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Sphingosine 1-phosphate-mediated α_{1B}-adrenoceptor desensitization and phosphorylation. Direct and paracrine/autocrine actions

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Abstract

Sphingosine-1-phosphate-induced α_{1B}-adrenergic receptor desensitization and phosphorylation was studied in rat-1 fibroblasts stably expressing enhanced green fluorescent protein-tagged adrenoceptors. Sphingosine-1-phosphate induced adrenoceptor desensitization and phosphorylation through a signaling cascade that involved phosphoinositide 3-kinase and protein kinase C activities. The autocrine/paracrine role of sphingosine-1-phosphate was also studied. It was observed that activation of receptor tyrosine kinases, such as insulin growth factor-1 (IGF-I) and epidermal growth factor (EGF) receptors increased sphingosine kinase activity. Such activation and consequent production of sphingosine-1-phosphate appears to be functionally relevant in IGF-I- and EGF-induced α_{1B}-adrenoceptor phosphorylation and desensitization as evidenced by the following facts: a) expression of a catalytically inactive (dominant-negative) mutant of sphingosine kinase 1 or b) $S1P_1$ receptor knockdown markedly reduced this growth factor action. This action of sphingosine-1-phosphate involves EGF receptor transactivation. In addition, taking advantage of the presence of the eGFP tag in the receptor construction, we showed that S1P was capable of inducing α_{1B}-adrenergic receptor internalization and that its autocrine/paracrine generation was relevant for internalization induced by IGF-I. Four distinct hormone receptors and two autocrine/paracrine mediators participate in IGF-I receptor- α_{1B}-adrenergic receptor crosstalk.

Keywords

α_{1B}-adrenoceptor; α_{1B}-adrenergic receptor; sphingosine-1-phosphate; EGF receptor; transactivation

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1. Introduction

The actions of adrenaline and noradrenaline (NA) are mediated through three families of receptors with three members each, i. e., the α_1- family (comprising theα_{1A}-, α_{1B}- and α_{1D}-adrenergic receptors (ARs); the α_2- family (α_{2A}-, α_{2B}- and α_{2C}-ARs) and the β-adrenergic family(β_1-, β_2- and β_3-ARs) [1].

The α_1- family participates in many of the physiological effects of these catecholamines and is also involved in the pathogenesis of some diseases [2–4]. α_{1B}-ARs were the first receptors to be cloned of this family [5] and have been studied in much greater detail. Evidence indicates that the function of these receptors is regulated through cycles of phosphorylation-dephosphorylation [2] and that protein kinases such as G protein coupled receptor kinases 2 and 3 [6, 7] and protein kinase C (PKC) play the central roles [8]. G protein coupled receptor kinases phosphorylate agonist-occupied receptors, being mainly responsible for homologous desensitization [6, 7] whereas PKC mediates α_{1B}-AR phosphorylation induced by phorbol esters and many non-adrenergic hormones and neurotransmitters participating in the heterologous desensitization of these ARs [9–18]. α_{1B}-AR phosphorylation sites have been determined at Ser404, Ser408, and Ser410 (for G protein receptor kinases) and at Ser394 and Ser400 (for PKC)[8]. Another key enzyme is the protein and phospholipid kinase, phosphoinositide 3-kinase (PI3K), which appears to act upstream of PKC [2]. Much less is known concerning the role of protein phosphatases in regulating α_{1B}-AR phosphorylation; but evidence indicates that inhibition of these enzymes increases the phosphorylation state of α_{1B}-ARs [19].

It is now known that G-protein coupled receptor-induced epidermal growth factor (EGF) receptor transactivation involves stimulation of metalloproteinases. These proteases cause shedding of HB-EGF from the plasma membrane and subsequent activation of EGF receptor intrinsic tyrosine kinase, resulting in the triggering of intracellular signaling [20, 21]. Surprisingly, this process is much more general than we could have anticipated and is involved in both homologous and heterologous desensitization and phosphorylation of α_{1B}-ARs [9, 10, 15, 16].

In our study on IGF-I-induced α_{1B}-AR desensitization and phosphorylation we observed that EGF receptor transactivation as well as pertussis toxin-sensitive G proteins were involved [16]; in fact, the action of other hormones/growth factors that act through receptor tyrosine kinase receptors also share these characteristics [16]. These data suggest the possibility that receptor tyrosine kinases could couple to pertussis toxin-sensitive G proteins and in this way activate metalloproteinases and the EGF receptor transactivation process [16]. G protein involvement in receptor tyrosine kinase action has been frequently observed, but the mechanisms have been elusive [22]. However, the possibility that receptor tyrosine kinases might couple to G proteins has been difficult to prove. At least one other possibility exists. It has been elegantly shown that many growth factors, hormones and neurotransmitters can activate sphingosine kinase-1 (SPHK-1), which generates sphingosine-1-phosphate (S1P) [23–26]; this phospholipid acts in an autocrine loop, activating cells through its own G protein-coupled receptors [27]. We have suggested the possibility that the connection between the IGF-I receptor and metalloproteinases could involve the generation and action of this sphingophospholipid [15, 28]. This possibility was tested and the results are presented here. Our results indicate that IGF-I-induced α_{1B}-AR desensitization and phosphorylation involves two autocrine/paracrine mediators (S1P and HB-EGF) and four receptor types (IGF-I receptors, $S1P_1$, EGF receptors and α_{1B}-ARs). Additionally, receptor phosphorylation is associated with internalization. Here we show that S1P and IGF-I induce α_{1B}-AR internalization and that their effects are interconnected.

2. Materials and Methods

2.1. Materials

Sphingosine-1-phosphate, (-)-noradrenaline, IGF-I, endothelin-1, tetradecanoyl phorbol acetate, staurosporine, wortmannin, bis-indolyl-maleimide I and protease inhibitors were purchased from Sigma Chemical Co. LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) and AG1478 were obtained from Calbiochem. Dulbecco's modified Eagle's medium, fetal bovine serum, trypsin, antibiotics, and other reagents used for cell culture were from Life Technologies. [γ-^{32}P]ATP (3000 Ci/mmol) and [^{32}P]Pi (8500–9120 Ci/mmol) were obtained from Perkin Elmer Life Sciences, while agarose-coupled protein A was from Upstate Biotechnology. DNA purification kits were obtained from Qiagen. Anti-HB-EGF neutralizing antibodies and chemiluminescence's kits were purchased from Pierce. The pDrive cloning system was purchased from QIAGEN and the pEGFP-N1 vector from Clontech. FM4-64 and Fura 2AM were obtained from Molecular Probes. Plasmids for expression of wild-type SPHK-1 and the catalytically inactive (dominant-negative) mutant were kindly provided to us by Dr. Stuart M. Pitson (The University of Adelaide, Australia) [29]. Primary antibodies against SPHK-1, β-actin and $S1P_1$ receptor were from Santa Cruz Biotechnology whereas those against Flag were from Sigma Chemical Co. BB94 was generously provided to us by Dr. S. Mobashery (University of Notre Dame, Notre Dame, IN, USA).

2.2. Cell line

The cell line used was described previously [15]. Briefly, a line of rat-1 fibroblast (American Type Culture Collection) stably expressing human α_{1B}-ARs tagged with enhanced green fluorescent protein (eGFP) was generated. Cells were cultured in a selection media: glutamine-containing high-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 300 μg/ml of the neomycin analog, G-418 sulfate, 100 μg/ml streptomycin, 100 U/ml penicillin, and 0.25 μg/ml amphotericin B at 37°C under a 95% air/5% CO_2 atmosphere. These cells express α_{1B}-ARs with a density of 900–1600 fmol/mg membrane cell protein (in the range observed in rodent hepatocytes [30]), with high affinity for [^{3}H]prazosin (Kd 0.2-0.3 nM). Photolabeled receptors were identified as a band of Mr ≈ 115–120 kDa and can be immunoprecipitated with an anti-eGFP antiserum generated in our laboratory with reasonably high efficacy (≈ 20%, as evidenced by radioactive photo-labeled receptor immunoprecipitation); these receptors are fully functional and have essentially the same pharmacological characteristics than wild-type receptors [15].

2.3. Intracellular calcium determinations

Cells were loaded with 2.5 μM of the fluorescent Ca^{2+} indicator, Fura-2/AM, in Krebs-Ringer-HEPES containing 0.05% bovine serum albumin, pH 7.4 for 1 h at 37°C and then washed three times to eliminate unincorporated dye. Fluorescence measurements were carried out at 340 and 380 nm excitation wavelengths and at 510 nm emission wavelength, with a chopper interval set at 0.5 sec, utilizing an AMINCO-Bowman Series 2 luminescence spectrometer (Rochester, NY, USA). Intracellular calcium ([Ca^{2+}]i) was calculated according to Grynkiewicz et. al. [31].

2.4. Phosphorylation of α_{1B}-adrenoceptors

The procedure employed to study α_{1B}-AR phosphorylation, has been previously described in detail [15, 16, 18]. In brief, cells were maintained overnight in phosphate-free Dulbecco's modified Eagle's medium without serum. The following day, cells were incubated in 1 ml of the same medium containing [^{32}P]Pi (50 μCi/ml) for 3 h at 37°C. Labeled cells were

stimulated as indicated, washed with ice-cold phosphate-buffered saline, and solubilized with 0.5 ml of ice-cold buffer containing 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 10 mM NaF, 1 mM Na_3VO_4, 10 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 1 mM p-serine, 1 mM p-threonine, 1 mM p-tyrosine, and protease inhibitors [15]. Cell lysates were centrifuged at 12,700 × *g* for 15 min at 4°C and supernatants were incubated overnight at 4°C with an anti-eGFP antiserum generated in our laboratory [15, 32] and protein A-Sepharose. After two washes with 50 mM Hepes, 50 mM NaH_2PO_4, 100 mM NaCl, pH 7.2, 1% Triton X-100, 0.1% SDS, and 100 mM NaF, pellets containing the immune complexes were boiled for 5 min in SDS-sample buffer containing 5% β-mercaptoethanol, and subjected to SDS-polyacrylamide gel electrophoresis. Gels were dried and exposed for 18–24 h and level of receptor phosphorylation was assessed with a Molecular Dynamics PhosphorImager using the Imagequant software (Amersham Biosciences). Data fell within the linear range of detection of the apparatus and were plotted using Prism 4 from GraphPad software.

2.5. Transfection for transient expression

Cells were transfected utilizing Lipofectamine 2000 following the manufacturer's instructions and were cultured as described previously. Repetition of transfection (2–3 times) increased efficiency as shown by Yamamoto et. al. [33] (from 20% to 40–50% in our experiments); cells were employed 3–4 days after transfection. Using this transfection repetition protocol the effect of the SPHK catalytically inactive (dominant-negative) mutant was evident for up to 7 days, decreasing afterward.

2.6. Construction of human/rat $S1P_1$ receptor short hairpin RNA

For gene knockdown we used the short hairpin RNA oligonucleotide 5′-TGCTGTTGACAGTGAGCGAGCTCTACCACAAGCACTATATTAGTGAAGCCAC AGATGTAATATAGTGCTTGTGGTAGAGCGTGCCTACTGCCTCGGA-3′ containing the sense/antisense target sequence against human/rat S1PR1. This oligonucleotide was employedas template for cloning the short hairpin RNA into the pSHAG MAGIC2 (pSM2) vector (Openbiosystems, Huntsville, AL) as reported by Paddison et al. [34]. In brief, we PCR amplified the oligonucleotide utilizing universal primers containing XhoI (5′-CAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCG-3′) and EcoRI (5′-CTAAAGTAGCCCCTTGAATTCCGAGGCAGTAGGCA-3′) sites. These PCR fragments were digested, cloned into the hairpin cloning site of pSM2 vector and transformed into PIR1-competent bacteria. After growth selection with chloramphenicol and kanamycin, we obtained the pSM2 vector containing the shRNA against $S1P_1$, which was tested for gene knockdown by transient transfection as described previously.

2.7 Detection of $S1P_1$ receptor expression by RT-PCR

Total RNA was isolated using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. For reverse transcription–PCR we used, Promega Access RT-PCR System A1250, Primers were as follows: to amplify $S1P_1$ receptor, forward primer 5′-GCTGCTTGATCATCCTAGAG and reverse primer 5′-GAAAGGAGCGCGAGCTGTTG-3′ [35] and to amplify GAPDH, forward primer 5′-GGTGTGAACCACGAGAAATATGAC-3′ and reverse primer 5′-CTCCAGGCGGCATGTCAGATCCAC-3′ [36] were synthesized at the Molecular Biology Unit of our Institute.

2.8. Western blot assays

Cells were washed with ice-cold phosphate-buffered saline and lysed for 1 h in buffer containing NaCl 150 mM, Tris 50 mM (pH 7.4), EDTA 1 mM and 1 % Nonidet P40 on ice.

Lysates were centrifuged at 12,700 × g for 15 min and proteins in supernatants were separated by electrophoresis on 10% SDS-PAGE. Proteins were electrotransferred to nitrocellulose membranes and immunoblottings were performed using the same membranes. Incubation with primary selective antibodies was conducted for 12 hs at 4 °C and with the secondary antibody for 30 min at room temperature. Super signal-enhanced chemiluminescence's kits were employed exposing the membranes to X-Omat X-ray films. Signals were quantified by densitometric analysis utilizing the Scion Image software from Scion Corporation (Frederick, MD, USA).

2.9. Sphingosine Kinase (SPHK) activity

Activity was assayed in cell extracts essentially as described by Olivera and Spiegel [37]. In brief cells were incubated in the presence of vehicle or the agents indicated for 15 min and extracts were obtained. SPHK activity was determined using D-sphingosine and [γ-^{32}P]ATP as substrates, reactions were initiated by addition of the cellular extracts and terminated with chloroform. Lipids were extracted and separated by thin-layer chromatography as described [37]. Reaction was linear with respect to time and amount of extract under the conditions employed.

2.10. Confocal microscopy

Confocal images were obtained using a Flowview FV 1000 laser confocal system (Olympus) attached/interfaced to an OLYMPUS IX81 inverted light microscope with a 40× glycerol-immersion objective; eGFP was excited using the 488-nm line of a krypton/argon laser and the emitted fluorescence detected with a 515–540-nm band pass filter. Operating the laser at a low power setting (97–99% attenuation) substantially reduced photobleaching and photodamage. Confocal images were viewed and processed using the FV10-ASW 1.6 software (Olympus). FM4-64 was used as a plasma membrane marker. To estimate receptor densities the area corresponding to the cell membrane (as guided by membrane marker FM4-64) was selected in each image and the amount of green fluorescence was quantified using ImageJ with Plugging Analyze Particles software [38].

2.11. Statistical Analysis

Statistical analysis between comparable groups was performed using ANOVA with Bonferroni's post-test and was performed with the software included in the GraphPad Prism program.

3. Results

We first wanted to determine wether S1P plays a role in α_{1B}-AR desensitization and phosphorylation. As shown in Fig. 1, 10 μM noradrenaline (NA) induced an immediate and robust ≈ 4-fold increase in intracellular calcium, which remained clearly above basal levels for more than 50 sec (upper left panel). In contrast, S1P (1 μM) induced a much smaller response (≈ 50% over basal values) that developed slowly and vanished after 30–40 seconds. When cells were incubated with S1P, for 15 min, and then challenged with NA the response to the adrenergic agent was markedly reduced (Fig. 1, representative tracings are shown in the left panel). Concentration-response curves in cells treated for 15 min with vehicle or S1P showed that the phospholipid treatment markedly reduced (by 40–50%) the maximal effect of NA without affecting its EC_{50} value (50–75 nM). The possibility of some calcium-storage depletion cannot be ruled out. However, 100 nM endothelin-1 induced a more robust effect than NA on this parameter and it was not decreased in cells treated with 1 μM S1P for 15 min, as compared with untreated cells (Supplementary Fig. S1).

Next we explored the possibility that functional α_{1B}-AR desensitization could be associated with adrenoceptor phosphorylation. As expected, S1P induced a concentration-dependent increase in the phosphorylation state of α_{1B}-ARs; the maximal increase was ≈ 2–2.5-fold with an EC_{50} of ≈ 0.5–1 μM (Fig. 2, left panel). S1P action was rapid inducing clearly detectable α_{1B}-AR phosphorylation 2 min after its addition, reaching a maximum at ≈ h10 min and remaining at a plateau for 60 min (Fig. 2, right panel).

The signaling pathways involved in S1P-induced α_{1B}-AR phosphorylation and desensitization were next explored. As anticipated, based on previous findings from our laboratory with other agents [2, 9–18], inhibitors of PI3K, such as wortmannin and LY294002, staurosporine, a general protein kinase inhibitor, and bis-indolyl-maleimide I, a selective PKC inhibitor, did not alter basal receptor phosphorylation but were able to block S1P-induced α_{1B}-AR phosphorylation (Fig. 3, upper panel). In addition, S1P-induced functional desensitization of these ARs (as reflected by the increase in intracellular calcium) was also clearly blocked by these inhibitors of protein and phospholipids kinases (Fig. 3, lower panel). Besides clarifying the signaling pathway involved in the action of S1P, the effect of the inhibitors also indicates that calcium-depletion was not a major player in the functional effect.

As mentioned previously, the effects of S1P can be mediated through a family of S1P receptors [27]. Interestingly, however, $S1P_1$ receptors are widely distributed among cells and tissues and appear to mediate the paracrine/autocrine action of S1P [23–26]. We tested the possibility that these receptors could be involved in S1P actions by knocking down $S1P_1$ expression using shRNA technology. Expression of $S1P_1$ at the level of mRNA and protein was decreased 30–40% as shown by both RT-PCR and Western blotting; GAPDH (RT-PCR) and β-actin (Western blots) were used as controls and no changes were observed (Fig. 4, bottom panel). The pSM2 empty vector did not alter $S1P_1$ receptor expression. None of these treatments changed the calcium response to 10 μM NA, 1 μM S1P or 100 ng/ml IGF-I (Fig. 4, upper left panel). Both S1P-mediated and IGF-mediated α_{1B}-AR desensitization were clearly observed in cells transfected with the pSM2 vector as shown by the diminished NA-mediated intracellular calcium increases (Fig. 4, upper right panel, white columns). In contrast, the response to NA was much less affected by preincubation with S1P or IGF-I in cells in which $S1P_1$ was knocked down (Fig. 4, upper right panel, solid columns). This was very clear in the calcium tracings depicted in Fig. 4 (middle panel).

Next, we studied α_{1B}-AR phosphorylation in control transfected and $S1P_1$-knocked down cells. As shown in Fig. 5 (left panel) 10 μM NA, 1 μM S1P and 100 ng/ml IGF-I increased α_{1B}-AR phosphorylation 2 to 3-fold in control transfected cells. In contrast, in $S1P_1$ knocked down cells, the effect of NA was slightly decreased but those of S1P and IGF-I were essentially abolished (Fig. 5, right panel). The effect of NA was studied at 5 min whereas those of the other agents (S1P and IGF-I) were examined at 15 min due to the different time-course of their effects [16, 18, 39].

The effect of hormonal stimulation on SPHK activity was assayed in cell extracts by in vitro phosphorylation of sphingosine. As shown in Fig. 6, extracts from cells treated with 1 μM S1P and 10 μM NA induced a consistently small (40%) and statistically insignificant increase in the enzyme's activity, while tetradecanoyl phorbol acetate (that was used as a positive control), IGF-I and EGF induced much greater, statistically significant 2-fold activity increases. The data are consistent with an autocrine/paracrine role of S1P in α_{1B}-AR phosphorylation.

In order to test this model further, we blocked S1P generation with an SPHK-1 catalytically inactive (dominant-negative) mutant and assayed for α_{1B}-AR desensitization by measuring

intracellular calcium concentration responses [29]; expression of the wild type SPHK-1 was used as a control. Expression of wild-type enzyme or of the dominant-negative mutant did not alter the effect of 10 μM NA on intracellular calcium (Fig. 7, upper left panel and bottom panel). As expected, neither expression of the wild-type enzyme nor that of the dominant-negative mutant altered α_{1B}-AR desensitization induced by a 15 min preincubation with 1 μM S1P (Fig. 7, left middle and bottom panels); but in agreement with previous findings, expression of the dominant- negative SPHK-1 mutant markedly inhibited IGF-I- and EGF-induced α_{1B}-AR desensitization (Fig 7, upper and middle right panels and bottom panel). The effect of the SPHK-1 dominant-negative mutant on α_{1B}-AR phosphorylation was also examined. In cells expressing wild-type SPHK-1, 10 μM NA, 1 μM S1P, 100 ng/ml IGF-I or 100 ng/ml EGF clearly increased (≈ 3-fold) α_{1B}-AR phosphorylation (Fig. 8, upper left panel); i. e., expression of the wild type kinase did not alter receptor phosphorylation induced by these agents. In contrast, in cells expressing the dominant-negative sphingolipid kinase, EGF-mediated α_{1B}-AR phosphorylation was markedly reduced, and that induced by IGF-I was nearly abolished (Fig. 8, upper right panel). Western blotting of cell extracts, with anti SPHK-1 and anti-flag antibodies, evidenced expression of the wild type enzyme and the dominant-negative mutant.(Fig 8, lower panel).

The possibility that S1P-induced α_{1B}-AR phosphorylation might involve EGF receptor transactivation was experimentally tested; to do this, the general metalloproteinase inhibitor BB94, a neutralizing anti-HB-EGF antibody and AG1478, an inhibitor of EGF receptor tyrosine kinase activity, were used. None of these agents alter basal α_{1B}-AR phosphorylation (data not shown, see [9, 10, 15, 16]). However, they essentially abolish S1P-indced adrenoceptor phosphorylation as shown in Fig. 9.

As indicated previously, the use of the human α_{1B}-ARs tagged with the enhanced green fluorescent protein (eGFP) allows *in cellulo* receptor visualization. As depicted in Fig. 10, α_{1B}-AR-eGFP fluorescence was localized to the plasma membrane and in intracellular vesicles; the membrane marker FM4-64 (red) allowed precise membrane localization (merge, yellow). Addition of 10 μM NA or 1 μM S1P induces receptor internalization as evidenced by a marked decrease of α_{1B}-AR-eGFP/FM4-64 colocalization (Fig. 10) (quantitative analysis is presented as Supplementary Fig. S2). A video is also included, as supplementary material (V1) showing the effect of S1P on receptor location. S1P induced marked changes in cell shape and receptor mobilization from the plasma membrane to intracellular vesicles. It is interesting that, at some time points, the receptors appear to be located in “lanes” and clusters.

Cells transfected with a shRNA vector exhibited receptor internalization in response to S1P whereas those transfected with an $S1P_1$ receptor shRNA did not (Supplementary Fig. S3). In cells transfected with wild-type SPHK-1, both 1 μM S1P and 100 ng/ml IGF-I induced α_{1B}-AR internalization (Supplementary Fig. S4). In contrast, in cells transfected with the dominant-negative SPHK-1 only S1P, but not IGF-I, induced internalization (Supplementary Fig. S5).

4. Discussion

In this manuscript we show that activation of S1P receptors induced α_{1B}-AR desensitization and phosphorylation. PI3K and PKC, are key enzymes involved in this action. We also demonstrate that activation of receptor tyrosine kinases, such as IGF-I and EGF receptors increased SPHK activity. Such activation and the consequent production of S1P appear to be functionally relevant in IGF-I- and EGF-induced α_{1B}-AR phosphorylation and desensitization as evidenced by the following facts: a) expression of a dominant-negative

mutant of SPHK or b) $S1P_1$ receptor knockdown, markedly reduced this growth factor action. These aspects are diagrammatically presented in Fig.11. In addition, taking advantage of the presence of the eGFP tag added in receptor construction, we showed that S1P was capable of inducing α_{1B}-AR internalization and that its autocrine/paracrine generation was relevant for the receptor internalization induced by IGF-I.

IGF-I is a relatively small (70 amino acid) peptide that is mainly produced in the liver in response to growth hormone and that mediates the majority of its actions, including those on anabolism, growth and development [40]. IGF-I signals through type 1 transmembrane tyrosine kinase receptors similar to the insulin receptors [41]. These receptors phosphorylate members of the insulin receptor substrate family of proteins that bind and activate PI3K and this enzyme can lead to activation of PKC through formation of 3′-phosphorylated phosphoinositides which activate PDK-1 and Akt/PKB [42]. As mentioned in the introduction, IGF-I-induced α_{1B}-AR phosphorylation involves EGF receptor transactivation and also pertussis toxin-sensitive G proteins [16]. The present work clarifies that the action of IGF-I including the autocrine/paracrine generation of S1P and its action through the G protein-coupled receptor, $S1P_1$.

S1P induced a small increase in intracellular calcium that was not observed when cells were stimulated with IGF-I [15, 16]. These data indicate that differences exist between direct agonist addition to the media and accumulation through autocrine/paracrine generation. Calcium signaling does not appear to be required for the actions studied here, but we can not discount the possibility that there was a very small effect of IGF-I on basal intracellular calcium concentration that was not detected.

Our current model shows that in order to induce α_{1B}-AR desensitization and phosphorylation, $S1P_1$ receptor stimulation leads to G protein-mediated EGF receptor transactivation, i. e., metalloproteinase activation, HB-EGF shedding from the plasma membrane and stimulation of EGF receptors triggering. This was experimentally shown to be the case, as it has been observed for the actions of other G protein-coupled receptors, such as those for LPA and endothelin-1 and NA [9–11]. It is note worthy that there is already evidence indicating that S1P can induce EGF receptor transactivation (for example see [43–46]). Our data knocking down $S1P_1$ receptors suggest that these receptors play a major role. However, we are unable to discard the possibility that other S1P receptor subtypes could be involved (although to a lesser extent). In this regard, it is important to mention that it has been shown that S1P induced EGF transactivation through $S1P_3$ receptors [46].

The ability to observe the eGFP-tagged receptor *in cellulo* and in real-time allowed us to define the importance of S1P action in α_{1B}-AR internalization. We previously showed that IGF-I induced α_{1B}-AR internalization, which was blocked by hypertonic sucrose and concanavalin I [15]; our present results showed that S1P generation and action on $S1P_1$ receptors play a central role. In addition, a correlation was observed among the functional sensitivity of ARs, their phosphorylation state and the receptor internalization process.

We were surprised by the intense action of S1P on cell morphology, receptor internalization and vesicular traffic; it was stronger in magnitude than that of IGF-I. The formation of “lanes” and “clusters” of receptors was noticeable. This observation suggests that internalized vesicles containing α_{1B}-ARs interact with cytoskeletal elements. There is already evidence indicating that such interactions might take place. G protein-coupled estrogen receptor 1 (GPR30) traffics intracellularly on cytokeratin intermediate filaments [47] and the transport of α_{2B}-ARs from the endoplasmic reticulum to the cell surface is controlled through their association with tubulin [48]. Certainly much more additional work

will be required to define how α_{1B}-ARs internalize and the molecular elements that participate in this.

In summary, our present work adds significantly to the mechanisms that participate in IGF-I receptor-α_{1B}-AR crosstalk. Taking into account the present information and that previously published [16] it is clear that four different receptors (IGF-I receptors, α_{1B}-ARs, $S1P_1$ receptors and EGF receptors) and two autocrine/paracrine mediators (S1P and HB-EGF) participate in this crosstalk. How general this process is remains to be determined. These two paracrine mediators appear to play very general roles, allowing crosstalks of receptor tyrosine kinases and G protein-coupled receptors, and in this manner taking part in the fine tuning of cell responsiveness. It is important to remember that the final target, i. e. α_{1B}-ARs, is phosphorylated by a second messenger-activated kinase, i. e., PKC. In principle, other receptors (or channels, or transporters) with functionally relevant consensus sequences for PKC-mediated phosphorylation, could be subjected to similar processes. Identification of the molecular elements that participate in regulatory processes provide by default potential sites for therapeutic intervention. It is worth remembering that all of these receptors and autocrine/paracrine messengers take part in health maintenance and that their dysfunction might be relevant for the development of diseases [23, 40, 49–52].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

NA	noradrenaline
AR	adrenergic receptor
PKC	protein kinase C
PI3K	phosphoinositide 3-kinase
S1P	sphingosine 1-phosphate
S1P	sphingosine 1-phosphate
SPHK-1	sphingosine kinase-1
eGFP	enhanced green fluorescent protein

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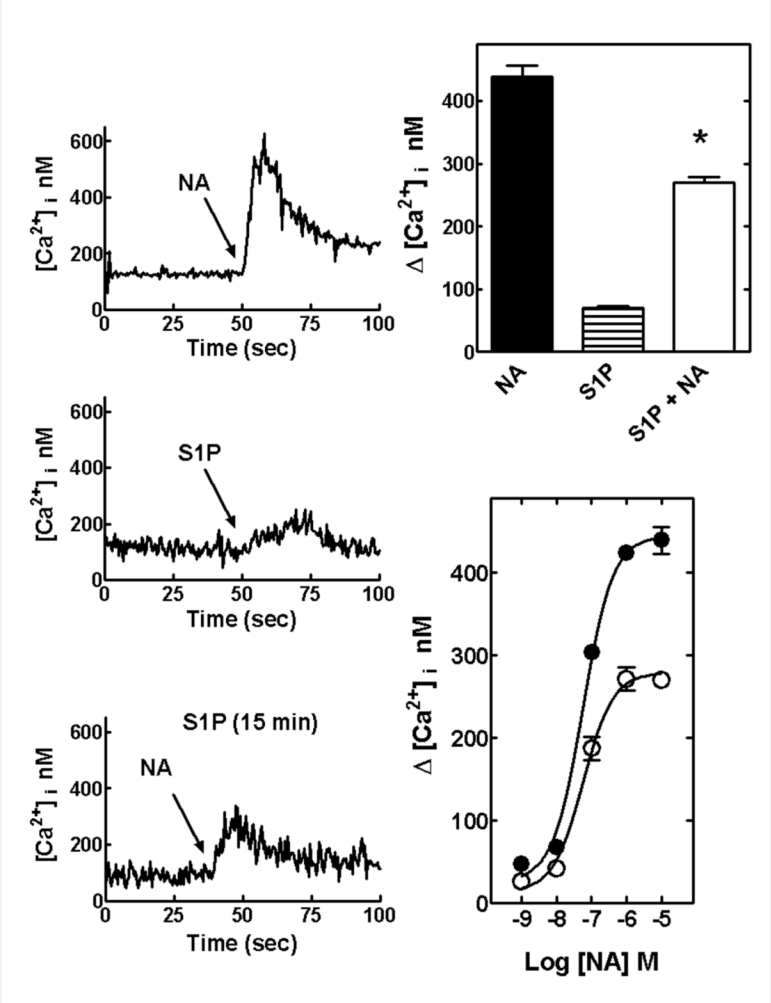

Fig. 1.

Effect of sphingosine-1-phosphate (S1P) on α_{1B}-adrenergic receptor (α_{1B}-AR) action. Left panels: representative tracings of intracellular free-calcium concentration ($[Ca^{2+}]_i$); upper tracing stimulation with 10 μM noradrenaline (NA), middle tracing with 1 μM S1P and lower tracing of cells preincubated with 1 μM S1P and challenged with 10 μM noradrenaline (NA). Upper right panel shows quantitative levels of intracellular calcium observed in cells challenged with 10 μM noradrenaline (NA), 1 μM S1P or 10 μM noradrenaline (NA) after a 15 min preincubation with 1 μM S1P (S1P + NA). Means are plotted with vertical lines representing the S.E.M. of 6 to 8 determinations using different cells preparations. Lower right panel: concentration- response curves of cells to noradrenaline (NA) preincubated for 15 min in the absence of any agent (solid circles) or presence of 1 μM S1P (open circles). Means are plotted with vertical lines representing the S.E.M. of 4 to 6 determinations using different cell preparations. * $p < 0.001$ vs. the other groups.

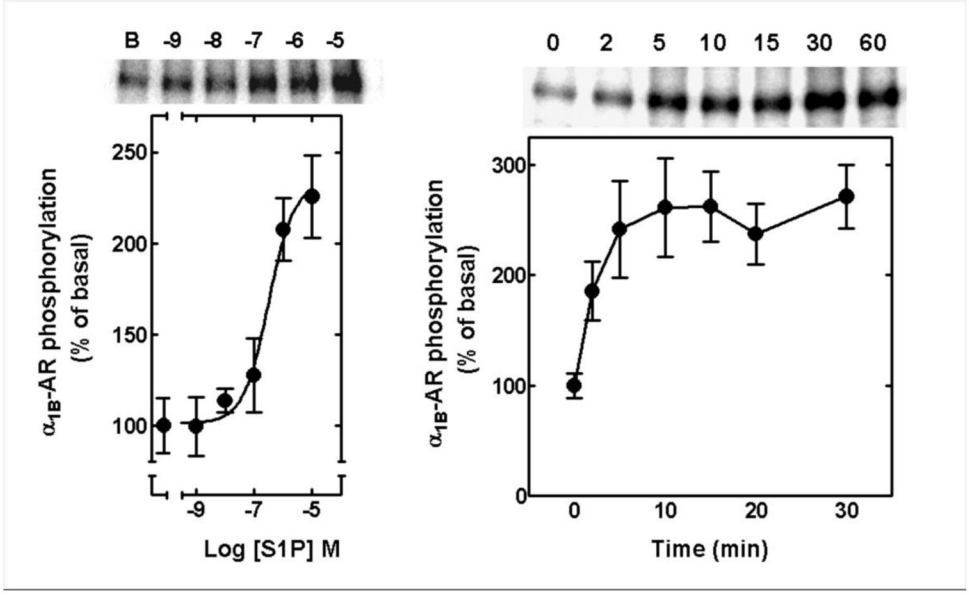

Fig. 2.

Effect of sphingosine-1-phosphate (S1P) on α_{1B}-adrenergic receptor (α_{1B}-AR) phosphorylation. Left panel: cells were incubated with the indicated concentration of SIP for 15 min. Right panel: cells were incubated in the presence of 1 μM S1P for the indicated times. Means are plotted with vertical lines representing the S.E.M. of 4 to 6 determinations using different cells preparations. Representative autoradiograms are shown. B, basal.

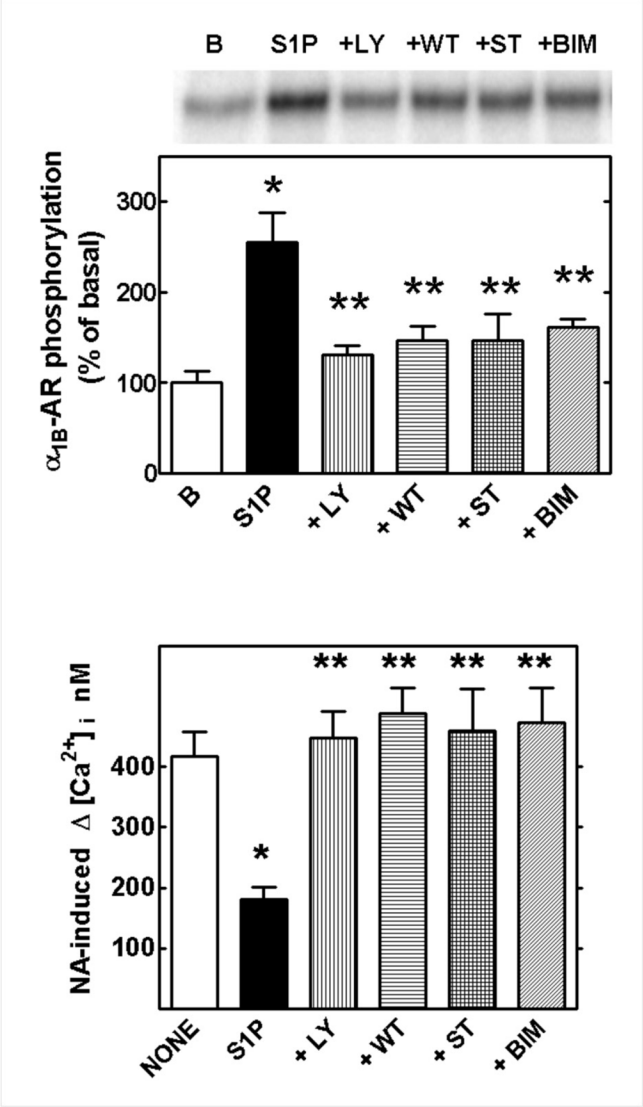

Fig. 3.

Effect of protein kinase C and phosphoinositide 3-kinase inhibitors on α_{1B}-adrenergic receptor (α_{1B}-AR) phosphorylation and action. Cells were preincubated in the absence or presence of the following inhibitors for 30 min 1μM LY294002 (+LY), 100 nM wortmannin (+WT), 100 nM staurosporine (+ST) or 1μM bis-indolyl-maleimide I (+BIM). To study (α_{1B}-AR) phosphorylation (upper panel), cells were incubated in the absence of any agent (B, basal) or challenged with 1 μM S1P (all of the remaining conditions) for 15 min. A representative autoradiogram is shown. To study the functional repercussion (lower panel): cells were preincubated with the inhibitors as indicated above and then no agent (NONE) or 1 μM S1P was added. The increase in intracellular calcium induced by 10 μM noradrenaline (NA) was recorded and the maximal effect observed is presented. Means are plotted with vertical lines representing the S.E.M. of 5 to 7 (receptor phosphorylation) or 12 to 15 (intracellular calcium) determinations using different cell preparations. *$p < 0.001$ vs basal (B, NONE), **$p < 0.001$ vs S1P.

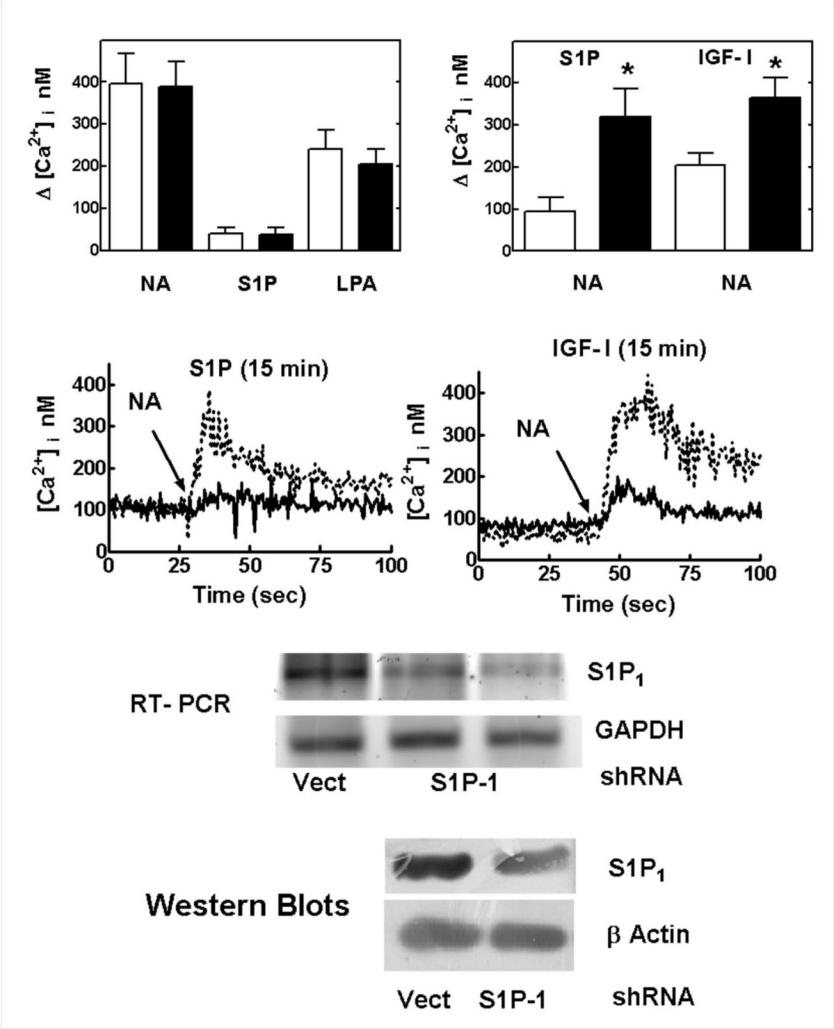

Fig. 4.

Effect of knocking down $S1P_1$ receptors on the α_{1B}-adrenergic receptor desensitization induced by S1P and IGF-I. Upper left panel: Cells transfected with the empty vector (open bars) or with $S1P_1$ shRNA (solid bars) were challenged with 10 μM noradrenaline (NA), 1 μM S1P or 1 μM lysophosphatidic acid (LPA). Upper right panel: Cells transfected with the vector (open bars) or with $S1P_1$ shRNA (solid bars) were preincubated for 15 min with 1 μM S1P or 100 ng/ml IGF-I and then challenged with 10 μM noradrenaline (NA). Means are plotted with vertical lines representing the S.E.M. of 5 to 10 determinations using different cell preparations. Middle panel: representative calcium tracings of intracellular free-calcium obtained from cells transfected with the empty vector (solid lines) or with $S1P_1$ shRNA (dotted lines); cells were preincubated with 1 μM S1P or 100 ng/ml IGF-I and then challenged with 10 μM noradrenaline (NA). Representative Reverse transcription (RT)-PCR determinations and Western blots are shown in the bottom panel.

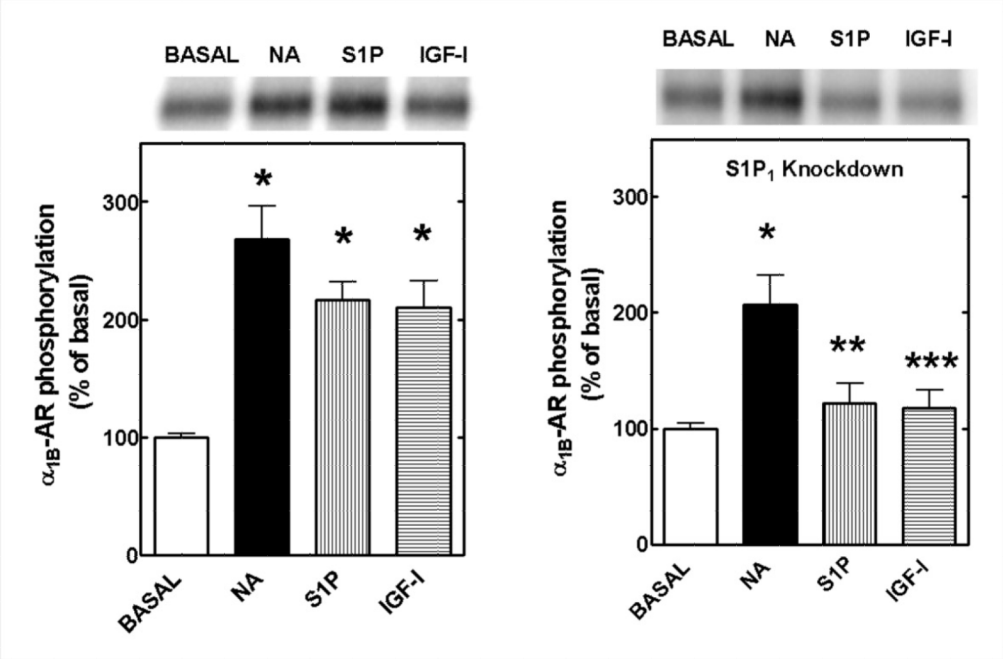

Fig. 5.

Effect of knocking down $S1P_1$ receptors on α_{1B}-adrenergic receptor (α_{1B}-AR) phosphorylation. Cells transfected with the empty vector (left panel) or with $S1P_1$ shRNA (right panel) were challenged with 10 μM noradrenaline (NA), 1 μM S1P or 100 ng/ml IGF-I. Means are plotted with vertical lines representing the S.E.M. of 6 to 9 determinations using different cell preparations. Representative autoradiograms are shown. *p <0.001 vs. basal; **p < 0.01 vs. S1P of vector-transfected cells; ***p <0.05 vs. IGF-I of vector-transfected cells.

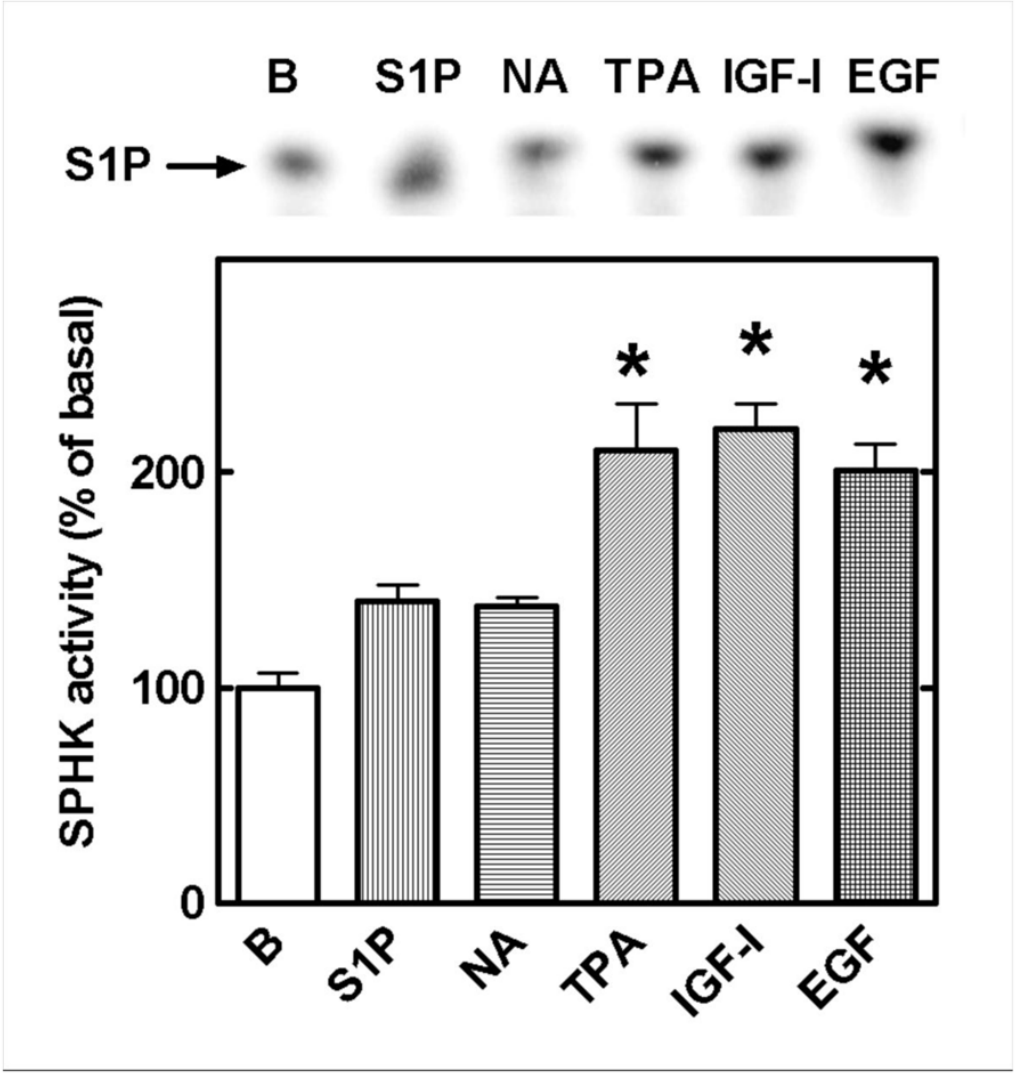

Fig. 6.

Sphingosine kinase (SPHK) activity in cell extracts. Cells were incubated for 30 min in the absence (B, basal) or presence of 1 μM S1P, 10 μM noradrenaline (NA), 1 μM tetradecanoyl phorbol acetate (TPA), 100 ng/ml IGF-I or 100 ng/ml EGF, extracts were prepared and enzyme activity assayed by determining sphingosine phosphorylation. Means are plotted with vertical lines representing the S.E.M. of 6 to 10 determinations using different cell preparations. A representative autoradiogram is presented. $p < 0.001$ vs basal (B).

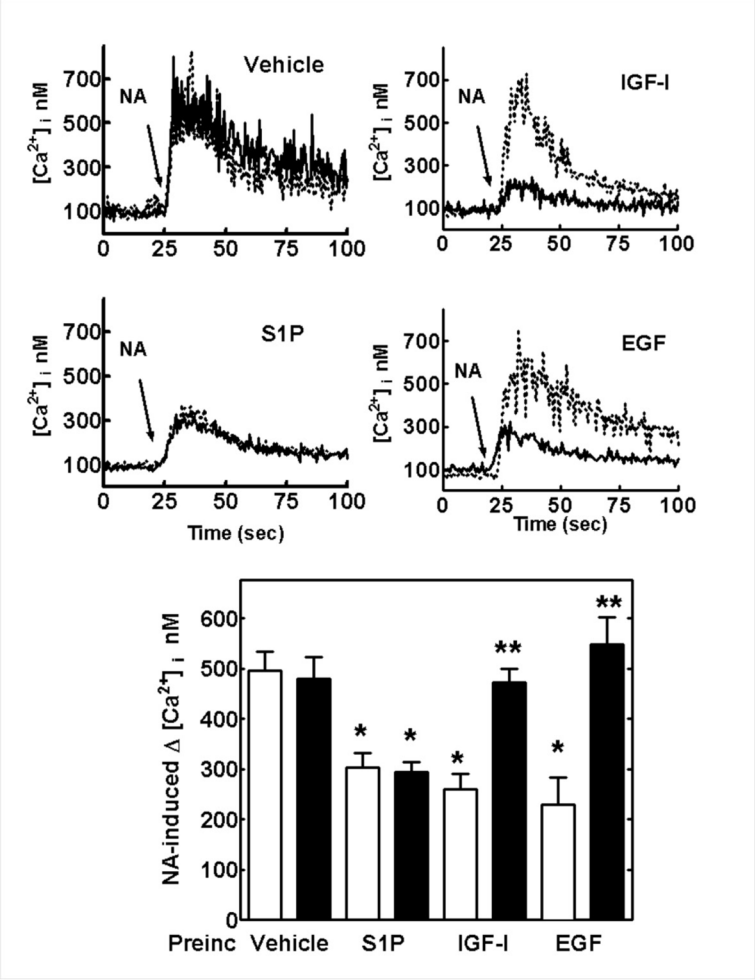

Fig. 7.

Effect of sphingosine kinase-1 (SPHK-1) dominant-negative expression on α_{1B}-adrenergic receptor desensitization induced by S1P and IGF-I. Upper and middle panels: representative calcium tracings of intracellular free-calcium obtained from cells transfected with wild type SPHK-1 (solid lines) or with the dominant-negative mutant (dotted lines); cells were preincubated for 15 min with vehicle (upper left panel) 1 μM S1P (middle left panel) 100 ng/ml IGF-I (upper right panel) or 100 ng/ml EGF (middle right panel) and then challenged with 10 μM noradrenaline (NA). Lower panel: Noradrenaline (10 μM)-induced increase in intracellular calcium of cells treated as described above; cells transfected with wild type SPHK-1 (open bars) or with the dominant-negative mutant (solid bars). Means are plotted with vertical lines representing the S.E.M. of 10 to 15 determinations using different cell preparations. *$p < 0.001$ vs. respective vehicle group; **$p < 0.001$ vs its respective EGF or IGF-I (wild type SPHK-1) group.

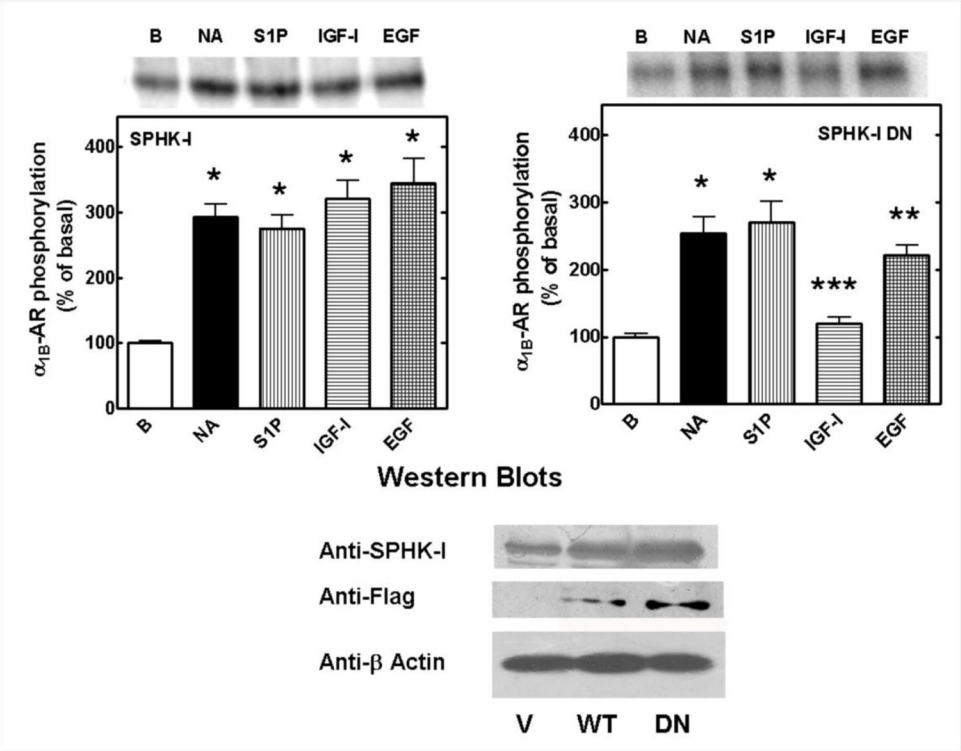

Fig. 8.

Effect of sphingosine kinase-1 (SPHK-1) dominant-negative expression on α_{1B}-adrenergic receptor (α_{1B}-AR) phosphorylation. Cells transfected with wild type SPHK-1 (left panel) or with the dominant-negative mutant (right panel) were challenged with vehicle (B, basal), 10 μM noradrenaline (NA), 1 μM S1P, 100 ng/ml IGF-I μM or 100 ng/ml EGF. Means are plotted with vertical lines representing the S.E.M. of 5 to 6 determinations using different cell preparations. Representative autoradiograms are shown. *p <0.001 vs. basal; **p < 0.01 vs. basal. p< 0.05 vs. EGF (wild type SPHK-1) group ***p < 0.01 vs IGF-I (wild type SPHK-1) group. Representative Western blots are shown in the bottom panel

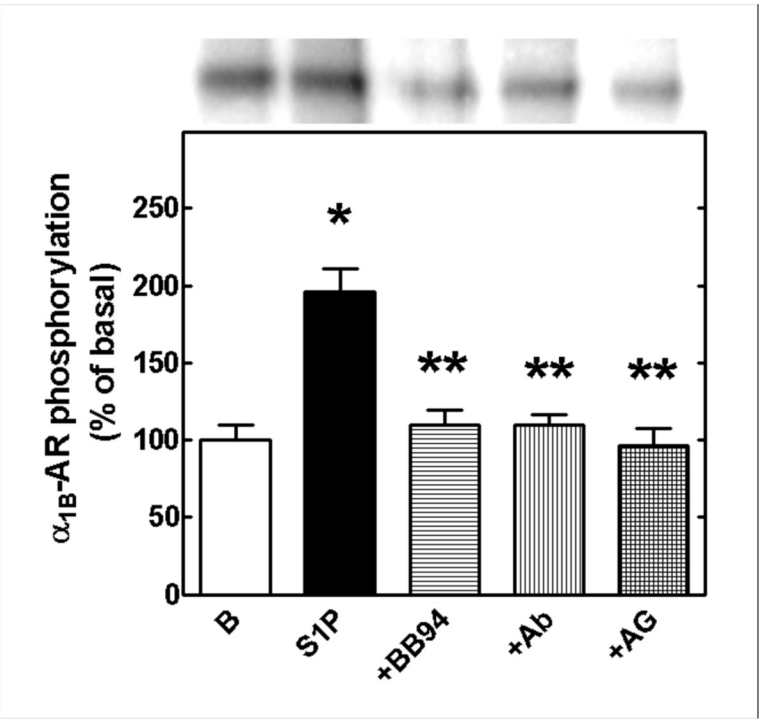

Fig. 9.

Effect of inhibitors of EGF receptor transactivation on sphingosine-1-phosphate (S1P)-mediated α_{1B}-adrenergic receptor (α_{1B}-AR) phosphorylation. Cells were preincubated for 30 min in the absence or presence of 10 μM BB94, 5 μg/ml of neutralizing anti HB-EGF antibody or 5 μM AG1478 and then further incubated for 15 min in the presence of 1 μM S1P. Means are plotted with vertical lines representing the S.E.M. of 6 determinations, using different cell preparations. * $p < 0.001$ vs. basal; ** $p < 0.001$ vs S1P alone. A representative autoradiograph is presented.

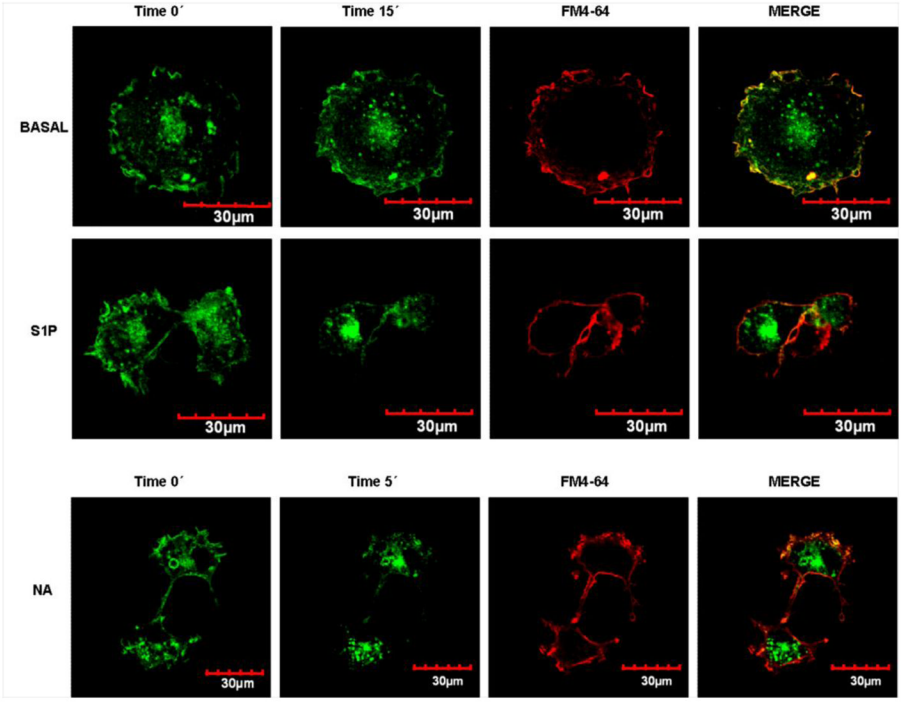

Fig. 10.

Confocal microscopy images of cells expressing α_{1B}-adrenergic receptor-enhanced green fluorescent protein construction. Images were taken at the beginning of incubation (Time 0′) and at the end of the incubation with the reagents (Time 5′ or Time 15′); immediately thereafter, membrane marker FM4-64 (red) was added and images were taken. Merged images (end of incubation-FM4-64) are presented for colocalization (yellow). Cells were incubated in the absence of any agent (BASAL) or presence of 1 μM S1P or 10 μM noradrenaline (NA). Images are representative of 3 to 4 observations using different cell preparations.

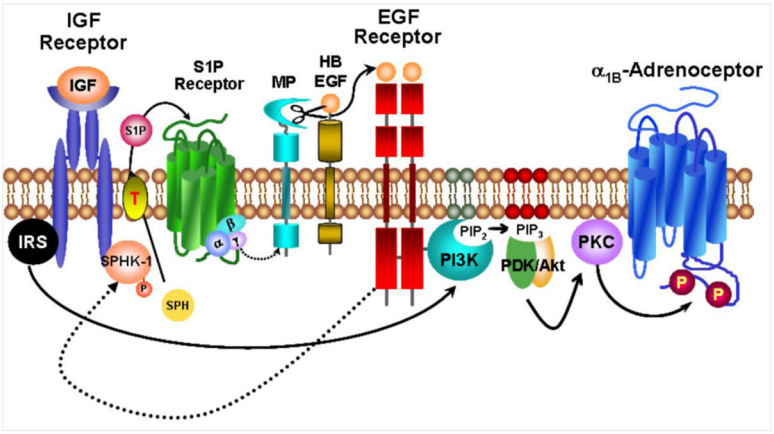

Fig. 11.

Model for IGF-I receptor-mediated α_{1B}-adrenergic receptor (α_{1B}-AR) phosphorylation. IGF, insulin-like growth factor I; IRS, insulin receptor substrate; SPHK1, sphingosine kinase 1; SPH, sphingosine; S1P, sphingosine-1-phosphate; MP, metalloproteinase; αβγ, G protein subunits; HB-EGF, heparin-binding EGF; EGF, epidermal growth factor; PI3K, phosphoinositide 3-kinase; PIP2, phosphatidylinositol (4,5)- bisphosphate; PIP3, phosphatidylinositol (3,4,5)- trisphosphate; PDK, phosphoinositide-dependent kinase; Akt, protein kinase B; PKC, protein kinase C; T, transporter.