HHS Public Access

Author manuscript

Cell Signal. Author manuscript; available in PMC 2016 June 01.

Published in final edited form as:

Cell Signal. 2015 June ; 27(6): 1178–1185. doi:10.1016/j.cellsig.2015.02.022.

Cytoskeletal rearrangement and Src and PI-3K-dependent Akt activation

Michelle T. Barati[a], **Janice Scherzer**[a], **Rui Wu**[a], **Madhavi J. Rane**[#a,*], and **Jon B. Klein**[#a,b,**]

[a]Department of Medicine, University of Louisville, Louisville, KY, USA

[b]Robley Rex VA Medical Center, Zorn Avenue, Louisville, KY 40206, USA.

[#] These authors contributed equally to this work.

Abstract

The γ-amino butyric acid (GABA) type B receptors ($GABA_BR$) function as chemoattractant receptors in response to $GABA_BR$ agonists in human neutrophils.

The goal of this study was to define signaling mechanisms regulating $GABA_BR$-mediated chemotaxis and cytoskeletal rearrangement. In a proteomic study we identified serine/threonine kinase Akt, tyrosine kinases Src and Pyk2, microtubule regulator kinesin and microtubule affinity-regulating kinase (MARK) co-immunoprecipitating with $GABA_BR$. To define the contributions of these candidate signaling events in $GABA_BR$-mediated chemotaxis, we used rat basophilic leukemic cells (RBL-2H3 cells) stably transfected with human $GABA_{B1b}$ and $GABA_{B2}$ receptors. The $GABA_BR$ agonist baclofen induced Akt phosphorylation and chemotaxis by binding to its specific $GABA_BR$ since pretreatment of cells with CGP52432, a $GABA_BR$ antagonist, blocked such effects. Moreover, baclofen induced Akt phosphorylation was shown to be dependent upon PI-3K and Src kinases. Baclofen failed to stimulate actin polymerization in suspended RBL cells unless exposed to a baclofen gradient. However, baclofen stimulated both actin and tubulin polymerization in adherent RBL-$GABA_BR$ cells. Blockade of actin and tubulin polymerization by treatment of cells with cytochalasin D or nocodazole respectively, abolished baclofen-mediated chemotaxis. Furthermore, baclofen stimulated Pyk2 and STAT3 phosphorylation, both known regulators of cell migration. In conclusion, $GABA_BR$ stimulation promotes chemotaxis in RBL cells which is dependent on signaling via PI3-K/Akt, Src kinases and on rearrangement of both microtubules and actin cytoskeleton. These data define mechanisms of $GABA_BR$-mediated chemotaxis which may potentially be used to therapeutically regulate cellular response to injury and disease.

Keywords

Baclofen; Chemotaxis; Src; Akt; Microtubules; $GABA_B$ receptor

[*]Correspondence to: Kidney Disease Program, University of Louisville, 570 S. Preston St. Room 102C, Louisville, KY 40202, USA. Tel.: +1 502 852 1159; fax: +1 502 852 4384. mjrane01@louisville.edu. [**]Correspondence to: 570 S. Preston St. Room 102, Louisville, KY 40202, USA. Tel.: +1 502 852 5239; fax: +1 502 852 4384. Jon.klein@louisville.edu. .

1. Introduction

Chemotaxis is essential to numerous biologic and pathophysiologic processes, including inflammation, neuronal development, and cancer cell metastasis [1–3]. Directed migration is accomplished by signaling of locally released chemoattractant molecules through G-protein coupled receptors (GPCR). The inhibitory neurotransmitter γ-amino butyric acid (GABA), has been shown to be released by growth cones and acts as a chemoattractant to embryonic neuronal cells [4,5]. We were first to report that in addition to the central nervous system, GABA type B receptors ($GABA_BR$) are present in and function as chemoattractant receptors in response to $GABA_BR$ agonists, in human neutrophils [6]. The functional significance of these receptors in neutrophils was demonstrated by decreased neutrophil recruitment to ischemic brain lesions in a rat stroke model by intraventricular pretreatment with a $GABA_BR$ antagonist [6].

The $GABA_BR$ is a metabotropic GPCR comprised of a heterodimer of $GABA_{B1b}$ and $GABA_{B2}$ subunits interacting via leucine zipper motifs in the C-terminal domains [7]. In the central nervous system, $GABA_BR$ inhibits neurotransmitter release from presynaptic endings by inhibition of voltage-gated calcium channels [8] whereas post-synaptically, they lead to inhibition of adenylate cyclase and activation of potassium channels [9]. Functional expression of $GABA_BR$ has also been demonstrated in airway smooth muscle cells, pancreas and adrenal medulla [10–12]. In our previous study we demonstrated that stimulation of $GABA_BR$ in neutrophils led to phosphatidylinositol-3 kinase (PI3-K)-dependent rearrangement of microtubules and neutrophil chemotaxis. A role for baclofen-stimulated actin polymerization in suspended neutrophils was not demonstrated [6].

A proteomic screen identified kinesins, known to regulate microtubules, and the kinases src, Pyk2, and Akt, co-immunoprecipitating with the $GABA_BR$ in neutrophils. The goal of this study was to determine the role of $GABA_BRs$ in the regulation of microtubules and actin in adherent and suspended cells. We utilized RBL-2H3 cells stably transfected with $GABA_{B1b}$ and $GABA_{B2}$ receptors (RBL-$GABA_BR$) to directly elucidate the role of $GABA_BRs$ and its downstream signaling events in the regulation of baclofen induced cytoskeletal reorganization and chemotaxis.

2. Methods

2.1. Culture of RBL-$GABA_BR$ cells

Stably transfected, cloned human $GABA_{B1b}$/$GABA_{B2}$ receptor-expressing RBL-2H3 cells were obtained from Chemicon International (Temecula, CA). The cells referred to as RBL-$GABA_BR$ cells, were grown in 4.5 g/L Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 1× non-essential amino acids, 10 mH HEPES, 0.25 mg/ml G418, 0.5 mg/ml hygromycin, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 5% CO_2 and 37 °C.

2.2. Chemotaxis assays

RBL-$GABA_BR$ cells were washed with Hanks balanced salt solution, trypsinized and resuspended in Krebs buffer. 6×10^5 suspended cells were placed in the upper chambers of

Transwell permeable support chambers with 8.0 μm polycarbonate membranes (Costar/Corning, Lowell, MA). Baclofen (Sigma, St. Louis, MO) was added to the lower chamber of the transwells and cells incubated for various time points to allow migration. To define the specific role of $GABA_BR$, kinase signaling, and cytoskeletal elements involved in baclofen-induced chemotaxis, suspended cells were either untreated or pretreated with 10 μM CGP-52432 (Tocris, Ellisville, MO), 10 μM PP2 (Calbiochem, San Diego, CA), 40 μM Akt inhibitor (1L-6-hydroxymethyl-*chiro*-inositol-2-[(R)-2-O-methyl-3-O-octadecylcarbonate]; Alexis, San Diego, CA), 10 μM LY294002 (Calbiochem), 100 nM nocodazole (Sigma), or 30 nM cytochalasin D (Sigma), for 30 min in the upper and lower chambers of Transwell permeable support chambers. Following pretreatments, 10 μM baclofen was added to the lower chamber of the transwells and cells were incubated for an additional 60 min, at 37 °C to allow cells to migrate. The polycarbonate membranes were removed, fixed, stained with hematoxylin & eosin, and dried at room temperature overnight. Membranes were cut and fixed on the glass slides keeping the bottom surface upright and viewed under a light microscope at 100× magnification. Cells within the scale that had passed through the pores and were at the focal plane of the pores were counted. Results were expressed as the mean ± SD of the number of cells migrating across a 6.5-mm diameter circle of the membrane.

2.3. Western blotting

RBL cell proteins were extracted by lysis with 1% Triton X-100, 1% NP40, 10% glycerol, 137 mM NaCl, 20 mM tris HCl, 1 ng/μl aprotinin, 1 ng/μl leupeptin, 4 mM PMSF, 20 mM NaF, and 1 mM Na_3VO_4. Samples were prepared by addition of 4× lithium dodecyl sulfate buffer supplemented with DTT to a final concentration of 50 mM, heating for 10 min at 70 °C, followed by electrophoresis on 4–12% bis–tris gels (Invitrogen, Carlsbad, CA) in 1× MES-SDS buffer. Proteins were electrophoretically transferred to PVDF membranes and blocked in Odyssey infrared imaging blocking buffer (Licor, Lincoln, NE). Mouse anti-phospho-Akt Ser473, rabbit anti-total Akt, and rabbit anti-phospho-STAT3 Ser727 antibodies were obtained from Cell Signaling (Danvers, MA). Mouse anti-phospho-Pyk2 Tyr 402 antibody was from Santa Cruz (Santa Cruz, CA). Primary antibodies were diluted in the Odyssey blocking buffer supplemented with 0.1% Tween 20 and incubated overnight at 4 °C. Proteins were visualized by incubation of membranes with either fluorescent-tagged anti-mouse and anti-rabbit antibodies (Molecular Probes, Carlsbad, CA) and scanning on an Odyssey Infrared Imager (Licor), or, incubation with HRP-linked secondary antibodies and use of enhanced chemiluminescent substrate (Pierce, Rockford, IL).

2.4. Confocal microscopy

For adherent cell experiments, 15,000 cells were seeded into 8-well LabTek chambered coverglass units. At the end of each experimental condition, cells were washed 2 times with PBS and fixed with 3.7% paraformaldehyde/PBS/pH 7.4, for 10 min at room temperature, followed by three 5 min washes with PBS. Cells were then permeabilized with 0.5% triton X-100/PBS for 10 min, at room temperature, followed by three 5 min washes with PBS. A solution of 2% BSA in PBS for 30 min at room temperature was used to block non-specific staining. Anti αβ-tubulin, rabbit polyclonal (Cell Signaling) at a dilution of 1:50 in the same blocking buffer was added to the cells and for overnight incubation at 4 °C. Following three 5 min washes with PBS, cells were incubated with anti-rabbit-rhodamine antibody (2 μg/ml)

and FITC phalloidin (1:1000, Invitrogen), in blocking buffer, for 1 h at room temperature. Images were acquired using the Zeiss Axiovert 100 confocal microscope and LSM 510 software. Images are stacked projections of z-sections acquired at 1 μm intervals. Phalloidin (F-actin) and tubulin immunofluorescence of adherent cells were quantified using Image-Pro 6.2 software (Media Cybernetics, Silver Spring, MD). Positive staining was defined in color range selection (tubulin), or automatic bright object detection (phalloidin), with the same profile applied to all images collected for each stain/label. Data collected were density–intensity sum and sum of total staining area. To normalize values between images, the ratio of density–intensity sum/sum of total staining area for each image was used. This normalized staining to total area of the image occupied by cells. For suspended neutrophils, the integrated density values for individual cells were collected by outlining each cell, using Image J. The integrated density values/experimental condition were averaged.

2.5. Tubulin assays

Separation of soluble tubulin from microtubules was carried out using the microtubule/tubulin in vivo kit from Cytoskeleton Inc. (Denver, CO), according to kit protocol. Briefly, cells were washed with warm (37 °C) PBS and lysed in microtubule stabilization buffer at 37 °C followed by centrifugation at 100,000 ×*g*, 37 °C, for 30 min, to separate microtubules from the free tubulin pool. The separated fraction samples were prepared for immunoblot analysis as described above. Membranes were blotted for tubulin and densitometry used to quantitate relative pixel intensity of each band. A ratio of the densitometric value of microtubules (Insoluble fraction) to free tubulin (soluble fraction) for each condition was calculated and divided by that of the unstimulated cells to achieve fold change induced by baclofen treatment.

2.6. Statistical analysis

For chemotaxis assays, the number of migrated cells was compared between conditions using two-tailed, unpaired t-test following ANOVA analysis. The same statistical analysis was used to compare relative immunoblot band pixel intensity between different cell culture conditions. Densitometry of immunoblot bands was carried out using Odyssey 2.0 software (Licor). Quantitation of pAktS-473 immunoblots developed using chemiluminescence peroxide substrate was performed using Image J. Densitometric values of pS473-Akt were normalized to that of total Akt in each lane. P values ≤0.05 were considered significant for two-tailed, unpaired t-test, after ANOVA analysis.

3. Results

3.1. Baclofen induces chemotaxis of RBL-GABA$_B$R cells

GABA$_B$ receptors have been shown to play a role in embryonic spinal and cortical neuronal cell migration [5,13] and neutrophil chemotaxis [6]. We utilized RBL cells stably transfected with GABA$_{B1}$R and GABA$_{B2}$R cDNA (RBL-GABA$_B$R cell) to elucidate contributions of actin polymerization and/or tubulin reorganization in regulating GABA$_B$R-mediated chemotaxis and to dissect signaling pathways controlling these events. First, the ability of baclofen to induce chemotaxis in RBL-GABA$_B$R cells was determined. Cells were placed in the top chamber of transwell chemotaxis chambers and migration analyzed

following 45 min and 1 and 2 hour exposure to a 10 μM baclofen gradient. Cell migration was maximal following 1 h exposure to baclofen (Fig. 1A). Next a concentration–response experiment was performed to analyze chemotaxis in response to varying concentrations of baclofen for 1 h. As shown in Fig. 1B, baclofen induced a concentration dependent increase in RBL-$GABA_BR$ cell chemotaxis up to a concentration of 10 μM. To confirm the role of $GABA_B$ receptors in the baclofen-induced chemotactic response, cells were incubated with CGP52432, a selective $GABA_BR$ receptor antagonist, placed in the chemotaxis chambers and exposed to a 10 μM baclofen gradient for 1 h. Selective blockade of the $GABA_B$ receptors reduced baclofen-induced chemotaxis (Fig. 1C).

3.2. Baclofen-induced Akt phosphorylation is dependent on activation of both PI3-K and Src kinases

To establish functional coupling of $GABA_B$ receptor to Akt signaling in RBL-$GABA_BR$ cells, we analyzed the ability of baclofen to promote Akt phosphorylation, a marker of its activation. Cells were treated with 10 μM baclofen for 10 min in the presence and absence of CGP52432 to selectively block $GABA_B$ receptors. RBL cell proteins were extracted and immunoblotted for phospho-Akt Ser473 and total Akt. Baclofen caused a significant increase in Akt ser473 phosphorylation and this response was completely abrogated by blockade of the $GABA_B$ receptors (Fig. 2A). Given that a number of Src kinases and Akt associated with $GABA_BRs$ we next determined if Akt phosphorylation was dependent on PI-3K and/or Src kinases. Cells were pretreated with PI-3K inhibitor (LY294002), Akt inhibitor, 1L-6-hydroxymethyl-*chiro*-inositol-2-[(R)-2-O-methyl-3-O-octadecylcarbonate (Akt-i), and Src kinase inhibitor (PP2), followed by stimulation with baclofen for 10 min. As shown in Fig. 2B, all inhibitors reduced baclofen-induced Akt Ser473 phosphorylation in RBL-$GABA_BR$ cells, and thus both PI3K and Src signaling are required for Akt Ser473 phosphorylation following $GABA_BR$ activation.

3.3. Baclofen-induced chemotaxis of RBL-$GABA_BR$ cells is dependent on PI3K, Akt and Src kinases

Mass spectrometric analysis of proteins in complex with $GABA_BR$ identified Akt and Src kinases. The contribution of these kinases in $GABA_BR$-chemotaxis is not known. To define these cell signaling pathways in $GABA_BR$-mediated chemotaxis, RBL-$GABA_BR$ cells were pretreated with LY294002, Akt-i, or PP2, to inhibit PI3-K, Akt, and Src kinases, respectively. As shown in Fig. 3, baclofen-induced chemotaxis was inhibited by all three inhibitors demonstrating that $GABA_B$ receptor-stimulated chemotaxis is dependent on activities of PI3-K, Akt, and Src kinases.

3.4. Baclofen causes reorganization of microtubules and actin filaments in adherent RBL-$GABA_BR$ cells

Rearrangement and polymerization of actin and/or microtubules is required for cell migration depending on the cell type [14,15]. Previous studies in suspended neutrophils demonstrated rearrangement of microtubules in response to baclofen [6]. Therefore, the effect of baclofen on actin filaments and microtubules was determined in RBL-$GABA_BR$ cells. Adherent cells were either untreated or treated with 10 μM baclofen for 10 and 30 min,

followed by fixation and processing for staining and analysis by confocal microscopy. As shown in Fig. 4A, cells were stained with FITC-conjugated phalloidin for visualization of filamentous actin (F-actin). In untreated cells, F-actin is mostly distributed to the cell periphery with some intracellular spanning of fibers. With baclofen, there is cell spreading and an immediate increase in F-Actin content, demonstrating increased actin polymerization, with increased cell periphery localization, increased localization to membrane ruffles, as well as dense focal bundles in the center of cells. As shown in Fig. 4B, unstimulated RBL-GABA$_B$R cells exhibit spindle-like projections dense with microtubules. Stimulation of cells with baclofen caused redistribution of microtubules. The images demonstrate separation of the cytoskeletal elements within the cells, with F-actin localized more to the cell periphery and microtubules exhibiting a net-like projection spanning from the microtubule organizing center (MTOC) throughout the cell, but below the level of the membrane. Quantification of FITC-phalloidin staining (Fig. 4C) demonstrates increased F-actin content in RBL-GABA$_B$R cells treated with baclofen. Alternatively, while tubulin was redistributed in response to baclofen, the total amount of tubulin immunostaining was unaltered by baclofen treatment (Fig. 4D).

To better define and quantify the effect of baclofen on microtubules, RBL-GABA$_B$R cells were fractionated to separate the free tubulin pool from polymerized microtubules. Fractions of unstimulated and baclofen-treated adherent RBL-GABA$_B$R cells were separated by electrophoresis and subject to tubulin immunoblot analysis. Stimulation of RBL-GABA$_B$R cells with baclofen for 30 min increased the amount of tubulin in the insoluble/pellet (P) fraction, indicating increased microtubule polymerization (Fig. 4E). As control experiments, treatment of the cell lysate with nocodazole, to prevent microtubule polymerization decreased amount of tubulin in the pellet fraction compared to unstimulated cells while treatment with taxol, to stabilize microtubules, increases amount of tubulin in the pellet fraction, in the absence of baclofen (Fig. 4E).

3.5. Baclofen induces microtubule and actin polymerization in suspended RBL-GABA$_B$R cells when exposed to a baclofen gradient

Having demonstrated baclofen induced actin polymerization in adherent RBL-GABA$_B$R cells, we next examined if baclofen could stimulate cytoskeletal rearrangement in suspended RBL-GABA$_B$R cells, the state the cells are in during chemotaxis. As shown in Fig. 5A, direct addition of baclofen failed to stimulate actin polymerization in suspended RBL-GABA$_B$R cells. Therefore, we next determined if actin polymerization occurs in suspended RBL-GABA$_B$R cells that are exposed to a baclofen gradient during a chemotaxis assay. Cells were loaded in the top well of the transwell chemotaxis chamber and following 5, 10, and 15 min exposure to a 10 μM baclofen gradient, cells were removed from the upper chambers of Transwell chemotaxis chambers, fixed and processed for F-actin staining and confocal microscopy. As shown in Fig. 5B, F-actin was distributed uniformly at membranes of unstimulated cells. Exposure to the baclofen gradient immediately caused aggregation of F-actin to one or two poles of the cells, likely to be leading edges formed during directed migration, and increased actin polymerization (Fig. 5C).

In an additional set of experiments, cells removed from the upper chambers of transwell units were processed for quantitation of free tubulin and microtubules. As shown in Fig. 5D, 20 min exposure to a baclofen gradient increased microtubule formation in suspended cells, whereas by 30 min of exposure, the amount of microtubules returned to baseline values. Therefore, $GABA_BR$-mediated chemotaxis is associated with actin and tubulin polymerization.

3.6. Baclofen induced chemotaxis of RBL-$GABA_BR$ cells is dependent on actin and microtubule polymerization

To determine if $GABA_BR$-mediated chemotaxis is dependent on tubulin and actin polymerization, the effect of nocodazole and cytochalasin D, to depolymerize microtubules and prevent actin polymerization, respectively, on baclofen-induced chemotaxis was determined. RBL-$GABA_BR$ cells were pretreated with 100 nM nocodazole or 30 nM cytochalasin D in chambers of transwell units for 30 min, followed by exposure to 10 μM baclofen placed in the lower chambers for 1 h. As shown in Fig. 6, nocodazole and cytochalasin alone did not alter chemotaxis, whereas both inhibited baclofen-induced chemotaxis in RBL-$GABA_BR$ cells. Therefore, $GABA_BR$-mediated chemotaxis requires intact, polymerized microtubules and actin polymerization.

3.7. Pyk2 and STAT3, regulators of cell migration are activated by baclofen in RBL-$GABA_BR$ cells

Similar to Akt and Src, the non-receptor tyrosine kinase Pyk2, was also identified to co-immunoprecipitate with $GABA_BR$ in neutrophils. Since Pyk2 interacts with and regulates function of Src, and also plays an important role in cell migration [16,17], we determined if it is activated following $GABA_BR$ stimulation. As shown in Fig. 7A, phospho-Pyk2 Tyr-402, is increased within 1 minute of exposure to baclofen, and remains significantly phosphorylated though 10 min exposure to baclofen. Src family kinases can regulate the cytoskeleton and cell migration through activation of STAT3 [18,19], and Pyk2 can amplify src-mediated STAT3 activation [20]. Thus, to further characterize functional coupling of $GABA_B$ receptor to src signaling, cytoskeletal regulation, and chemotaxis, we analyzed the ability of baclofen to promote STAT3 activation. Phosphorylation of STAT3 on Ser727 required for transactivation [21] is increased following 5 min of exposure to baclofen (Fig. 7B).

4. Discussion

Directed cell migration is critical to normal function in many cell types and is accomplished by activation of multiple cell signaling pathways including coordination of protein phosphorylation and dephosphorylation events. This study showed that $GABA_B$ receptor mediated chemotaxis is accomplished by PI3-K- and src-dependent Akt activation and actin and tubulin polymerization. Furthermore, this study identified association with and functional coupling of $GABA_B$ receptors to mediators of Src signaling, Pyk2 and STAT3, critical to cell cytoskeletal regulation and cell migration.

Baclofen via binding to $GABA_BRs$ induced chemotaxis in RBL-$GABA_BR$ cells. The data are in agreement with our previous findings on the ability of baclofen to induce chemotaxis in human neutrophils and other studies in embryonic cortical neurons and spinal neuroblasts [5,6]. The $GABA_BR$ is a GPCR and asymmetric stimulation of GPCR-linked chemoattractant receptors leads to localized activation of PI3-K and PIP3 generation in the leading edge of cells undergoing chemotaxis. Our previous studies showed association of $GABA_B$ receptors with Akt in neutrophils [6], and other studies have shown functional coupling of the receptors to Gi proteins and activation of Src tyrosine kinases and ERK [22–24]. In this study we found that Src kinase- and PI3-K-dependent Akt phosphorylation are required for $GABA_BR$-mediated chemotaxis. The role of PI3-K in $GABA_BR$-mediated chemotaxis is in agreement with our previous studies on baclofen-induced chemotaxis in human neutrophils [6], and other studies on CXCL12 induced chemotaxis [25]. Signaling via all three kinases has also been shown to regulate activated CXCR3-mediated chemotaxis in hepatic stellate cells [26]. Other reports in neutrophils have shown Src kinases inhibitors to have either no effect or augment fMLP-induced chemotaxis [27,28]. Additional studies suggested that Src kinase regulation of fMLP-induced actin polymerization in response to fMLP decreases the ability of cells to transmigrate through a chemotaxis chamber pore size of 1 μm, while transmigration through a 3 μm pore is unaffected [29]. Alternatively, MIP-2 induced neutrophil emigration requires Src kinases [30]. While our study and other reports have used different types and concentrations of Src kinase inhibitors and/or cell lines from specific Src kinase knockout mouse models, the results suggest differential requirement of chemoattractant receptors and cell lines for signaling pathways regulating directed cell migration. Therefore, this is the first study to establish a requirement for Src-dependent Akt phosphorylation in promoting, $GABA_BR$-induced chemotaxis.

This study established functional coupling of $GABA_B$ receptor to Akt signaling shown by a significant increase in Akt ser473 phosphorylation in response to baclofen. Activated $GABA_BR$ can activate of Akt via transactivation of the IGF1 receptor [31], and this study found baclofen-induced Akt activation to be dependent on both PI3K and Src. While PI3-K and Src kinases have been shown to regulate Akt activity in other cell types [32,33], this is the first study demonstrating functional coupling of the Src signaling pathway to Akt activation, following activation of $GABA_BR$ in RBL-$GABA_BR$ cells.

Rearrangement and polymerization of actin and/or microtubules are required for cell migration depending on the cell type [14,15]. In this study, baclofen caused microtubule and actin reorganization in adherent RBL-$GABA_BR$ cells and polymerization of both cytoskeletal elements when suspended cells were exposed to a baclofen gradient. The role of each cytoskeletal component during $GABA_BR$-mediated directed migration was confirmed by the ability of nocodazole and cytochalasin D to inhibit baclofen-induced chemotaxis. While treatment of cells with nocodazole did not alter baclofen induced actin polymerization in adherent cells (data not shown), decreased chemotaxis may have been due to the requirement for intact microtubules for receptor asymmetrical redistribution to the baclofen gradient, as has been shown for GABA-induced $GABA_A$ receptor redistribution [34]. Additionally, nocodazole-induced microtubule disruption may have altered calcium influx [35] required for migration in response to baclofen [5]. Furthermore, these studies indicate that actin polymerization alone is not sufficient for baclofen-induced chemotaxis. Likewise

in neutrophils we have demonstrated that tubulin reorganization was necessary for baclofen induced neutrophil chemotaxis [6]. A previous report showed that colchicine-induced microtubule disruption in neutrophils increased cell migration which was inhibited by taxol, demonstrating that microtubule reorganization in general is significant to neutrophil migration [36]. In addition, the cytoskeleton was differentially regulated in adherent and suspended cells. Compared to adherent cells, 20 minute exposure to a baclofen gradient increased microtubule formation in suspended cells, whereas by 30 min of exposure, the amount of microtubules returned to baseline values. The differences in time of microtubule formation in adherent versus suspended cells may be due to re-distribution of $GABA_BR$ toward the baclofen gradient, as has been shown for $GABA_AR$ of dendritic cells exposed to a gradient [37], and increased migration of cells. Therefore in the presence of a chemoattractant gradient, cell signaling and Ca^{++} signaling mechanisms required for directed migration are activated, whereas overall exposure to the $GABA_B$ receptor agonist, while it activates the receptor, may not be sufficient to fully engage signaling required for activation of the cytoskeletal machinery necessary for chemotaxis. Similarly, as early as a 5 min exposure to the baclofen gradient caused aggregation of F-actin to one or two poles of the cells, likely to be leading edges formed during directed migration, whereas no rearrangement of actin occurred in suspended cells directly treated with baclofen. Other studies have shown a link between $GABA_BR$ and tubulin by direct interaction with marlin-1 and kinesin which regulates receptor distribution [38]. Similarly, proteomic analysis identified kinesin associating with $GABA_BR$ in neutrophils in the current study. The current study found activation of STAT3 in response to baclofen and STAT3 is known to be required for microtubule stabilization through interaction with and inhibition of microtubule destabilizing protein stathmin [39]. STAT3 is activated during Src signaling induced cytoskeletal regulation and cell migration [18,19]. In addition, phosphorylation of Pyk2 on Tyr 402, or Pyk2 autophosphorylation, following baclofen stimulation in this study, is required for src binding, further activation of pyk2 [40,41], and Pyk2 can amplify src-mediated STAT3 activation [20]. This is the first study to demonstrate increased microtubule polymerization following $GABA_BR$ stimulation, specifically. Together, the latter findings with Pyk2 and STAT3 demonstrate functional coupling of $GABA_B$ receptor to src signaling components critical in cytoskeletal regulation and chemotaxis.

In conclusion, this study has identified $GABA_BR$ mediated chemotaxis to be dependent on Src and PI3-K dependent Akt kinase phosphorylation. Furthermore, $GABA_BR$-mediated chemotaxis is dependent on polymerization of actin and microtubule filaments in RBL cells. Signaling pathways contributing to $GABA_BR$-induced chemotaxis may provide therapeutic options to regulate inflammatory cell recruitment.

Acknowledgments

This work was supported by the Department of Veterans Affairs Merit grant (J.B.K.), and National Institutes of Health grant R01A1075212-01A1 (M.J.R.).

References

[1]. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Nat. Rev. Immunol. 2007; 7:678–689. [PubMed: 17717539]

[2]. Hamasaki T, Goto S, Nishikawa S, Ushio Y. Brain Res. Brain Res. Rev. 2003; 41:1–12. [PubMed: 12505644]

[3]. Cheng GZ, Zhang W, Wang LH. Cancer Res. 2008; 68:957–960. [PubMed: 18281467]

[4]. Gordon-Weeks PR, Lockerbie RO, Pearce BR. Neurosci. Lett. 1984; 52:205–210. [PubMed: 6527836]

[5]. Behar TN, Li YX, Tran HT, Ma W, Dunlap V, Scott C, Barker JL. J. Neurosci. 1996; 16:1808–1818. [PubMed: 8774448]

[6]. Rane MJ, Gozal D, Butt W, Gozal E, Pierce WM Jr. Guo SZ, Wu R, Goldbart AD, Thongboonkerd V, McLeish KR, Klein JB. J. Immunol. 2005; 174:7242–7249. [PubMed: 15905570]

[7]. Marshall FH, Foord SM. Adv. Pharmacol. 2010; 58:63–91. [PubMed: 20655478]

[8]. Pitler TA, Alger BE. J. Neurophysiol. 1994; 72:2317–2327. [PubMed: 7884461]

[9]. Yamada K, Yu B, Gallagher JP. J. Neurophysiol. 1999; 81:2875–2883. [PubMed: 10368404]

[10]. Osawa Y, Xu D, Sternberg D, Sonett JR, D'Armiento J, Panettieri RA, Emala CW. Am. J. Physiol. Lung Cell. Mol. Physiol. 2006; 291:923–931.

[11]. Brice NL, Varadi A, Ashcroft SJ, Molnar E. Diabetologia. 2002; 45:242–252. [PubMed: 11935156]

[12]. Castro E, Oset-Gasque MJ, Cañadas S, Gimenez G, González MP. J. Neurosci. Res. 1988; 20:241–245. [PubMed: 2845114]

[13]. Behar TN, Schaffner AE, Tran HT, Barker JL. J. Neurosci. Res. 1995; 42:97–108. [PubMed: 8531231]

[14]. Sasaki AT, Janetopoulos C, Lee S, Charest PG, Takeda K, Sundheimer LW, Meili R, Devreotes PN, Firtel RA. J. Cell Biol. 2007; 178:185–191. [PubMed: 17635933]

[15]. Xu J, Wang F, Van Keymeulen A, Rentel M, Bourne HR. Proc. Natl. Acad. Sci. U. S. A. 2005; 102:6884–6889. [PubMed: 15860582]

[16]. Soe NN, Ishida T, Ishida M, Sawano M, Abe K, Miho N, Chayama K, Kihara Y, Yoshizumi M. J. Atheroscler. Thromb. 2009; 16:230–238. [PubMed: 19556728]

[17]. Baruzzi A, Caveggion E, Berton G. Cell. Mol. Life Sci. 2008; 65:2175–2190. [PubMed: 18385944]

[18]. Jougasaki M, Ichiki T, Takenoshita Y, Setoguchi M. Br. J. Pharmacol. 2010; 159:1294–1303. [PubMed: 20136831]

[19]. Badgwell DB, Lu Z, Le K, Gao F, Yang M, Suh GK, Bao JJ, Das P, Andreeff M, Chen W, Yu Y, Ahmed AA, Liao WS-L, Bast RC Jr. Oncogene. 2012; 31:68–79. [PubMed: 21643014]

[20]. Shi CS, Kehrl JH. J. Biol. Chem. 2004; 279:17224–17231. [PubMed: 14963038]

[21]. Shen Y, Schlessinger K, Zhu X, Meffre E, Quimby F, Levy DE, Darnell JE Jr. Mol. Cell. Biol. 2004; 24:407–419. [PubMed: 14673173]

[22]. Padgett CL, Slesinger PA. Adv. Pharmacol. 2010; 58:123–147. [PubMed: 20655481]

[23]. Richman RW, Tombler E, Lau KK, Anantharam A, Rodriguez J, O'Bryan JP, Diversé-Pierluissi MA. J. Biol. Chem. 2004; 279:24649–24658. [PubMed: 15047708]

[24]. Tu H, Rondard P, Xu C, Bertaso F, Cao F, Zhang X, Pin JP, Liu J. Cell. Signal. 2007; 19:1996–2002. [PubMed: 17582742]

[25]. Floridi F, Trettel F, Di Bartolomeo S, Ciotti MT, Limatola C. J. Neuroimmunol. 2003; 135:38–46. [PubMed: 12576222]

[26]. Bonacchi A, Romagnani P, Romanelli RG, Efsen E, Annunziato F, Lasagni L, Francalanci M, Serio M, Laffi G, Pinzani M, Gentilini P, Marra F. J. Biol. Chem. 2001; 276:9945–9954. [PubMed: 11136732]

[27]. Nijhuis E, Lammers JW, Koenderman L, Coffer PJ. J. Leukoc. Biol. 2002; 71:115–124. [PubMed: 11781387]

[28]. Zen K, Liu Y. Immunobiology. 2008; 213:13–23. [PubMed: 18207024]

[29]. Fumagalli L, Zhang H, Baruzzi A, Lowell CA, Berton G. J. Immunol. 2007; 178:3874–3885. [PubMed: 17339487]

[30]. Sarraj B, Massberg S, Li Y, Kasorn A, Subramanian K, Loison F, Silberstein LE, von Andrian U, Luo HR. J. Immunol. 2009; 182:7190–7200. [PubMed: 19454716]

[31]. Tu H, Xu C, Zhang W, Liu Q, Rondard P, Pin JP, Liu J. J. Neurosci. 2010; 30:749–759. [PubMed: 20071540]

[32]. Liang J, Slingerland JM. Cell Cycle. 2003; 2:339–345. [PubMed: 12851486]

[33]. Chen R, Kim O, Yang J, Sato K, Eisenmann KM, McCarthy J, Chen H, Qiu Y. J. Biol. Chem. 2001; 276:31858–31862. [PubMed: 11445557]

[34]. Coyle JE, Qamar S, Rajashankar KR, Nikolov DB. Neuron. 2002; 33:63–74. [PubMed: 11779480]

[35]. Oka T, Hori M, Ozaki H. J. Immunol. 2005; 174:4584–4589. [PubMed: 15814680]

[36]. Niggli V. J. Cell Sci. 2003; 116:813–822. [PubMed: 12571279]

[37]. Bouzigues C, Morel M, Triller A, Dahan M. Proc. Natl. Acad. Sci. U. S. A. 2007; 104:11251–11256. [PubMed: 17592112]

[38]. Vidal RL, Ramírez OA, Sandoval L, Koenig-Robert R, Härtel S, Couve A. Mol. Cell. Neurosci. 2007; 35:501–512. [PubMed: 17532644]

[39]. Ng DC, Lin BH, Lim CP, Huang G, Zhang T, Poli V, Cao X. J. Cell Biol. 2006; 172:245–257. [PubMed: 16401721]

[40]. Park SY, Avraham HK, Avraham S. J. Biol. Chem. 2004; 279:33315–33322. [PubMed: 15166227]

[41]. Bruzzaniti A, Neff L, Sandoval A, Du L, Horne WC, Baron R. Mol. Cell. Biol. 2009; 29:3644–3656. [PubMed: 19380485]

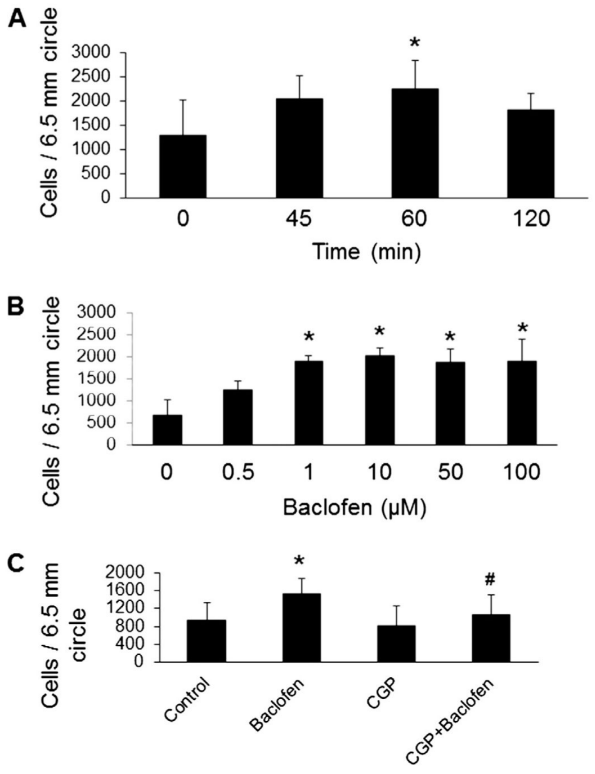

Fig. 1.

The $GABA_BR$ specific agonist baclofen induces chemotaxis of RBL-$GABA_BR$ cells. A, RBL-2H3 cells stably transfected with cloned human $GABA_{B1b}/GABA_{B2}$ receptor (RBL-$GABA_BR$ cells) were placed in transwell chemotaxis chambers and exposed to 10 μM baclofen for various time points (n = 5). Chemotaxis was determined by counting the number of cells on the opposite side of the transwell membrane. B, RBL-$GABA_BR$ cells were prepared for chemotaxis assay as in A and exposed to 0.5–100 μM baclofen for 1 h. C, RBL-$GABA_BR$ cells were prepared for chemotaxis assays with or without pretreatment with CGP-52432 (10 μM) for 30 min, then exposed to 10 μM baclofen for 1 h (n = 9). Data are average number of migrated cells ± SD. *$P < 0.05$ by unpaired, two-tailed t-test compared to control or 0, and $^{\#}P < 0.05$ compared to baclofen.

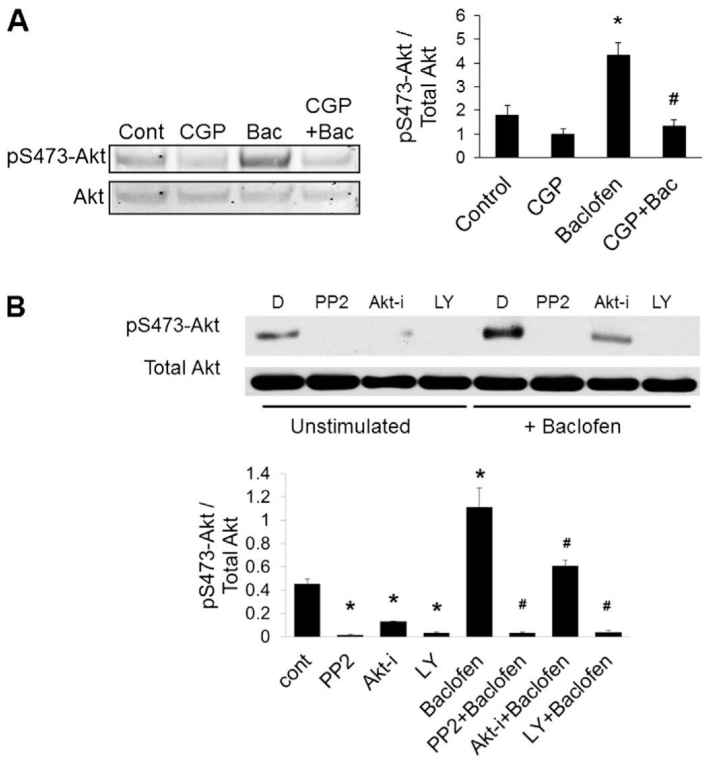

Fig. 2.

Both PI3-K and Src tyrosine kinases regulate activated $GABA_BR$-mediated Akt phosphorylation. A, RBL-$GABA_BR$ cells were either pretreated with 10 μM CGP52432 or untreated for 30 min prior to stimulation with 10 μM baclofen for 10 min. Lysate was subject to immunoblot analysis for phospho-Akt Ser 473 and total Akt. Data in graph are average of phospho-Akt/total Akt band pixel intensity ratio ± SEM (n = 7). *P < 0.05 by unpaired, two-tailed t-test compared to control. #P < 0.05 compared to baclofen treated. B, RBL-$GABA_BR$ cells were pretreated with 10 μM PP2, 10 μM LY294002, 40 μM Akt inhibitor (Akt-i), or dmso (D; vehicle control) for 30 min, followed by stimulation with 10 μM baclofen for 10 min. Lysate was subject to immunoblot analysis for phospho-Ser473-Akt and total Akt (n = 3). Data in graph are average of phospho-Akt-S473/total Akt band pixel intensity ratio ± SEM. *P < 0.05 by unpaired, two-tailed t-test compared to control. #P < 0.05 compared to baclofen treated.

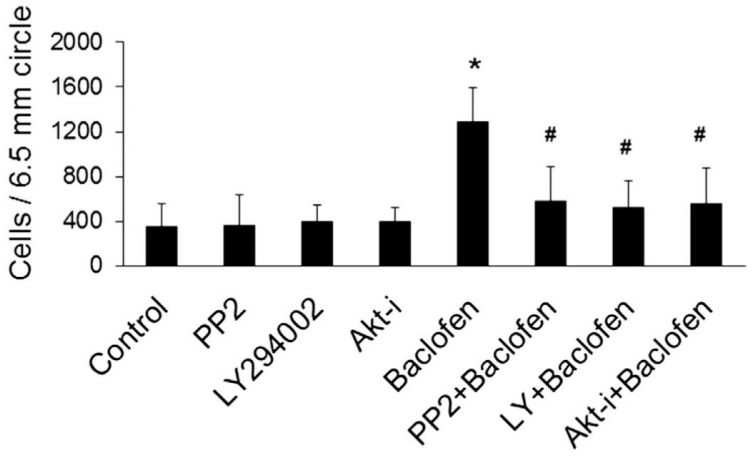

Fig. 3.

Src tyrosine kinases, PI3-K and Akt control baclofen-induced chemotaxis of RBL-GABA$_B$R cells. 6×10^5 suspended cells were prepared for chemotaxis assays and pretreated with 10 μM PP2, 10 μM LY294002, 40 μM Akt inhibitor, or dmso (vehicle control) for 30 min, and exposure to 10 μM baclofen for 1 h. Data are average number of migrated cells ± SD (n = 5–9). *P < 0.05 compared to control and $^{\#}$P < 0.05 compared to baclofen treated, by unpaired two-tailed t-test.

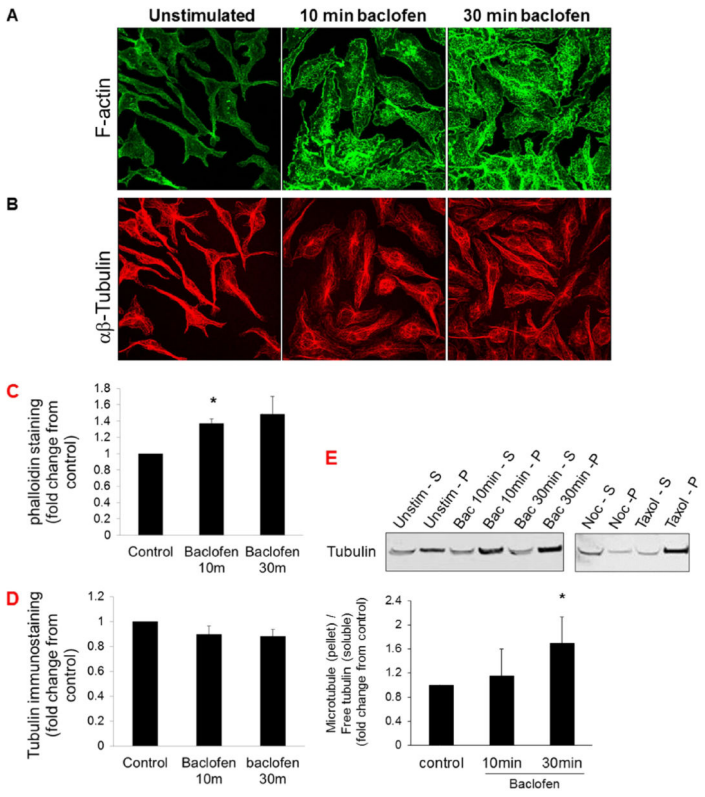

Fig. 4.

Baclofen induces microtubule and actin polymerization in adherent RBL-GABA$_B$R cells. Adherent RBL-GABA$_B$R cells were treated with 10 μM baclofen for 10 or 30 min, followed by fixation and immunofluorescence staining of A, F-actin with FITC-phalloidin (green) or, B, tubulin using anti-αβ tubulin (red). C, quantification of FITC-phalloidin staining. D, quantification of tubulin immunofluorescence staining. E, Adherent RBL-GABA$_B$R cells were treated with 10 μM baclofen for 10 or 30 min, followed by lysis/fractionation and immunoblot analysis of soluble (S; free tubulin) and pellet (P; insoluble/polymerized tubulin). Bar graph, average of pellet (microtubule)/soluble (free tubulin) tubulin immunoblot band pixel intensity ratio ± SD (n = 6). *P < 0.05 by unpaired, two-tailed t-test compared to control.

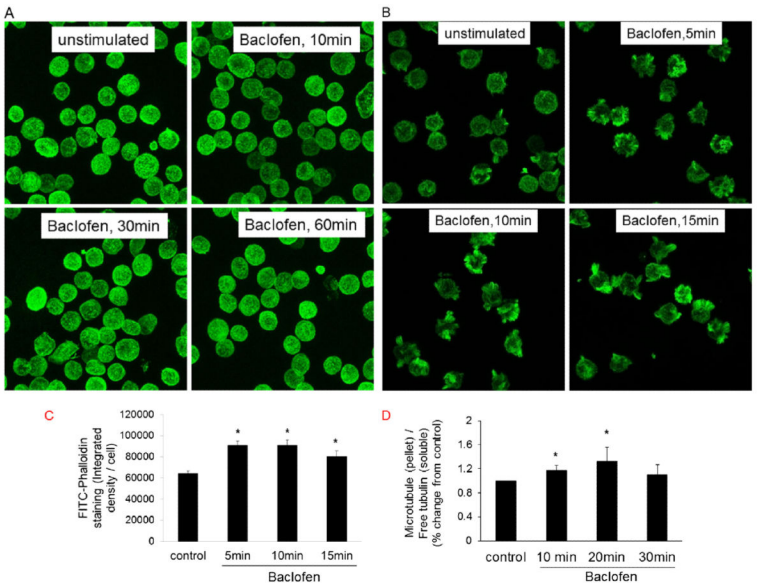

Fig. 5.

Baclofen induces microtubule and actin polymerization in suspended RBL-GABA$_B$R cells when exposed to a baclofen gradient. A, suspended RBL-GABA$_B$R cells were directly treated with 10 μM baclofen for various time points, then fixed and stained with FITC phalloidin for analysis of actin polymerization. B, suspended RBL-GABA$_B$R cells were placed in upper chambers of transwell chemotaxis chambers and exposed to a 10 μM baclofen gradient for various times, followed by removal of the cells for fixation and staining with FITC-phalloidin and visualization of F-actin. C, quantification of FITC-phalloidin-stained cells in B. D, suspended RBL-GABA$_B$R cells were placed in upper chambers of transwell chemotaxis chambers and exposed to a 10 μM baclofen for various time points, removed from the well and cell extracts fractionated for separation of soluble and polymerized tubulin by immunoblot analysis. Average of pellet (microtubule)/soluble (free tubulin) tubulin immunoblot band pixel intensity ratio ± SD (n = 4). *P < 0.05 by unpaired, two-tailed t-test compared to control.

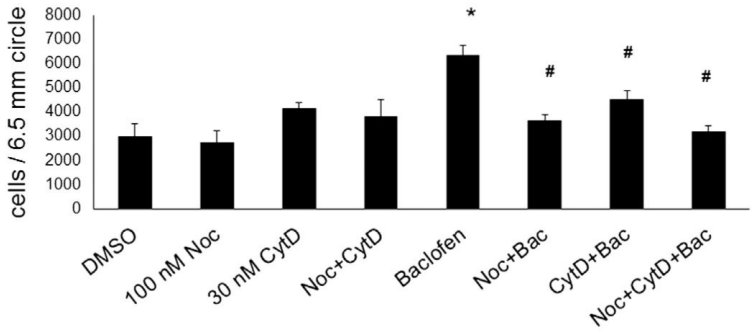

Fig. 6.

Baclofen induced chemotaxis of RBL-GABA$_B$R cells is dependent on actin and microtubule polymerization. Suspended RBL-GABA$_B$R cells were pretreated with 100 nM nocodazole, 30 nM cytochalasin, both, or Dmso (vehicle control) for 30 min prior to placement in transwell chambers for analysis of chemotaxis to 10 μM baclofen for 1 h. Data are average number of migrated cells ± SEM (n = 6). *P < 0.05 by unpaired, two-tailed t-test compared to control, or # compared to baclofen.

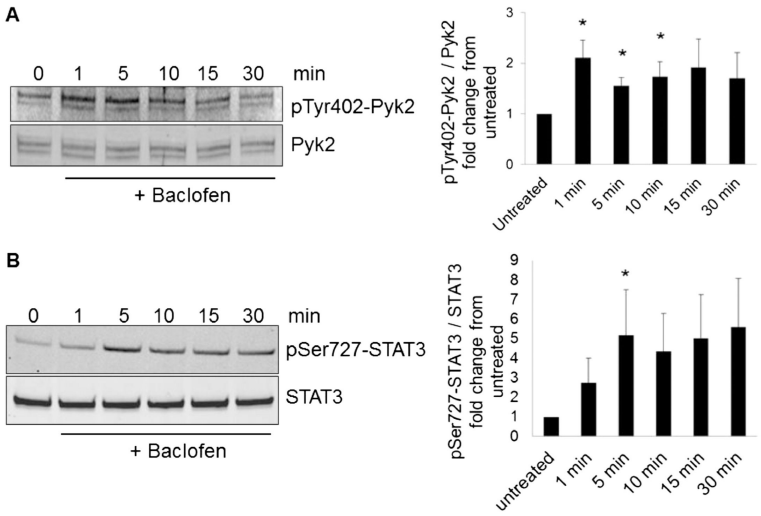

Fig. 7.

Baclofen stimulates Pyk2 and STAT3 phosphorylation RBL-GABA$_B$R cells were stimulated with 10 μM baclofen for various time points. Lysate was subject to immunoblot analysis A, for phospho-Tyr402-Pyk2 and total Pyk2 and B, phospho-Ser-7270STAT3 and total STAT3. Data in bar graphs are average of phospho-Pyk2/total or phospho-STAT3/total STAT3 band pixel intensity ratio/lane, represented as fold change from untreated, ±SEM (n = 5). *P < 0.05 by unpaired, two-tailed t-test compared to control.