of an upstream Src–related tyrosine kinase activity, in accord with the classical two-step model described for immune receptors. Finally, we provide evidence that this Syk/hSlo interaction does not affect the electrical features of BK channels in osteosarcoma cells. With these data, we would like to suggest the new notion that besides its conductance function, hSlo channel can behave in bone cells, as a true transduction protein intervening in the bone remodeling induced by PGE_2. (J Bone Miner Res 2002;17:869–878)

Key words: prostaglandin E_2, Slo potassium channel, Syk, Src, osteoblast

INTRODUCTION

PROSTAGLANDINS (PGs) are key mediators of inflammation with an autocrine/paracrine role in bone metabolism. However, the role of PGs in bone appears complex, because they have been shown to have both stimulatory and inhibitory effects on bone formation and bone resorption.[1] Cyclo-oxygenases (COXs) catalyze the first step in the conversion of arachidonic acid (AA) to prostanoids, including PGs. Recent studies suggest that the inducible isoform COX-2 mediates increased prostaglandin E_2 (PGE_2) production in bone in response to various stimuli including parathyroid hormone (PTH), mechanical strain, or inflammatory cytokines such as tumor necrosis factor (TNF) α, interleukin (IL) 1β, IL-6, IL-11, and IL-17.[2–9]

PGE_2 has been studied extensively in the past and like many resorptive cytokines exhibits pleiotropic effects on bone metabolism. PGE_2 acts directly on the physiology of osteoblasts with indirect consequences on the osteoclastic bone resorption. Recently, PGE_2 has been shown to stimulate the expression of the osteoclast differentiation factor (ODF)/receptor activator of nuclear factor κB (NF-κB) li-

The authors have no conflict of interest.

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gand (RANKL)/osteoprotegerin ligand (OPGL)/TNF-related activation cytokine (TRANCE) in osteoblasts.[10] Moreover, PGE_2 can inhibit bone resorption by a direct action on mature osteoclasts.[11]

In osteoblasts, PGE_2, PTH, and mechanical loading stimulate an increase in intracellular calcium, likely via the production of inositol triphosphate, diacylglycerol, and cyclic adenosine monophosphate (cAMP).[12–14] This increase in intracellular Ca^{2+} has been shown to trigger the opening of Ca^2-dependent K^+ channels of high conductance (BK).[15,16] It is noteworthy, in the context of bone remodeling, that this type of channel also has been shown to exhibit mechanosensitive properties in osteoblasts and odontoblasts.[16,17]

In contrast to other voltage-dependent K^+ channels, the pore-forming α-subunit of BK channels is encoded by a single gene *slo* (*slowpoke*),[18,19] that undergoes extensive, hormonally regulated, alternative RNA splicing.[20,21] These splice variants give rise to channels with different functional properties, including calcium sensitivity, single-channel conductance, and regulation by intracellular signaling pathways.[22–25] In addition, association of the Slo α-subunit with accessory subunits modulates channel characteristics or their cellular distribution.[26–29] Large-conductance calcium-activated potassium channels also are subject to modulation by protein kinases,[26,30–32] phosphatases,[33,34] and other signaling proteins.[35–37]

In this study we address the question of the involvement of BK channels in the triggering and propagation of signaling, via PGE_2, in a human osteoblastic model. We provide the first evidence that on its Ca^{2+}-activated form, the hSlo α-subunit of BK channels is phosphorylated by the Src kinases on an immunoreceptor tyrosine-based activation motif (ITAM) consensus sequence that allows the subsequent recruitment and activation of Syk, a tyrosine kinase known to play a crucial role in the activation of various cell types.[38–40] This leads to the concept that BK channels can behave not only as ion pores but also as true transducing proteins. This newly identified role of BK channels is likely to have important implications in the understanding of the early biochemical events intervening in the activation of osteoblasts.

MATERIALS AND METHODS

Antibodies and chemicals

The antibody (Ab) to hSlo was generated by immunizing rabbits with a glutathione *S*-transferase (GST) fusion protein containing the hSlo amino sequence from residue 805–975 (GST-hSlo). Specific Ab's to hSlo were purified first through an affinity column containing the GST protein to eliminate anti-GST Ab's, followed by a second affinity column containing the GST-hSlo protein. Ab's to hSlo ultimately were eluted by an acid shock (0.1 M of HCl/glycine at pH 2.6). The antiphosphotyrosine (anti-Ptyr; 4G10), the anti-cSrc (GD11), and the anti-Syk (4D10.1) monoclonal Ab's (mAb's) were from Upstate Biotechnology (Lake Placid, NY, USA). The anti-c-myc mAb 9E10 was from Roche Diagnostics, Indianapolis, IN, USA). Piceatannol and 4-amino-5-(4-chlorophenyl)-7-(t. butyl) pyrazolo [3,4-d] pyrimidine (PP2) were from Calbiochem (La Jolla, CA, USA) and all other chemicals were from Sigma (St. Louis, MO, USA).

Cell lines, cell transfection, and preparation of cell lysates

The human MG63 osteosarcoma cell line was obtained from American Type Culture Collection (ATCC; Rockville, MD, USA). Cells were cultured in RPMI 1640 supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS) (Biowhittaker, Gagny, France), 2 mM of L-glutamine, 50 IU/ml of penicillin, and 50 μg/ml of streptomycin in 5% CO_2-air–humidified atmosphere at 37°C. Cells were starved 16 h in RPMI 1640 before exposure to various effectors. The human CAL-72 osteosarcoma cell line that we have previously characterized[41] was maintained like MG63 cells. CAL-72/Syk stable transfectants were established using the Transfast Promega kit (Promega, Madison, WI, USA). Briefly, 10^6 cells were plated onto petri dishes 16 h before transfection with pEF-Bos/Syk (8 μg) + pcDNA3 (2 μg) according to the manufacturer's protocol. Cells were cultured for 48 h and then selected in RPMI complete medium containing 1 mg/ml of G418 (Life Technologies, Paisley, UK). Clones were isolated and screened by Western blotting for Syk expression.

COS cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) heat-inactivated FCS (Life Technologies), L-glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (100 μg/ml) and were transiently transfected by the diethylaminoethyl (DEAE)-dextran method with complementary DNA (cDNA) plasmids. Briefly, cells (10^6 cells) were plated onto petri dishes 16 h before transfection. Cells were washed twice with serum-free DMEM and incubated for 3 h at 37°C in the transfection medium (0.8 μg/ml of DNA, 0.1 μM of chloroquine, and 500 μg/ml of DEAE-dextran in DMEM supplemented with 10% [vol/vol] NU serum [Pharmingen, San Diego, CA, USA]). Then, cells were treated with 10% dimethyl sulfoxide in phosphate-buffered saline (PBS) for 2 minutes at room temperature and cultured in DMEM supplemented with 10% (vol/vol) FCS for 48 h.

Stimulated MG63 cells and transfected CAL-72 or COS cells were washed twice with PBS and harvested by lysis in ice-cold 1% NP-40 buffer containing 150 mM of NaCl, 0.8 mM of $MgCl_2$, 5 mM of EGTA, 1 mM of phenylmethylsulfonyl fluoride (PMSF), 15 μg/ml of leupeptin, 1 μM of pepstatin, 1 mM of Na_3VO_4, and 50 mM of HEPES at pH 7.5 (lysis buffer). The crude lysates were centrifuged at 18,000*g* for 20 minutes at 4°C; the supernatants (lysates) were removed and the protein concentration was assayed using the Lowry method (Bio-Rad Laboratories, Hercules, CA, USA).

Plasmids, DNA constructs, and mutagenesis

Bluescript II KS + plasmid containing hSlo cDNA, alternative splice exon free, (Genebank access number U13913) was provided by Dr. Steven Dworetzky (Walling-

ford, CO, USA). For a more efficient protein expression, hSlo cDNA was subcloned into pRcCMV plasmid in the *Hin*dIII and *Not*I sites. hSlo mutants were generated by site-directed mutagenesis of double-stranded DNA using the Gene Editor system kit (Promega). Tyrosine 1061, tyrosine 1072, or both were replaced by phenylalanine residues (mutants hSlo F_{1061}, hSlo F_{1072}, and hSlo $F_{1061–1072}$, respectively). All mutations were verified by DNA sequencing analysis. The pSGT-cSrc plasmid was a kind gift of Dr S. Roche (Montpellier, France). The pEF-Bos expression vector containing the full-length cDNA coding for human Syk was a kind gift from Dr. V. Imbert (Nice, France). The porcine Myc epitope–tagged Syk expression vector was generated by cloning the full-length Syk cDNA from PM-Syk in the *Eco*RI and *Xho*I sites of the pCS3 vector.[42]

Electrophysiology

Three types of recording modes were used: cell-attached and excised inside-out and outside-out patches. For outside-out patch recordings, the pipette solution contained 140 mM of KCl, 3 mM of $MgCl_2$, and 10 mM of HEPES, adjusted at pH 7.3, with KOH. The free Ca^{2+} concentration was adjusted to 1 μM (5 mM of EGTA and 4.7 mM of $CaCl_2$). The external solution was either a 5-mM K^+ solution containing 140 mM of NaCl, 5 mM of KCl, 3 mM of $MgCl_2$, 1 mM of $CaCl_2$, 5 mM of glucose, and 10 mM of HEPES, adjusted to pH 7.4, with NaOH or a K^+-rich solution that contained 140 mM of KCl instead of 140 mM of NaCl. For cell-attached and inside-out configurations, the pipette solution was the 5-mM K^+ external solution and the bath solution was the 140-mM K^+ external solution.

The cell-surrounding medium was changed rapidly by a local microperfusion system consisting of a series of stainless tubes (100-μm inner diameter) connected to different test solutions and capped by a tapered plastic tube (500-μm tip inner diameter), flowing at a constant rate of about 0.2 ml/minute. The fluid was maintained at a constant level by direct suction.

The experiments were done at room temperature. Data were recorded using an RK400 path-clamp amplifier (Bio-Logic, Claix, France). Single channel currents were monitored and stored continuously using a DAT recorder. Data were replayed, sampled, and analyzed using pClamp software (Axon Instruments, Union City, CA, USA).

Immunoprecipitation and kinase assay

Cell lysates (0.5–1 mg of proteins) were precleared with rabbit nonimmune serum prebound to protein A–Sepharose (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA). Then hSlo was immunoprecipitated with 1 μg of polyclonal anti-hSlo, followed by incubation with protein A–Sepharose for 1 h. Immunopellets were washed twice with lysis buffer and twice with kinase buffer (10 mM of $MgCl_2$, 10 mM of $MnCl_2$, 1 mM of paranitrophenylphosphate, and 50 mM of HEPES, pH 7.4).

For in vitro kinase assay, immunoprecipitates were incubated in 50 μl of kinase buffer containing 10 μCi of [γ-^{32}P]adenosine triphosphate (ATP; 370 MBq/ml**;** ICN, Costa-Mesa, CA, USA) for 15 minutes at 30°C. Reactions were stopped by the addition of Läemmli sample buffer, boiled at 95°C for 5 minutes, and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

For immunoblotting analysis, immunoprecipitates were dissolved in SDS sample buffer, boiled at 95°C for 5 minutes, and directly resolved by 10% SDS-PAGE.

Immunoblotting

SDS-PAGE resolved samples were transferred onto Immobilon-P membranes (Millipore, Bedford, MA, USA) as detailed previously.[43] The immunoblots were incubated overnight at 4°C with rabbit anti-hSlo polyclonal Ab, anti-Src, anti-Syk, anti-c-myc, or anti-Ptyr mAb's. After, three washes with 10 mM of Tris at pH 7.4, 150 mM of NaCl, and 1% NP-40, blots were incubated with secondary horse radish peroxidase (HRP)–conjugated goat anti-rabbit (GAR) or HRP-conjugated rabbit anti-mouse (RAM) Ab's (Dako, Copenhagen, Denmark) and Ab-bound proteins were detected by the Amersham enhanced chemiluminescence (ECL) system. In some experiments, autoradiographs were quantified by densitometric scanning using a laser densitometer equipped with ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

RESULTS

MG63 cells express a PGE_2-responsive Ca-activated K conductance of the BK type

Patch-clamp experiments performed on MG63 human osteosarcoma cells revealed the presence of a Ca^{2+}-sensitive high conductance (Fig. 1A). Ca^{2+} sensitivity was assessed by exposing inside-out patches to various internal Ca^{2+}concentrations. Channel openings were both rare and brief in the absence of Ca^{2+}, while they were frequent and maintained in 1 μM of Ca^{2+}. Current-voltage relationships obtained from an outside-out patch configuration in physiological (5 mM of K^+) and symmetrical (140 mM) K^+ gradients showed reversal potentials of -75 mV and 0 mV, respectively, in accord with a K^+ selectivity for this channel (Fig. 1B). The calculated unitary conductance values for this channel were 95 pS (in 5 mM) and 235 pS (in 140 mM). Addition of PGE_2 (10 nM) in a recording configuration of silent cell-attached patches held at 0 mV induced a frequent and sustained activation of large conductance K^+ channels after a delay of a few seconds (Fig. 1D). Altogether these data support the idea that in MG63 cells, PGE_2 induces the opening of a Ca^{2+}-sensitive K^+ channel of BK (big conductance) type. Interestingly, in this cell type, the Ca^{2+}-dependent K^+ channel was insensitive to classical blockers of BK channels such as charybdotoxin and iberiotoxin, as shown in Fig. 1C. However, it was totally blocked by application of 1 mM of tetraethyl ammonium (TEA), in accord with previous data on the sensitivity of BK channels toward this drug.[44,45]

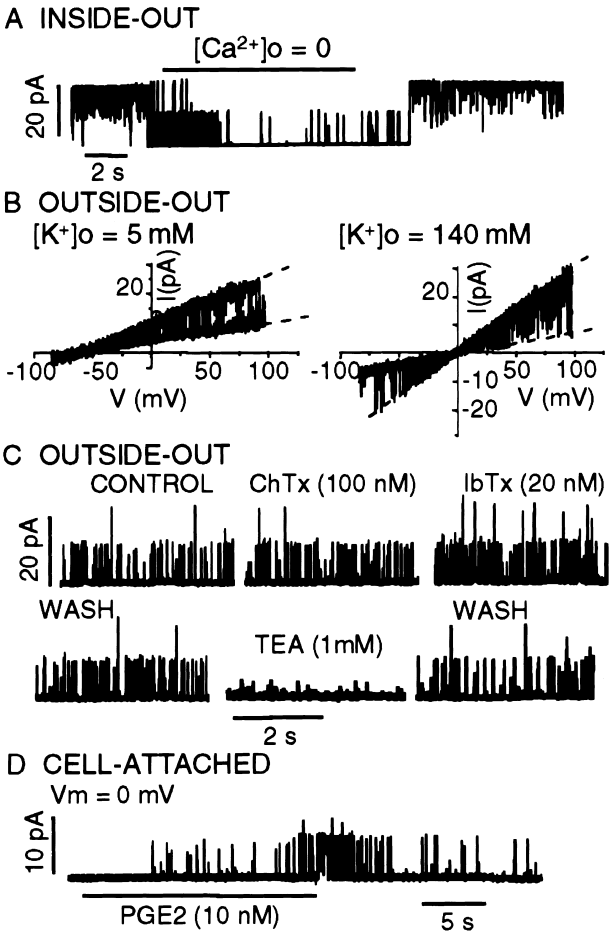

FIG. 1. Functionnal expression of high-conductance K^+ channel (BK) in MG63 osteosarcoma cells. (A) Calcium sensitivity of the BK channel recorded in the inside-out configuration at +50 mV. Replacing the intracellular solution containing 1 μM of free Ca^{2+} with one without Ca^{2+} reversibly blocks channel activities. (B) Successive traces from an outside-out patch containing a BK channel. I-V relationships obtained with voltage ramp (200 mV/s) between −80 and +100 mV. Bath solution was either the external 5 mM of K^+ solution (upper traces) or the K^+-rich (140 mM of K^+) external solution (lower traces); internal free Ca^{2+} concentration was adjusted to 1 μM. Dotted lines were drawn through the open- and closed-currents levels. Unitary conductances in low and high K^+ external concentration were estimated at 95 pS and 235 pS, respectively. (C) Pharmacologic properties of the BK channel. In a typical experiment in the outside-out configuration, neither charybdotoxin (ChTx; 100 nM) nor iberiotoxin (IbTx; 20 nM) blocks channel activity. Tetraethylammonium (TEA; 1 mM) reversibly inhibits the channel activity (holding potential, +50 mV). (D) Activation of BK channels by PGE_2 (10 nM) in a cell-attached patch with a low basal activity (bath solution, 140 mM of K^+-external solution; pipette solution, 5 mM of K^+ external solution; holding potential, 0 mV). Application of PGE_2 is indicated by a horizontal bar.

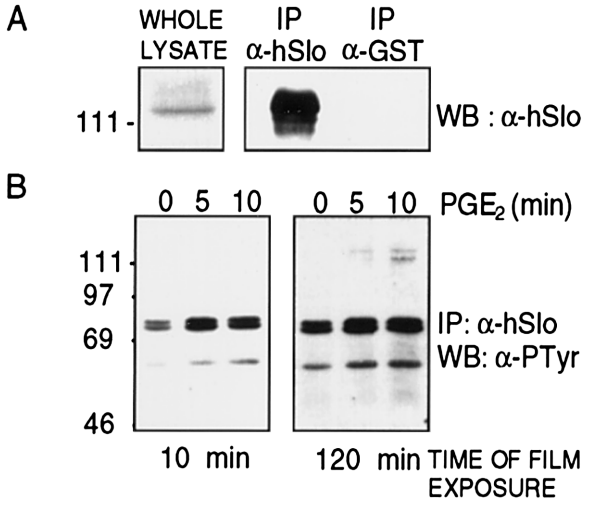

FIG. 2. PGE_2 induces the recruitment of tyrosine phosphorylated proteins on hSlo in MG63 cells. (A) Immunodetection of hSlo α-subunit of BK channel in MG63 osteosarcoma cells. Whole lysates and anti-hSlo or anti-GST immunoprecipitates prepared from MG63 cells were subjected to immunoblot analysis with an anti-hSlo Ab as described in the Materials and Method section. (B) Coimmunoprecipitation of hSlo channel with tyrosine phosphorylated proteins under PGE_2 stimulation. MG63 cells were either untreated or treated with PGE_2 (10 nM) for 5 minutes or 10 minutes. Then, cell lysates were prepared and subjected to immunoprecipitation using an anti-hSlo Ab. Tyrosine phosphorylated proteins coimmunoprecipitated with hSlo channels were analyzed by Western blotting with an anti-Ptyr Ab. Left and right panels correspond to different times of ECL signal detection.

Given that BK conductances have been reported to depend on the expression of the hSlo protein,[22,24,46] we produced polyclonal Ab's to the recombinant C terminus part of hSlo to assess the expression of this protein in MG63 cells. As shown in Fig. 2A, a protein migrating around 110–120 kDa, consistent with the expected molecular mass of hSlo, was both specifically immunoprecipitated and revealed by Western blotting using these Ab's.

hSlo constitutively interacts with Src and recruits Syk on PGE_2 stimulation in MG63 cells

PGE_2 has been reported to increase the cAMP/protein kinase A (PKA) and phospholipase C (PLC) pathways resulting in the phosphorylation of various substrates. To gain information on the mechanisms through which modulation of the BK channel activity occurs, we analyzed, by Western blotting, the pattern of phosphotyrosine proteins associated with hSlo/BK channels in response to PGE_2. To this end, MG63 cells were exposed to PGE_2 for various periods of time before being lysed. Thereafter, hSlo α-subunit of BK channels was immunoprecipitated with specific polyclonal Ab's and immunopellets were subjected to electrophoresis, transferred onto a nylon membrane, and Western blotted with Ab's to phosphotyrosine. As shown in Fig. 2B several proteins interacted with hSlo in response to PGE_2 in a time-dependent manner, exhibiting various levels of phosphorylation, with molecular mass ranging from 60 to 120 kDa.

Recently, A direct interaction of *Drosophila* and mouse Slo with the Src tyrosine kinase has been reported.[30,47] This prompted us to investigate whether this kinase also interacted with hSlo in our system. To address this question, lysates from MG63 cells preexposed to PGE_2 for various

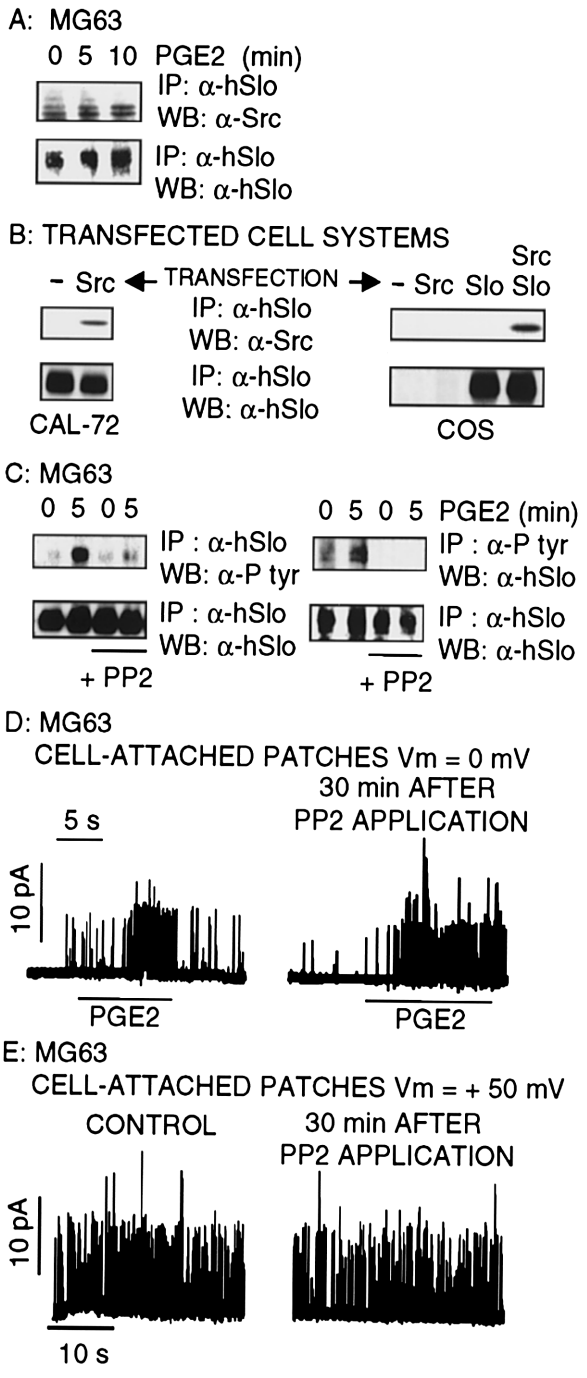

FIG. 3. cSrc is associated with hSlo and participates in its tyrosine phosphorylation on PGE_2-stimulated MG63 cells. (A) Effect of PGE_2 on Src/hSlo interaction. MG63 cells were treated for the indicated times with PGE_2 (10 nM). Cell lysates were prepared and immunoprecipitated with anti-hSlo Ab. Immune complexes were blotted with anti-cSrc Ab. Membranes were stripped and reprobed with anti-hSlo Ab to normalize immunoprecipitation efficiency. (B) Reconstitution of Src/hSlo interaction in CAL-72 and COS transfected cells. CAL-72 osteosarcoma cells, which expressed a high level of native hSlo, were transiently transfected with control vector or cDNA coding for cSrc. COS cells were transfected with cDNA coding for hSlo and Src, alone or together. After 48 h, cells were lysed and subjected to immunoprecipitation with anti-hSlo Ab, and immunoprecipitates were analyzed by immunoblotting with an anti-cSrc Ab. Then, membranes were stripped and reprobed with anti-hSlo. (C) Effect of PGE_2 and PP2 on hSlo tyrosine phosphorylation in MG63 cells. Cells were preincubated (or not) for 30 minutes with the Src-specific inhibitor PP2 (10 μM) and then stimulated with PGE_2 (10 nM) for 5 minutes. Immunoprecipitation of hSlo or phosphotyrosine-containing proteins were performed and analyzed by Western blotting with 4G10 or anti-hSlo Ab's, respectively. Immunoblots were stripped and reprobed with anti-hSlo Ab. (D) Activation of BK channel by PGE_2 (10 nM) in the absence or presence of 10 μM of PP2 in a cell-attached patch (bath solution, 140 mM of K^+ external solution; pipette solution, 5 mM of K^+ external solution; holding potential, 0 mV). Application of PGE_2 is indicated by a horizontal bar. (E) BK channel activity was measured in cell-attached patches, holding potential near +50 mV, in the absence or presence of 10 μM of PP2. Bath and pipette solutions were 140 mM of Na^+ and 2 mM of Ca^{2+}.

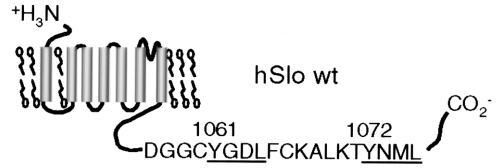

FIG. 4. An ITAM is present in the cytoplasmic tail of hSlo. The minimal consensus ITAM sequence (underlined) consists of two YxxL/I repeats (one letter code of amino acids, where "x" stems for any residue), 6–8 residues apart.

periods of time were subjected to coimmunoprecipitation experiments. As shown in Fig. 3A, Src was constitutively associated with hSlo and was not recruited further on PGE_2 treatment. Consistently, we showed that in CAL-72 osteosarcoma cells, which expressed high endogenous level of hSlo, the overexpression of Src resulted in the formation of a constitutive Src-hSlo complex (Fig. 3B). Along the same line, when Src and Slo were coexpressed in COS cells, we did observe the same kind of association (Fig. 3B). Based on the observation that PGE_2 increased the extent of tyrosine phosphorylation of a band traveling with an apparent M_r of 120 (Fig. 2B), we sought to determine whether this band corresponded to a Src-dependent phosphorylated form of hSlo. Indeed, we found that the level of tyrosine phosphorylation of the 120-kDa band, immunoprecipitated by anti-hSlo Ab's, was increased by exposure to PGE_2 whereas PP2, a potent inhibitor of Src family tyrosine kinases,[48] abrogated this phosphorylation (Fig. 3C, left panel). The Src-dependent phosphorylation of hSlo was confirmed by the dramatic decrease induced by PP2 on the antiphosphotyrosine immunoprecipitable fraction of hSlo (Fig. 3C, right panel). Contrary to numerous reports on modulation of K^+ channel activities by tyrosine kinases,[32] we found that the Src-dependent phosphorylation of hSlo had no significant effect on the electrical features of BK channels in MG63 cells when stimulated either by PGE_2 (Fig. 3D) or by depolarization (Fig. 3E).

The analysis of the hSlo sequence reveals the presence of an ITAM motif in the C terminus tail of the protein (Fig. 4). This consensus sequence has been proven to be essential for the docking of Syk tyrosine kinase on im-

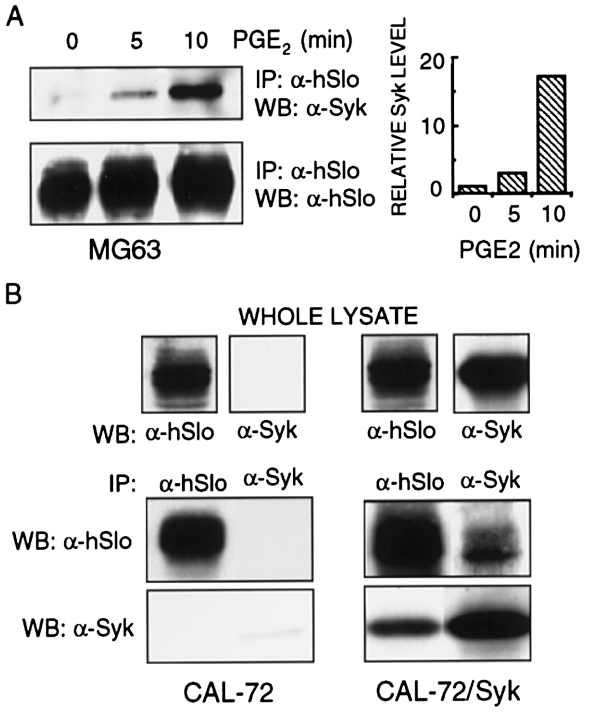

FIG. 5. Syk tyrosine kinase interacts with hSlo. (A) PGE_2 stimulates Syk/hSlo interaction in MG63 cells. Cells were treated with 10 nM of PGE_2 for the indicated times and lysed. Then, cell lysates were subjected to immunoprecipitation using anti-hSlo Ab and the immune complexes were analyzed by Western blotting for the presence of Syk. The same membrane was stripped and reprobed for hSlo. The diagram shows the densitometric scanning quantification of Syk signals normalized to hSlo. (B) Reconstitution of Syk/hSlo interaction in CAL-72–transfected cells. CAL-72 cells stably expressing Syk were established as described in the Materials and Methods section. The upper panel shows the Western blot analysis of native hSlo and exogenous Syk expression in whole lysates from parental and Syk-transfected CAL-72 cells, respectively. In the lower panel, cell lysates from both cell types were immunoprecipitated with anti-hSlo or anti-Syk Ab's and analyzed by successive anti-hSlo or anti-Syk hybridizations.

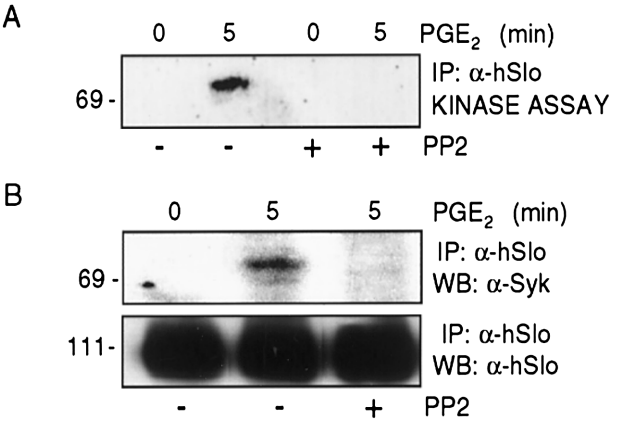

FIG. 6. A Src-like tyrosine kinase is necessary for Syk/hSlo interaction. (A) MG63 cells were preincubated or not preincubated for 30 minutes with PP2 (10 μM) before stimulation in the presence of PGE_2 for 5 minutes. Then, cell lysates were immunoprecipitated with anti-hSlo Ab and an in vitro kinase assay was performed as described in the Materials and Methods section. (B) Cells were processed as in A and hSlo immunoprecipitates were blotted with anti-Syk Ab. The blot was reprobed with anti-hSlo as a loading control.

mune receptors.[49] Furthermore, the major tyrosine phosphorylated band associated with hSlo exhibited an apparent M_r of 70–72. Taken together, these data prompt us to investigate the possibility that this protein corresponded to $p72^{Syk}$. In fact, Syk is expressed mainly in immune cells,[50] although recent studies have reported also expression of this nonreceptor tyrosine kinase in other cell types.[51,52] We observed that Syk was indeed expressed in MG63 osteosarcoma cells by Western blotting with anti-Syk Ab's. Furthermore, when MG63 cells were exposed to PGE_2, we detected a time-dependent recruitment of Syk in anti-hSlo immunopellets (Fig. 5A) after kinetics compatible with those observed for the PGE_2 triggered association with tyrosine phosphorylated cellular substrates (Fig. 2B). In an attempt to reveal a direct hSlo/Syk interaction, we made use of CAL-72 cells that do not express Syk while they exhibit high level of hSlo. When CAL-72 cells were stably transfected with p72Syk cDNA, we detected the constitutive presence of Syk in anti-hSlo immunoprecipitates and, reciprocally, the presence of hSlo in anti-Syk immunopellets (Fig. 5B).

An Src-like tyrosine kinase activity is necessary for the docking of Syk on hSlo ITAM

Syk recruitment on ITAM of immunoreceptors has been shown to require prior phosphorylation of the two tyrosine residues of this motif by Src-related tyrosine kinases.[50] In this regard, it is of particular interest that the Src inhibitor PP2 altered the level of PGE_2-induced hSlo/Syk association, as evidenced by kinase assay (Fig. 6A) or anti-Syk immunoblotting experiments (Fig. 6B) performed on anti-hSlo immunoprecipitates. To better delineate the mode of interaction of Syk on the hSlo sequence, we carried out separate or combined site-directed mutagenesis of the two tyrosine residues located within the C-terminus ITAM of hSlo. COS cells were transfected with both hSlo and myc-tagged Syk constructions. In this model, ectopic high expression of Syk-myc and hSlo resulted in a constitutive association between the two partners. Data presented in Fig. 7A show that single substitution of Tyr 1061 or Tyr 1072 for Phe was without any effect on hSlo/Syk-myc association. In contrast, combined mutation of both Tyr 1061 and 1072 residues markedly diminished the association of Syk with hSlo, showing the importance of phosphorylation of these two residues in the control of Syk/hSlo interaction.

Futhermore, we showed that substitution of Tyr 1061 and 1072 for Phe was without any effect on the open probability of BK channels when expressed in COS cells (Fig. 7B).

At this stage, it was of interest to investigate whether the intrinsic tyrosine kinase activity of Syk also participated in the control of Syk/hSlo interaction. As shown in Fig. 7C, we observed, in the same doubly transfected COS cells, that addition of piceatannol, a potent specific inhibitor of Syk

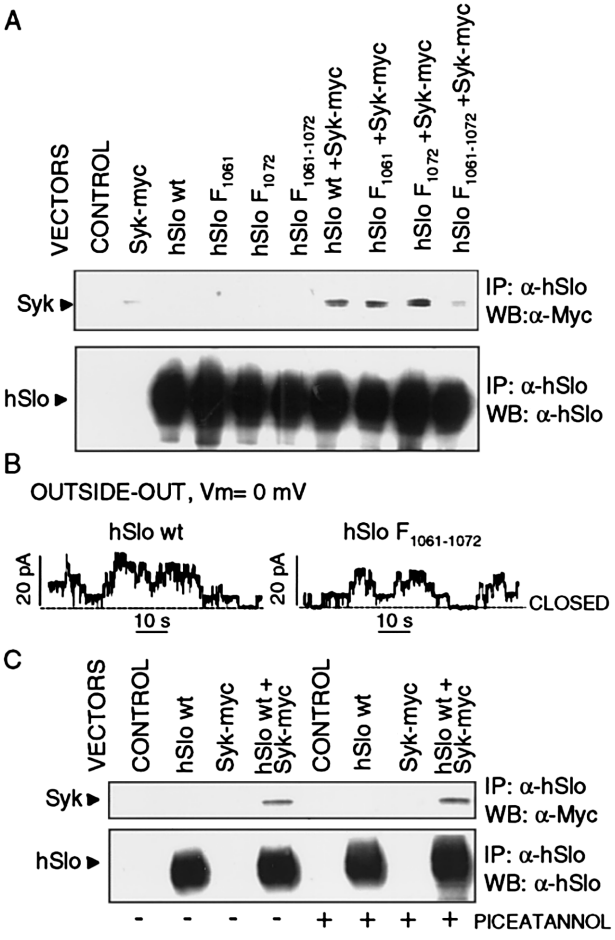

FIG. 7. hSlo ITAM sequence is required to permit Syk/hSlo interaction. (A) Role of hSlo ITAM motif in the interaction with Syk. hSlo ITAM mutations were generated by site-directed mutagenesis as described in the Materials and Methods section. COS cells were transiently transfected with vectors coding for the indicated cDNA (Syk-myc corresponding to a myc-tagged form of Syk). Cell lysates were subjected to anti-hSlo immunoprecipitation and successively immunoblotted with anti-Myc and anti-hSlo Ab's. (B) Representative traces of BK channel activity in the outside-out configuration at +50 mV in COS cells transiently transfected with vectors coding for hSlo wt or mutated hSlo $F_{1061-1072}$. Bath solution was the external 5-mM K^+ solution; the 140-mM K^+ pipette solution contained 1 μM of Ca^{2+}. (C) Role of Syk tyrosine kinase activity on Syk/hSlo interaction. COS cells transiently transfected with the indicated vectors were treated or not treated with the Syk-specific inhibitor piceatannol (5 μg/ml) for 1 h before being lysed. Cell lysates were immunoprecipitated with anti-hSlo Ab and immunoblotted with anti-Myc Ab. Then, the membrane was stripped and reprobed with the anti-hSlo Ab.

activity,[53] was without any effect on Syk recruitment, ruling out the possibility that the intrinsic tyrosine kinase activity of Syk intervened, by itself, in the control of its interaction with hSlo. Moreover, Syk overexpression did not modify the electrical features of BK channels in stably transfected Syk-expressing CAL-72 cells (Fig. 8A). Conversely, Syk inhibition by piceatannol also was without any effect on the aperture of BK channels (Fig. 8B). Taken together, these data suggest that BK channel activity is not

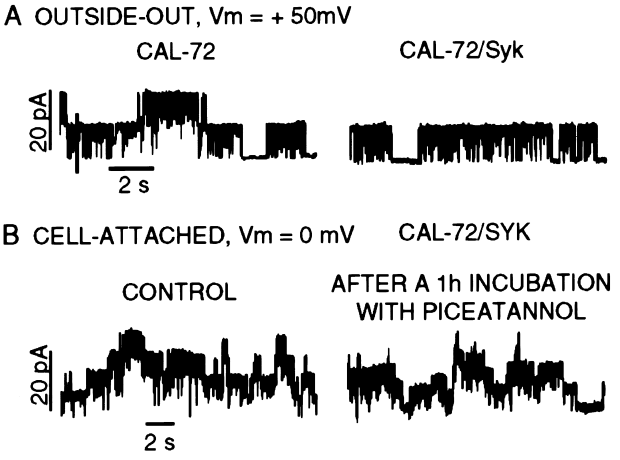

FIG. 8. Syk tyrosine kinase does not affect BK channel activity in osteosarcoma cells. (A) Representative traces of BK channel activity recorded in the outside-out configuration at +50 mV from parental or stably Syk-transfected CAL-72 cells. Bath solution was the external 5-mM K^+ solution; the 140-mM K^+ pipette solution contained 1 μM of Ca^{2+}. (B) CAL-72/Syk cells were preincubated or not preincubated with piceatannol (5 μg/ml) for 1 h and BK channel activity recorded in the cell-attached configuration at 0 mV. Bath and pipette solutions were the 5-mM K^+ external solution.

modulated through Syk/hSlo interaction in osteosarcoma cells.

DISCUSSION

PGE_2 has been shown to play an important role in the orchestration of bone remodeling, especially through its activating effect on osteoblasts. Interestingly, PGE_2 has been shown to increase the intracellular calcium concentration and, consequently, the opening of Ca^{2+}-dependent potassium channels.[15] In this study, we focused on the Ca^{2+}-dependent large conductance potassium channel, because of its expression in osteoblasts, and its ability to open in response to PGE_2. We first verified that the product of the *hslo* gene, responsible for the BK conductance, was indeed expressed in the MG63 osteosarcoma cell line. Exposure of these cells to PGE_2 triggered the opening of a potassium conductance that exhibited all the electrical features and sensitivity to TEA that hallmark a Ca^{2+}-dependent K^+ channel of BK type, with the exception that it was insensitive toward the two blockers iberiotoxin and charybdotoxin. This particularity distinguishes it from most of the BK channels studied in other tissues.[45] A possible explanation lies in the fact that BK channels are organized as a multimer complex of α-subunits carrying the pore function and β-regulatory subunits that have been shown recently to modulate both the electrical and the pharmacologic features of the channels.[54] Indeed, we verified that all the β-subunits known ($\beta1$–$\beta4$) were expressed in MG63 cells (data not shown), allowing us to think that the resulting $\alpha\beta$-oligomers mixture may generate channels displaying peculiar tissue-specific features.

Syk interacts with an ITAM located in the C terminus of the hSlo sequence

The large intracytoplasmic C-tail of hSlo is a distinctive feature among the members of the K_{Ca} channel family. Analysis of this domain revealed that it contains a well-conserved ITAM sequence, which consists of two YXX(L/I) cassettes separated by 6–8 amino acids. This was an unexpected observation, because this kind of motif has only been reported so far to be part of the sequence of immune receptors such as the ζ-chain of the T-cell receptor, the α- and β-chains of the B-cell receptor, and the Fcγ-receptor of macrophages.[49] In immune cells, engagement of these receptors has been shown to activate Src-related kinases that phosphorylate the two tyrosine residues in the ITAM sequence. This phosphorylation process confers to ITAM the ability to interact with the two tandem Src homology 2 domains of Syk or Zap 70.[55] Syk is a protein tyrosine kinase (PTK) that is widely expressed in hematopoietic cells, whereas its congener Zap 70 is exclusively expressed in T lymphocytes and natural killer (NK) cells.[56] Both of them are involved in the coupling of activated immunoreceptors to downstream signaling events in relation with proliferation, differentiation, and phagocytosis.[57–59] Recently, Syk was shown to be activated on adhesion of platelets through the $\alpha_{IIb}\beta_3$-integrin.[60] In addition to immune cells, Syk expression has been detected in epithelial cells,[51] fibroblasts, and adipocytes.[52] We verified that this nonreceptor tyrosine kinase also was expressed largely in MG63 osteosarcoma cells. Exposure of osteosarcoma cells to PGE_2 induced the recruitment on hSlo of a panel of tyrosine phosphorylated proteins, including a protein with an apparent M_r of 72, which corresponds to Syk.

Potassium channels have been shown to be targets for various protein kinases.[32] Nevertheless, the effect of these phosphorylations in terms of open probability is still a matter of debate. Some potassium channels are intimately associated with their kinases. It is the case of the voltage-dependent delayed rectifier potassium channel (Kv1.3), which was reported to interact with the SH3 domain of Src.[61] More recently, *Drosophila* and mouse Slo channels have been shown to interact constitutively with c-Src and the catalytic subunit of PKA.[30,47] In this context, it was tempting to explore the exciting hypothesis that the state of hSlo opening could control its interaction with tyrosine kinases. Unlike the Src-related members, the Syk/Zap 70 family is devoid of prenylation sites, destining it to a cytoplasmic location. In MG63 cells, we observed a slight constitutive association of Syk with hSlo, which was increased markedly in response to PGE_2, suggesting that the potassium channel needs to adopt an open conformation to interact with Syk.

Recruitment of Syk on hSlo ITAM requires activation of a Src-related kinase

Recruitment of Syk to immune receptors has been shown to be strictly dependent on phosphorylation of ITAM by Src-related kinases. Nevertheless, intrinsic Syk kinase activity also has been shown to be required, in conjunction with Src-like kinases, for its interaction with integrins.[60] We verified that piceatannol, an inhibitor of Syk activity, was without any effect on the Syk/hSlo interaction, indicating that docking of Syk to hSlo did not imply an autocatalytic process.

Based on the two-step Src-dependent process described in immune cells, we verified that Src was constitutively associated to hSlo in osteosarcoma cells.[30] Moreover, we found that PP2, a potent inhibitor of Src-related kinases, abolished the interaction of Syk with hSlo, suggesting strongly that phosphorylation of the tyrosine residues of the ITAM motif by an Src-like kinase was necessary for docking Syk onto the cytoplasmic domain of hSlo. To further assess the molecular basis of the hSlo/Syk interaction, each or both of the tyrosine residues located within the ITAM motif were replaced for phenylalanine. It turned out that the two tyrosine residues needed to be mutated at the same time to significantly alter, but not abrogate, the hSlo/Syk association. In contrast with studies carried out in immune cells,[49] but in accord with data performed in platelets aiming to study the interaction of Syk with the $\alpha_{IIb}\beta_3$-integrin,[60] these data suggest that other domains of the C terminus of hSlo might participate in the interaction with Syk.

Recently, a large body of evidence has been accumulated, showing that serine/threonine kinases as well as tyrosine kinases could regulate the open probability of ion channels.[32] In this regard, of particular interest is the demonstration, using Syk-deficient DT40B mutants,[62] that B cell receptor (BCR)-mediated Ca^{2+} response requires Syk activation. Thus, the question raised is to know whether, in our model, Syk could exert a negative feedback on the Ca^{2+}-induced opening of hSlo channels. We found that inhibition of Syk by piceatannol had no effect on BK channel activity in osteosarcoma cells. Moreover, in transfected CAL-72/Syk cells stably expressing a high level of Syk, the permanent association of hSlo with Syk failed to modify the electrical features of BK channels.

Taking our data together, we would like to bring forward the new concept that besides their ion conductance function, hSlo channels also can be regarded as true transducing proteins that, in their open state, can activate a Src-related/Syk PTK cascade, leading to the phosphorylation of a series of cellular substrates. Identification of those substrates and elucidation of the mode of activation of the Src activity will help in understanding the means by which PGE_2 controls (in osteoblastic cells) the expression of genes important for bone homeostasis. In this context, BK channels might appear as important targets for the development of new drugs aimed at modulating bone remodeling.

ACKNOWLEDGMENTS

The authors are indebted to E. Van Obberghen for critical reading of this study. This work was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM) and the Association pour la Recherche sur le Cancer (ARC; grant 5417). Roger Rezzonico was a recipient of a fellowship from ARC.

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Received in original form March 12, 2001; in revised form December 4, 2001; accepted December 21, 2001.