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Exposure to TARC alters β_2-adrenergic receptor signaling in human peripheral blood T lymphocytes

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Heijink, Irene H., Edo Vellenga, Jaap Oostendorp, Jan G. R. de Monchy, Dirkje S. Postma, and Henk F. Kauffman. Exposure to TARC alters β_2-adrenergic receptor signaling in human peripheral blood T lymphocytes. *Am J Physiol Lung Cell Mol Physiol* 289: L53–L59, 2005. First published March 4, 2005; doi:10.1152/ajplung.00357.2004.—The β_2-adrenergic receptor (β_2-AR) negatively regulates T cell activity through the activation of the G_s/adenylyl cyclase/cAMP pathway. β_2-AR desensitization, which can be induced by its phosphorylation, may have important consequences for the regulation of T cell function in asthma. In the present study we demonstrate that the C-C chemokine thymus and activation-regulated chemokine (TARC) impairs the ability of β_2-agonist fenoterol to activate the cAMP downstream effector cAMP-responsive element binding protein (CREB) in freshly isolated human T cells. The TARC-induced activation of Src kinases resulted in membrane translocation of both G protein-coupled receptor kinase (GRK) 2 and β-arrestin. Moreover, TARC was able to induce Src-dependent serine phosphorylation of the β_2-AR as well as its association with GRK2 and β-arrestin. Finally, in contrast to CREB, phosphorylation of Src and extracellular signal-regulated kinase was enhanced by fenoterol upon TARC pretreatment. In summary, we show for the first time that TARC exposure impairs β_2-AR function in T cells. Our data suggest that this is mediated by Src-dependent activation of GRK2, resulting in receptor phosphorylation, binding to β-arrestin, and a switch from cAMP-dependent signaling to activation of the MAPK pathway. We propose that aberrant T cell control in the presence of endogenous β-agonists promotes T cell-mediated inflammation in asthma.

cAMP-responsive element binding protein phosphorylation; Src kinases; G protein-coupled receptor kinase 2; β-arrestin; thymus and activation-regulated chemokine

THE β_2-ADRENERGIC RECEPTOR (β_2-AR) is expressed on a variety of cell types, including T lymphocytes, and belongs to the family of cell surface receptors referred to as G protein-coupled receptors (GPCR). Upon binding of β-agonists (e.g., epinephrine) to their cognate receptor, the β_2-AR associates with the G_s protein, which triggers adenylyl cyclase (AC) activity and subsequently results in the formation of intracellular cAMP. The cAMP-dependent pathway is widely known as a negative regulator of T cell activity. Upon prolonged or repeated agonist stimulation, uncoupling of the β_2-AR from the AC/cAMP system can be induced. This desensitization is regulated by receptor phosphorylation and subsequent receptor internalization, which proceeds slower than phosphorylation. Internalization is not strictly required for β_2-AR desensitization and is thought to be an early step in β_2-AR downregulation and/or resensitization (25, 33, 41). Phosphorylation of the β_2-AR can be induced by several kinases. Most described candidates are the G-coupled receptor kinases (GRK) and protein kinase A (PKA), which are activated upon stimulation of the β_2-AR (13). β_2-AR phosphorylation mediated by GRK2 and GRK3, also called βARK1 and βARK2, creates a binding site for β-arrestin (3, 13, 17, 23), which inhibits β_2-AR function by preventing association with the G_s protein (17). Furthermore, β-arrestin has been described to recruit c-Src to the receptor, thereby linking the receptor to Ras/Raf/extracellular signal-regulated kinase (ERK) activation (24). In addition to βARK and PKA, various heterologous stimuli (e.g., growth factors and cytokines) have been described to phosphorylate the β_2-AR, for example by the activation of PKC, tyrosine kinases, or phosphatidylinositol 3-(PI3)-kinase (35, 28, 12, 9).

Because the β_2-AR system functions to suppress T cell-mediated responses, β_2-AR desensitization may have important implications for the pathophysiology of atopic asthma. Impaired function of the β_2-AR system has been observed in peripheral blood lymphocytes during allergen-induced inflammatory reactions in asthma (7, 8, 26). The origin of this β_2-adrenergic dysfunction is still largely unclear; however, it is unlikely to be caused by enhanced endogenous β_2-agonist levels (21). It has been proposed that the release of proinflammatory mediators from the lung into the circulation is responsible for the β_2-AR dysfunction in peripheral lymphocytes in asthma (27). In this respect, chemokines may be of interest, since the release of certain chemokines [e.g., C-C chemokine thymus and activation-regulated chemokine (TARC)] is thought to play a crucial role in airway inflammation in asthma. There is growing evidence that TARC plays an important role in the recruitment of T helper (Th) 2 cells to the lung tissue and that TARC is released upon allergen challenge in asthma (4, 6, 18, 22, 29, 34), whereas the role of other Th2-directing chemokines appears less important (4, 6, 29, 34). Most chemokine receptors are linked to the G_i protein. Activation of G_i proteins can induce Rho GTPases, PI3-kinase, and Src/son of sevenless (SOS)-dependent signaling, leading to changes in the cytoskeleton and cellular responses required for directional migration (14). Additionally, G_i activity is known to counterregulate the function of the G_s protein and has been implicated in β_2-AR dysfunction (39). Moreover, chemokines have been demonstrated to induce activation of GRKs and β-arrestin, which may be critical for cytoskeletal reorganization and migration (5, 11, 37). We hypothesized that this may not only induce cytoskeletal

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changes and homologous desensitization of chemokine receptors (1), but also β_2-AR desensitization. To investigate this, we analyzed the effects of TARC on β_2-AR signaling in freshly isolated human T cells.

MATERIALS AND METHODS

Isolation of the T cells. Peripheral blood mononuclear cells from healthy volunteer platelet donors, of whom for ethical reasons no further information was available, were isolated by Ficoll-Hypaque (Lymphoprep; Nycomed, Oslo, Norway) density-gradient centrifugation. The Human Subject Review Board of the University of Groningen has approved the protocol. T cells were isolated by rosetting with 2-aminoethylisothioronium bromide-treated sheep red blood cells (SRBC). The SRBC were lysed with 155 mmol/l NH_4Cl, 10 mmol/l $KHCO_3$, and 0.1 mmol/l EDTA. The remaining cell preparations contained >98% T cells, as assessed by flow cytometric analysis after staining with α-CD2 (Becton Dickinson, Erebodegem-Aalst, Belgium). T cells were incubated overnight at 37°C in RPMI 1640 (BioWhittaker, Verviers, Belgium) containing 0.5–1% fetal calf serum (FCS; Hyclone, Logan, UT), supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin.

Immunodetection by Western blotting. The phosphorylation of cAMP-responsive element binding protein (CREB), ERK, and Src family kinases was analyzed by Western blotting. T cells were pretreated with 10 μM PP2 (Calbiochem, Omnilabo International, Breda, The Netherlands) or 100 ng/ml pertussis toxin (PTX, Calbiochem, Omnilabo International) for 1 and 3 h, respectively, and subsequently incubated with or without TARC (250 pg/ml to 100 ng/ml) for 4–6 h. Next, cells were stimulated with 10 μM fenoterol (Sigma, St. Louis, MO) or 10 mM NaF (Sigma) for 0, 10, 30, 60, 90, or 180 min. Cells were harvested and spun down at maximum speed for 30 s. Total cell lysates were obtained by resuspension of the pellets in 1× sample buffer (containing 2% SDS, 10% glycerol, 2% 2-mercaptoethanol, 60 mM Tris·HCl pH 6.8, and bromphenol blue) and boiling for 5 min. Samples were loaded on an SDS 10% PAGE gel (acrylamide-bisacrylamide 173:1) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). Immunodetection of phospho-CREB, phospho-ERK, phospho-Src (New England Biolabs, Hitchin, Herts, UK), pan-ERK or actin (Santa Cruz Biotechnology, Santa Cruz, CA) was performed by standard procedures, and the detection was performed according to the manufacturer's guidelines (ECL; Amersham, Buckinghamshire, UK). Relative protein levels were quantified using the gelscan program ImageMaster (Pharmacia, Uppsala, Sweden) and normalized for total protein levels.

cAMP assay. T cells were rested overnight in RPMI 1640 containing 2% FCS, preincubated with or without TARC (100 ng/ml) for 6 h, and incubated at 2.5×10^6 cells/300 μl with 100 μM 3-isobutyl-1-methyl-xanthine (IBMX) to prevent cAMP degradation for 10 min at 37°C. Next, T cells were stimulated with 10 μM fenoterol for 10 min. The reactions were terminated with 300 μl ice-cold 3.5% perchloric acid and placed on ice for at least 30 min. After centrifugation of precipitated protein, the samples were neutralized by the addition of 180 μl of 50% saturated $KHCO_3$. cAMP levels were measured by a competitive protein-binding assay as described previously (36). In short, 200 μl of sample were incubated with 200 μl of buffer containing 50 mM Tris·HCl, 4 mM EDTA, 160 μg cAMP binding protein, 1 mg bovine serum albumin, and 190 nM [^{3}H]cAMP (30 Ci/mmol) at 4°C for at least 2 h. The reaction was terminated by adding 1,000 μl of charcoal suspension (Norit A special, 3.5 g/l) followed by centrifugation at 3,000 rpm for 15 min to remove the excess of unbound [^{3}H]cAMP. Radioactivity in the supernatant was measured by scintillation counting.

Migration assay. The migration assay was performed using a microchamber Transwell system with 5-μM pores (Corning Costar). T cells were incubated in RPMI 1640/1% FCS with or without 10 μM PP2 and 100 ng/ml PTX for 1 and 3 h, respectively. Next, 2×10^5 T cells were applied to the upper compartment of the chamber. Migration was induced by 5, 20, 100, or 500 ng/ml TARC (R&D Systems, ITK diagnostics, Uithoorn, the Netherlands) in RPMI 1640/1% FCS present in the lower compartment of the chamber. T cells were allowed to migrate to the lower compartment for 2 h at 37°C. The upper well was removed, and the T cells in the lower well were counted and expressed as percentage of the total amount of T cells added to the upper well.

Cytosolic and membrane fractionation. T cells (10×10^6) were pretreated with PP2 (10 μM) or SU-6656 (1 μM) for 1 h in RPMI 1640/0.5% FCS and subsequently incubated with or without TARC for 1 to 4 h. Cells were harvested and washed with ice-cold PBS containing 1 mM sodium orthovanadate and 1 mM PMSF and lysed in 200 μl of hypotonic lysis buffer [10 mM Tris pH 7.4, 1.5 mM $MgCl_2$, 5 mM KCl, 1 mM DTT, 1 mM sodium orthovanadate, 10 U/ml aprotonin, 1 μM pepstatin, Complete (Roche, Basel, Switzerland), and 1 mM PMSF] at 4°C. Cell lysates were homogenized by sonification. After the removal of intact cells, nuclei, and cell debris by centrifugation at 800 *g* for 8 min at 4°C, the membrane fractions were spun down at maximum speed for 60 min at 4°C. The cytosol fraction was suspended in 5× sample buffer, boiled for 5 min, and stored at −80°C. The sedimented membrane fraction was washed with hypotonic lysis buffer, spun down at maximum speed for 30 s, and resuspended in 1× sample buffer, boiled for 5 min, and stored at −80°C. Samples were loaded on SDS 10% PAGE gels and blotted to a PVDF membrane. Immunodetection was performed with antibodies against βARK1/GRK2, β-arrestin-2, IL-1R, and tubulin (Santa Cruz Biotechnology) using ECL according to the manufacturer's guidelines.

Immunoprecipitation. T cells (10×10^6) were pretreated with 10 μM PP2 for 1 h in RPMI 1640/0.5% FCS and subsequently incubated with TARC (100 ng/ml) for 1–4 h. Cells were harvested, washed with ice-cold PBS containing 1 mM sodium orthovanadate and 1 mM PMSF, and subsequently lysed in 500 μl of lysis buffer (50 mM Tris, pH 7.4, 10% glycerol, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM DTT, 1 μM pepstatin, 1 μg/ml aprotonin, Complete, 1 mM PMSF, and 1 mM sodium orthovanadate) for 10 min on ice. Cell lysates were clarified at 10,000 *g* for 15 min and precleared with 30 μl of protein A Sepharose beads (in a 50% slurry) for 1 h at 4°C. After 1-min centrifugation at 300 *g*, the cell lysates were subjected to immunoprecipitation with antibodies specific for β_2-AR, β-arrestin-2 (Santa Cruz Biotechnology), or GRK2-agarose conjugate (GRK2-AC, Santa Cruz Biotechnology) and incubated rotating at 4°C for 3 h or overnight. In case of immunoprecipitation with β_2-AR and β-arrestin, protein A Sepharose beads were added to each sample and incubated rotating overnight to bind to the primary antibodies. Next, immune complexes were washed three times with lysis buffer. The precipitates were subjected to SDS 10% PAGE, immunoblotted on PVDF membrane, and immunocomplexes were detected by ECL, which was performed according to the manufacturer's guidelines. The phosphoserine antibody was purchased from Zymed Laboratories (SANBIO, Uden, The Netherlands).

Statistical analyses. We used the Wilcoxon signed-rank test for paired observations in case of the cAMP measurements. The Student's *t*-test for paired observations was used in case of the protein levels or measurements of T cell migration. Data are expressed as means ± SE. Statistical significance of the secretion data was set at $P < 0.05$.

RESULTS

TARC preincubation reduces the ability of β_2-agonist fenoterol to activate CREB. We first examined whether TARC is able to induce alterations in β_2-AR signaling by studying the effect on cAMP downstream effector CREB in freshly isolated T cells. By flow cytometry we observed that 18.5 ± 6% of the peripheral T cells from healthy donors expresses the TARC

receptor CCR4 ($n = 4$, data not shown). In freshly isolated T cells from healthy donors, incubation with fenoterol (10 μM) resulted in a clear induction of CREB phosphorylation. This was observed after just 10 min of stimulation, with a maximal response (fivefold induction) between 60 and 90 min (Fig. 1*A*). Incubation with TARC alone for 60 min (data not shown) or 6 h (Fig. 1*B*) had no effect on CREB phosphorylation. When T cells were preincubated with TARC for 6 h and subsequently stimulated with fenoterol for 60 min, the ability of fenoterol to induce CREB phosphorylation was markedly reduced (approximately twofold, Fig. 1*B*). This effect of TARC was also observed at a concentration of 250 pg/ml. At 50 pg/ml, the suppressive effect of TARC could no longer be observed (Fig. 1*C*). The effect of TARC may not be mediated by direct inhibition of the G_s protein, since 5 min of preincubation with TARC did not alter the ability of fenoterol to induce CREB phosphorylation (Fig. 1*B*). To investigate whether TARC directly affects the AC system or induces alterations at receptor level, we used NaF for direct activation of the G protein. NaF (10 mM) induced a strong increase in CREB phosphorylation (Fig. 1*A*), which was not reduced upon preincubation with TARC (Fig. 1*D*). This suggests that the effect of TARC is mediated upstream from the G_s protein at the receptor level, probably by reducing the association of the β_2-AR with the G_s/AC/cAMP pathway.

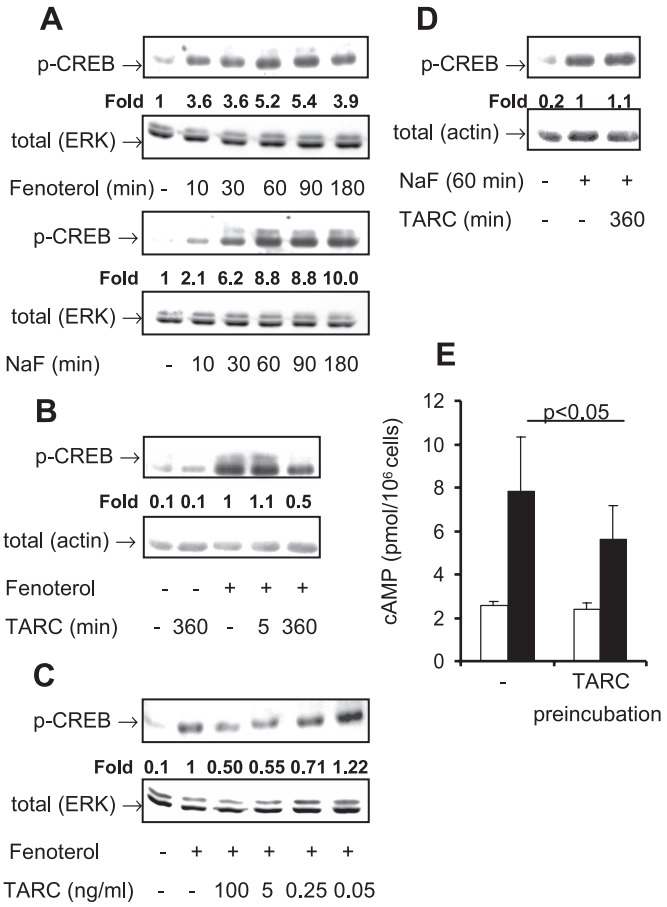

Fig. 1. The ability of β_2-agonist fenoterol and NaF to induce cAMP-dependent signaling. Freshly isolated T cells were stimulated with fenoterol or NaF for 0, 10, 30, 60, 90, or 180 min. Total cell lysates were prepared, and phosphorylated cAMP-responsive element binding protein (p-CREB) was detected by Western blotting ($n = 2$) (*A*). T cells were preincubated with 100 ng/ml thymus and activation-regulated chemokine (TARC) for 5 min or 6 h and subsequently stimulated with fenoterol for 60 min. Total cell lysates were prepared, and p-CREB was detected by Western blotting ($n = 3$) (*B*). T cells were preincubated with 50 pg/ml, 250 pg/ml, 5 ng/ml, or 100 ng/ml TARC for 6 h and subsequently stimulated with fenoterol for 60 min. Total cell lysates were prepared, and p-CREB was detected by Western blotting ($n = 3$) (*C*). T cells were incubated with TARC for 6 h and subsequently stimulated with NaF for 60 min. Total cell lysates were prepared and p-CREB was detected by Western blotting ($n = 3$) (*D*). p-CREB is depicted in the *top* panels and total ERK or actin levels are shown in the *bottom* panels as a control for equal loading (marked by arrows). Representative blots are shown. T cells were pretreated with or without TARC (100 ng/ml) for 6 h, incubated with IBMX for 10 min, and subsequently stimulated with fenoterol for 10 min. Intracellular cAMP accumulation was measured by a competitive protein-binding assay ($n = 8$) (*E*).

TARC preincubation reduces the ability of β_2-agonist fenoterol to induce cAMP formation. To test whether TARC indeed reduces β_2-AR-coupled AC activity, we studied intracellular cAMP formation by β_2-AR stimulation upon TARC pretreatment. In freshly isolated T cells, we observed that 10 μM fenoterol induced a significant (approximately threefold) increase in intracellular cAMP accumulation after 10 min of stimulation (Fig. 1*E*). When T cells were first incubated with TARC for 6 h and then stimulated with fenoterol, cAMP accumulation was significantly reduced (by ~25%, $P < 0.05$, $n = 6$), whereas TARC preincubation had no significant effect on basal cAMP levels (Fig. 1*E*). This indicates that TARC preincubation reduces β_2-AR coupling to the G_s/AC/cAMP system.

Immunoblotting and RT-PCR studies demonstrated no change in β_2-AR expression and β_2-AR mRNA levels, indicating that TARC does not alter β_2-AR function by degradation or downregulation of receptor expression (data not shown).

TARC induces G_i activation and Src kinase activity. To further identify the mechanism of TARC-induced desensitization of the β_2-AR, we investigated the pathways induced by TARC stimulation. Most chemokine receptors are coupled to G_i proteins, leading to $G_{\beta\gamma}$ release and Src activation upon chemokine binding (14). We tested the involvement of the G_i protein and Src family kinases in TARC-induced T cell migration by using the G_i inhibitor PTX and the selective Src family kinase inhibitor PP2 in the migration assay. As depicted in Fig. 2*A*, T cells show some spontaneous migration, but chemotaxis is considerably enhanced in the presence of TARC, with a maximal response at 100 ng/ml (3.5-fold induction). Preincubation of the cells with both PTX and PP2 blocked the migratory response to TARC (Fig. 2*B*), indicating the requirement of G_i signaling and Src kinase activation.

In Fig. 3*A* the involvement of Src family kinases is further supported, showing an increase in Src kinase phosphorylation in T cells after 5 min of TARC stimulation. Phosphorylated Src was completely absent in the presence of PP2 (Fig. 3*A*). We used PTX in these experiments to underscore the involvement of G_i activity. The TARC-induced Src phosphorylation could be blocked by treatment with PTX (Fig. 3*B*). In accordance, PTX and PP2 pretreatment blocked the TARC-induced phosphorylation of ERK (data not shown).

Activity of Src family kinases is critically involved in TARC-induced β_2-adrenergic dysfunction. Next, we studied whether or not Src kinase activation is critical for the suppressive effect of TARC on fenoterol-induced CREB phosphorylation. To establish the role of Src kinases in this process, T cells were preincubated with PP2, followed by exposure to TARC. As demonstrated in Fig. 3*C*, the fenoterol-induced phosphoryla-

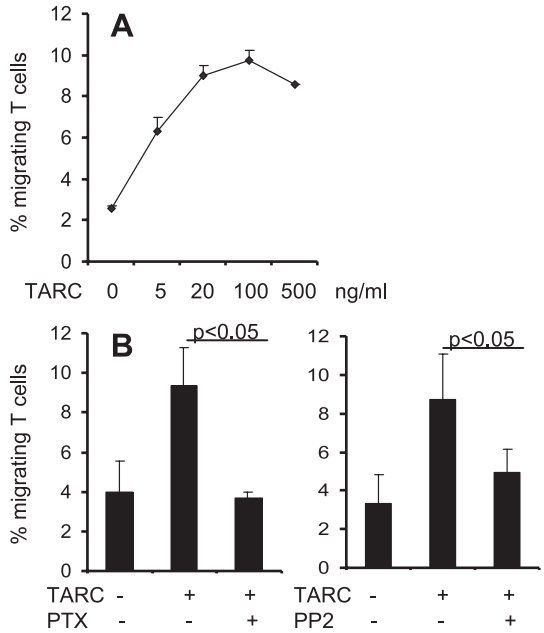

Fig. 2. TARC-induced T cell migration. T cells were added to the upper well of a Transwell system. Migration was induced by the presence of 0, 5, 20, 100, or 500 ng/ml TARC in the lower well (*A*). Migrating T cells are expressed as percentage of the total amount of T cells added to the upper well. T cells were incubated with or without Src inhibitor PP2 or G_i inhibitor pertussis toxin (PTX) for 1 and 3 h, respectively. Migration was induced by the presence of 100 ng/ml TARC. T cell migration is expressed as percentage of the total amount of T cells added to the upper well (*B*). Experiments were carried out in duplicate with T cells from 4 healthy donors, and means ± SE are shown.

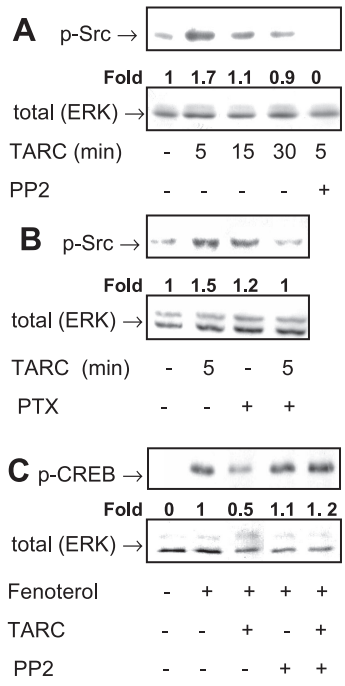

Fig. 3. TARC induces G_i-dependent activation of the Src kinases and this is involved in the suppressive effect on β_2-adrenergic receptor (AR) function. Freshly isolated T cells were stimulated with TARC (100 ng/ml) for 5, 15, and 30 min. One hour before stimulation, PP2 was added. Total cell lysates were prepared and phosphorylated (p)-Src was detected by Western blotting ($n = 3$) (*A*). T cells were stimulated with TARC (100 ng/ml) for 5 min. Before stimulation, PTX was added. Total cell lysates were prepared. p-Src was detected by Western blotting ($n = 5$) (*B*). Freshly isolated T cells were preincubated with 100 ng/ml TARC for 6 h. Before TARC incubation, PP2 was added. Next, T cells were stimulated with fenoterol for 60 min. Total cell lysates were prepared, and p-CREB was detected by Western blotting ($n = 4$) (*C*). p-Src and p-CREB are depicted in the *top* panels, and total ERK levels are shown in the *bottom* panels (marked by arrows).

tion of CREB was suppressed by TARC (0.51 ± 0.03-fold, $n = 4$), yet this was no longer observed when T cells were pretreated with PP2 (1.1 ± 0.16-fold, $n = 4$). This indicates that Src kinase activity in response to TARC is critically involved in the suppressive effect of TARC on the fenoterol-induced activation of the cAMP-dependent pathway. PP2 incubation alone did not result in stronger fenoterol-induced CREB phosphorylation or higher basal CREB phosphorylation. This suggests that fenoterol does not induce (sufficient) Src activation to reduce β_2-AR signaling (at least not within 60 min) and that basal Src activity does not affect β_2-AR function.

TARC induces Src kinase-dependent membrane translocation of GRK2 and β-arrestin. It is conceivable that Src kinase activity is involved in β_2-AR desensitization, since Src kinases may phosphorylate and subsequently activate GRK2 (10, 33). Therefore, we tested whether TARC was able to induce Src-dependent activation and membrane translocation of GRK2. In resting T cells, GRK2 was localized in both cytosol and membrane fractions. TARC exposure reduced the expression of GRK2 in the cytoplasm and increased the expression in the membrane fraction (Fig. 4). As control for the purity of the subcellular fractions, IL-1R was used as membrane marker, and tubulin was used as cytosol marker. The increased membrane translocation of GRK2 was already seen after 1 h of TARC exposure (data not shown) and remained for at least up to 4 h (Fig. 4). The effect of TARC on GRK2 location was inhibited by both PP2 (Fig. 4) and the selective Src inhibitor SU-6656 (data not shown), indicating that TARC-induced Src kinase activity is responsible for the membrane translocation of GRK2. Interestingly, incubation with TARC triggered the membrane translocation of β-arrestin as well (Fig. 4), which was again blocked by PP2 pretreatment. This membrane translocation of GRK2 and β-arrestin may be involved in the TARC-induced effect on β_2-AR function.

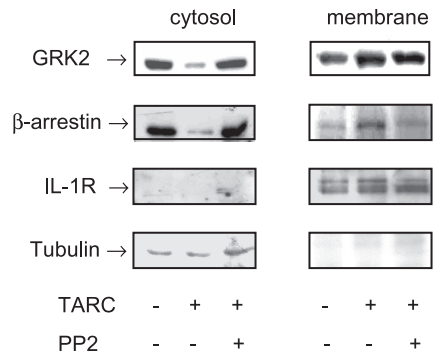

Fig. 4. TARC exposure induces Src kinase-dependent membrane translocation of G-coupled receptor kinase (GRK) 2 and β-arrestin. Freshly isolated T cells were challenged with 100 ng/ml TARC for 4 h. Before TARC incubation, PP2 was added. Cells were harvested and subjected to subcellular fractionation. The expression of β-arrestin and GRK2 in cytosol and membrane fractions was detected by Western blotting and is indicated by arrows. IL-1R and tubulin indicate purity of the membrane and cytosolic fractionation and were used to establish equal loading. A representative blot of 4 independent experiments is shown.

TARC exposure induces serine phosphorylation of the β_2-AR and association to GRK2 and β-arrestin. Next, we questioned whether the effects of TARC as described above lead to alterations in the phosphorylation status of the β_2-AR. To this end, we determined the phosphoserine content of the β_2-AR after immunoprecipitation. As demonstrated in Fig. 5*A*, the levels of serine-phosphorylated β_2-AR were increased by ~1.5-fold when T cells were exposed to TARC (3 h), which was observed in absence of a β_2-agonist. In addition, we studied the association of GRK2 and β_2-AR by coimmunoprecipitation. As depicted in Fig. 5*B*, the association between β_2-AR and GRK2 was markedly increased when T cells were exposed to TARC, supporting a role for GRK2 in the β_2-AR serine phosphorylation. Furthermore, by coimmunoprecipitation of β-arrestin and β_2-AR we observed that TARC induces recruitment of β-arrestin to the β_2-AR (Fig. 5*C*). In accordance to the effects as described in the previous paragraphs, the TARC-induced β_2-AR phosphorylation and recruitment of βARK and β-arrestin were blocked by PP2 (Fig. 5, *A–C*). Together, these data suggest that the activation of Src family kinases by TARC induces GRK2-mediated phosphorylation of the β_2-AR and subsequent complex formation with β-arrestin in an agonist-independent manner.

Finally, we tested whether the TARC-induced recruitment of β-arrestin to the β_2-AR results in a switch to activation of the

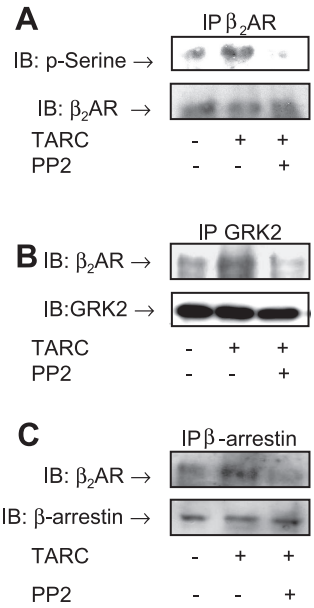

Fig. 5. Exposure to TARC increases serine phosphorylation of the β_2-AR and association of the β_2-AR with GRK2 and β-arrestin. T cells were incubated with TARC for 3 h in the presence and absence of PP2. Whole cell lysates were prepared and subjected to immunoprecipitation (IP) with anti-β_2-AR. Immune complexes were subjected to SDS 10% PAGE, and immunoblots (IB) were stained with anti-phospho-serine and anti-β_2-AR to establish equivalent loading. A representative blot of 2 independent experiments is shown ($n = 3$) (*A*). T cells were incubated with TARC for 3 h in the absence and presence of PP2. Whole cell lysates were prepared and subjected to IP with anti-GRK2 (agarose conjugated). Immune complexes were subjected to SDS 10% PAGE, and IB were stained with anti-β_2-AR and anti-GRK2 to establish equivalent loading ($n = 4$) (*B*). T cells were incubated with TARC for 3 h in the absence and presence of PP2. Whole cell lysates were prepared and subjected to IP with antibody against β-arrestin. Immune complexes were subjected to SDS 10% PAGE, and IB were stained with anti-β_2-AR as well as anti-β-arrestin to establish equivalent loading ($n = 4$) (*C*). Representative blots are shown.

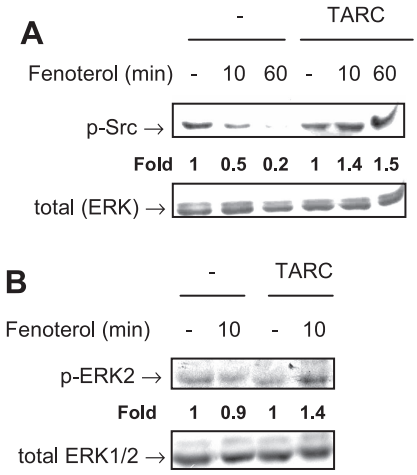

Fig. 6. TARC preincubation induces the ability of β_2-agonist fenoterol to enhance Src and ERK phosphorylation. Freshly isolated T cells were incubated with 100 ng/ml TARC for 6 h and subsequently stimulated with fenoterol for 10 or 60 min. Total cell lysates were prepared and p-Src was detected by Western blotting ($n = 3$) (*A*). Freshly isolated T cells were incubated with 100 ng/ml TARC for 6 h and subsequently stimulated with fenoterol for 10 min. Total cell lysates were prepared, and p-ERK was detected by Western blotting ($n = 3$) (*B*). Representative blots are shown.

Src/MAPK pathway. To this end, we studied whether or not fenoterol was able to induce phosphorylation of Src family kinases and ERK in T cells that were exposed to TARC (3 h). In resting T cells, basal Src kinase activity was inhibited by fenoterol after 10 and 60 min of incubation (Fig. 6*A*). In contrast, fenoterol induced a modest increase in Src phosphorylation when T cells were first exposed to TARC. Similarly, fenoterol enhanced ERK phosphorylation after preincubation with TARC (Fig. 6*B*). This indicates that TARC exposure switches β_2-AR function from activation of the G_s/AC/cAMP system, which negatively regulates the Src/SOS/MAPK pathway, to activation of the Src/MAPK pathway. Thus, after TARC exposure, T cell activity may be enhanced instead of inhibited by the β_2-AR system.

DISCUSSION

The β_2-AR is linked to the G_s protein and AC/cAMP activation, thereby providing negative feedback control over various proinflammatory cells. Prolonged or repeated stimulation of the β-agonist as well as exposure to various heterologous stimuli (e.g., growth factors and cytokines) may induce desensitization of the β_2-AR, resulting in impaired activation of the cAMP-dependent pathway (12, 35). We have previously demonstrated that allergen exposure in asthma reduces β_2-AR function for 40–50% in the total pool of peripheral blood lymphocytes, resulting in a loss of control over Th2-like cytokines (16, 26, 27). This loss of β_2-AR function is thought to be the result of allergen-induced release of proinflammatory mediators and is not observed during stable asthma (27). However, the extrinsic factor(s) responsible for the β_2-AR desensitization has remained unidentified. In the present study we demonstrate for the first time that TARC-induced activation of the C-C chemokine receptor CCR4, which is expressed on ~50% of the memory Th cells (19), results in desensitization of the β_2-AR in peripheral blood T lymphocytes. Our results demonstrate that TARC induces Src-dependent membrane

translocation of GRK2 and β-arrestin, resulting in β_2-AR phosphorylation and defective cAMP-dependent signaling. This observation may have important implications for T cell-mediated immune responses, including airway inflammation in asthma, since T cell activity is normally controlled by the β_2-AR/AC system. Indeed, we have previously demonstrated that Th1 and Th2 cytokine expression in α-CD3/α-CD28-stimulated, freshly isolated human T cells is inhibited by the β_2-agonist fenoterol (15). Interestingly, we have recently observed that the allergen-induced loss of β_2-AR control over Th2 cytokines could be mimicked by TARC exposure in T cells from healthy donors (16). In addition, we observed that TARC has a similar effect in stable asthma (data not shown). These data indicate that TARC exposure may induce important alterations in the regulation of T cell function.

In this report we demonstrate that TARC exposure reduces AC activity and cAMP-dependent signaling upon stimulation with the β_2-agonist fenoterol in peripheral T cells. The effect of TARC on cAMP accumulation was not as pronounced as the effect on CREB phosphorylation. Recently, it has been shown that cAMP accumulation and CREB phosphorylation do not always correlate. Partial β-agonists that hardly increased intracellular cAMP were shown to induce an increase in CREB phosphorylation (2). This may be explained by the fact that it is not the total quantity of cAMP is responsible for CREB activation, but cAMP's sustained turnover (2). cAMP turnover can be regulated by β-arrestin, since β-arrestin has been shown to target cAMP degradation to the receptor by its binding to phosphodiesterase (PDE) 4 (30). Thus TARC might affect cAMP accumulation by the recruitment of β-arrestin/PDE4 to the β_2-AR, thereby simultaneously reducing cAMP accumulation by desensitization and increasing the rate of cAMP degradation at the membrane. Because we used IBMX in the cAMP assay to prevent cAMP degradation and to obtain sufficient high cAMP accumulation, the possible additional effect of PDE4-mediated degradation could not be observed.

The results with NaF indicate that the effect of TARC on β_2-adrenergic signaling is most likely mediated upstream from the G_s/AC system at receptor level and not by direct inhibition of the AC system. Furthermore, our data suggest the involvement of G_i-dependent activation of Src family kinases in reduced β_2-AR function. We observed that TARC induces Src-dependent membrane translocation of GRK2 and β-arrestin. Although $G_{\beta\gamma}$ subunits are thought to be essential for the membrane anchoring of GRK2 (31), our results indicate that Src activation is also required for the TARC-induced membrane translocation of GRK2 and subsequent β_2-AR phosphorylation. This is in accordance with the findings of Sarnago et al. (33), demonstrating that Src activation induces GRK2 phosphorylation and activation. In addition, the findings of Fan et al. (10) demonstrate that Src activation precedes GRK2-mediated receptor phosphorylation and binding to β-arrestin. Src is known to associate with β-arrestin upon β_2-AR stimulation (24), but the role of Src kinases in β-arrestin membrane translocation has to our knowledge not been described before. In the present study we show that Src kinase activation by TARC induces the membrane translocation and recruitment of β-arrestin to the β_2-AR. The membrane translocation of β-arrestin may be a direct effect of Src or a consequence of β_2-AR phosphorylation. Either way, receptor phosphorylation is required to induce binding of β-arrestin to the receptor. Subsequently, β-arrestin may promote the complex formation of β_2-AR/Src (24), which might be responsible for the observed switch of β_2-AR signaling to activation of the MAPK pathway. The positive β_2-AR signaling observed after TARC exposure may enhance the activation profile of T cells. In accordance to the findings concerning β-arrestin, it has recently been reported that β-arrestin-2 is crucial for Th2 migration to the lung in a mouse model of asthma. β-Arrestin-2 knockout mice did not accumulate T cells in the lung and had defective macrophage-derived chemokine-mediated T cell migration (40).

Our findings suggest that the activation of GRK2 and β-arrestin by TARC does not facilitate agonist-induced β_2-AR desensitization but induces β_2-agonist-independent desensitization of the β_2-AR. So far, it has been assumed that βARK mediates homologous desensitization of GPRCs, as GRK2 preferentially phosphorylates the agonist-occupied receptor. However, agonist-independent phosphorylation of the β_2-AR has been demonstrated in other cell systems. For instance, insulin can induce agonist-independent phosphorylation of the β_2-AR in A431 cells (9). In addition, it has recently been described in HEK-293 cells that EGF receptor internalization is triggered by β_2-AR stimulation in the absence of EGF, in a GRK2- and β-arrestin-dependent manner (20). These findings clearly illustrate that GRK2-mediated desensitization is not restricted to homologous desensitization. Furthermore, chemokines have been demonstrated to induce cross-desensitization of another GPCR, i.e., the μ-opioid receptor, yet the mechanism remained undefined (38). The chemokine-induced desensitization of the β_2-AR may be an important mechanism in the amplification of inflammation, as it may result in enhanced T cell activity in presence of endogenous β_2-agonists. In addition to TARC, we observed that the pretreatment with the chemokines monocyte chemoattractant protein-1, IL-8, and 10-kDa interferon-γ-inducible protein similarly reduced β_2-AR signaling in T cells (data not shown), indicating that chemokines in general have an important role in the regulation of β_2-AR function. Our findings may have implications for additional cell types, including mast cells, eosinophils, and smooth muscle cells, which are known to be under control of the β_2-AR system and express various chemokine receptors as well.

In summary, we demonstrate that exposure of T cells to TARC alters the function of the β_2-AR, resulting in impaired ability to activate the cAMP-dependent pathway and a switch to Src/MAPK activation. This may be mediated by the Src-dependent membrane translocation of GRK2, phosphorylation of the β_2-AR, and recruitment of β-arrestin and result in dysregulated and enhanced activity of T cells by circulating endogenous β_2-agonists.

GRANTS

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