Pannexin 1 Channels Link Chemoattractant Receptor Signaling to Local Excitation and Global Inhibition Responses at the Front and Back of Polarized Neutrophils

Received for publication, April 10, 2013, and in revised form, June 14, 2013. Published, JBC Papers in Press, June 24, 2013, DOI 10.1074/jbc.M113.476283

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Background: Chemotaxis requires excitatory and inhibitory signals at the front and back of cells. PANX1 and P2Y2 receptors provide local excitation at the front.

Results: We found that PANX1 triggers A2A receptor activation and inhibits chemotactic signaling at the back.

Conclusion: PANX1 thus integrates excitatory and inhibitory signals that regulate chemotaxis at the front and back of cells.

Significance: These findings suggest new strategies to modulate neutrophil chemotaxis.

Neutrophil chemotaxis requires excitatory signals at the front and inhibitory signals at the back of cells, which regulate cell migration in a chemotactic gradient field. We have previously shown that ATP release via pannexin 1 (PANX1) channels and autocrine stimulation of P2Y2 receptors contribute to the excitatory signals at the front. Here we show that PANX1 also contributes to the inhibitory signals at the back, namely by providing the ligand for A2A adenosine receptors. In resting neutrophils, we found that A2A receptors are uniformly distributed across the cell surface. In polarized cells, A2A receptors redistributed to the back where their stimulation triggered intracellular cAMP accumulation and protein kinase A (PKA) activation, which blocked chemoattractant receptor signaling. Inhibition of PANX1 blocked A2A receptor stimulation and cAMP accumulation in response to formyl peptide receptor stimulation. Treatments that blocked endogenous A2A receptor signaling impaired the polarization and migration of neutrophils in a chemotactic gradient field and resulted in enhanced ERK and p38 MAPK signaling in response to formyl peptide receptor stimulation. These findings suggest that chemoattractant receptors require PANX1 to trigger excitatory and inhibitory signals that synergize to fine-tune chemotactic responses at the front and back of neutrophils. PANX1 channels thus link local excitatory signals to the global inhibitory signals that orchestrate chemotaxis of neutrophils in gradient fields.

Efficient chemotaxis is an essential feature of neutrophils that allows these cells to fulfill their central role in immune defense. Neutrophil chemotaxis involves a complex set of coordinated processes that are triggered by chemoattractant receptors that are expressed across the cell surface. The first of these coordinated processes, termed gradient sensing, allows neutrophils to recognize various threats as well as their position within chemoattractant gradient fields emanating from tissues harboring such threats. In response to this information, neutrophils polarize and align in the gradient field. They assume an elongated cell shape and form a leading and a receding edge. Cell polarization results in the accumulation of specific sets of intracellular signaling molecules at the leading and receding edges at the front and back of the cell. These signaling molecules are needed for efficient migration of cells upstream of chemotactic gradient fields until the cells reach their final goal, the infected or inflamed site where chemoattractants are released from invading microbes or inflamed tissues (1).

Mathematical modeling suggests that these coordinated chemotactic processes require local excitatory and global inhibitory mechanisms at the front and back of cells, respectively (2). Various local excitation and global inhibition (LEGI) models of chemotaxis were developed in attempts to explain how such excitatory and inhibitory feedback mechanisms might translate external chemotactic cues into the complex sequence of cellular events that regulate gradient sensing, polarization, and migration of neutrophils in a chemotactic gradient field (3–6). These models are based on experimental evidence that has revealed that the excitatory mechanisms at the front involve molecules such as G0s, subunits of heterotrimeric G protein-coupled receptors (GPCRs), the small GTPases Rac and Cdc42, as well as phosphoinositide 3-kinase (PI3K) (6–8). Although less information is available about the makeup of the inhibitory signaling mechanisms that regulate cell responses at the back, recent evidence has emerged to suggest that cAMP accumu-
tion and PKA signaling are involved (7, 9). These findings elucidated the intracellular signaling events that regulate chemotaxis, but the upstream processes that trigger and integrate these opposing signaling processes at the front and back of cells are not fully understood (6).

We have previously reported that chemoattractants such as formylated peptides elicit the release of cellular ATP from neutrophils and that the released ATP drives autocrine feedback mechanisms that stimulate P2Y₂ and A3 receptors and thereby contribute to the excitatory signals that promote chemotactic responses at the front of cells (10). Chemotactic receptors can induce ATP release by triggering the opening of pannexin 1 (PANX1) channels, which are related to gap junction proteins. We found that ATP release through PANX1 channels and feedback through P2Y₂ receptors are absolute requirements for neutrophil activation and represent key events that initiate chemotaxis (11).

Extracellular ATP can regulate cell functions by stimulating a large family of purinergic receptors that are widely expressed and found in all mammalian cell types. Purinergic receptors include three different families, the P2Y, P2X, and P1 receptors. Mammalian cells can express various combinations of the eight known P2Y receptor subtypes that belong to the GPCR superfamily, the seven P2X receptors that act as ATP-gated ion channels, and the four different adenosine (P1) receptors that are GPCRs and can couple to Gα₃₁ (A1 and A3) or Gα₅₂ subunits (A₂A and A₂B). The diversity of the purinergic receptor subtype profiles that can be present in mammalian cells allows purinergic signaling mechanisms to promote or inhibit cell responses through the different downstream signaling events they can trigger (12–14).

In human neutrophils, A₂A receptors are more abundantly expressed than any other purinergic receptor subtype, including the P2Y₂ and A3 receptors whose autocrine stimulation is known to regulate neutrophil chemotaxis (10). Because A₂A receptors can trigger cAMP/PKA signaling (15–17), which was recently implicated in the control of chemotaxis (9), we wished to investigate whether and how autocrine signaling through A₂A receptors contributes to the chemotaxis of human neutrophils. Our findings have revealed that A₂A receptors are stimulated during chemotaxis, that they trigger cAMP and PKA signaling, and that autocrine A₂A receptor activation provides global inhibition that offsets local excitatory signals at the front of polarized neutrophils.

### EXPERIMENTAL PROCEDURES

**Materials**—Apyrase, carbenoxolone (CBX), dibutyryl cAMP-AM, forskolin, isobutylmethylxanthine, H89, fMLP, dextran, and all other reagents were from Sigma-Aldrich unless otherwise stated. Percoll was from Amersham Biosciences. The PANX1 inhibitory peptide 10panx1, a scrambled 10panx1 control peptide (−10panx1), CGS21680, 8-(3-chlorostyryl) caffeine (CSC), and SCH58261 were from Tocris Bioscience (Ellisville, MI).

**Human Neutrophil Isolation**—The Institutional Review Board of the Beth Israel Deaconess Medical Center (BIDMC) approved all studies. Neutrophils were isolated from peripheral blood of healthy volunteers as described previously using dextran sedimentation followed by Percoll gradient centrifugation (10). Cell preparations were kept pyrogen-free, and osmotic shock lysis of red cells was omitted to avoid mechanical stimulation.

**Cell Culture**—HL-60 cells were maintained as described previously (11). HL-60 cells stably expressing YFP-tagged actin were a kind gift from Dr. Orion Weiner at the University of California, San Francisco, and maintained following the protocol described previously (18, 19). For differentiation, HL-60 or HL-60/YFP-actin cells were treated with 1.3% dimethyl sulfoxide (DMSO) for 3 days. Differentiated neutrophil-like HL-60 (dHL-60) cells transiently expressing an A₂A receptor-EYFP fusion protein construct were generated by electroporation (Neon transfection system, Invitrogen) with an expression plasmid kindly provided by Dr. Oliver Kudlacek from the Medical University of Vienna, Austria.

**Immunofluorescence Staining**—Freshly purified human neutrophils (2.5 × 10⁶/ml) were allowed to adhere to flamed and fibronectin-coated 25-mm glass coverslips for 10 min at room temperature (Fisher Scientific). The coverslips were coated with 40 μg/ml human fibronectin as described previously (10). Then the cells were pretreated with different reagents for 10 min and stimulated with 1 nM fMLP for another 10 min. The cells were fixed for 15 min with 3.7% paraformaldehyde in HBSS (Irvine Scientific, Irvine, CA), rinsed with fresh HBSS, and permeabilized for 30 s using HBSS containing 0.01% Triton X-100. The cells were treated with 5% human serum in HBSS for 1 h and then incubated for 1 h with rabbit anti-human A₂A receptor antibodies (1:200 dilutions; Abcam, Cambridge, MA). Then the cells were incubated for 30 min in the dark with secondary antibodies (1:1,000; Alexa Fluor 488 goat anti-rabbit Invitrogen). F-actin was stained with phalloidin using 5 units/slide and the methods suggested by the supplier (Invitrogen). Fluorescence and bright field images were acquired using a Zeiss LSM 510 Meta confocal microscope configured to excite both fluorescent dyes with multi-track mode using the 488-nm and the 543-nm laser lines.

**Chemotaxis Assays**—Chemotaxis was assessed using the life cell microscope system described previously (10). Briefly, freshly isolated human neutrophils or dHL-60/YFP-actin cells (2.5 × 10⁶/ml) were plated onto 25-mm glass coverslips (Fisher Scientific) coated with 40 μg/ml human fibronectin and placed into a temperature-controlled stage incubator (Harvard Apparatus, Holliston, MA) at 37°C. Cells were treated with or without reagents as described for each individual experiment and exposed to a chemoattractant gradient field generated by a micropipette loaded with 100 nM fMLP. The tip of the micropipette was placed in proximity to cells to be studied, and cell migration was tracked by obtaining 20 sequential images at 20-s intervals (3 frames min⁻¹). From these images, the speed and migration paths of individual cells toward the point source of fMLP were analyzed using Image Pro Plus software (Media Cybernetics, Bethesda, MD). Each trace shown in the associated figures corresponds to the path of a single cell from its origin (assigned coordinate x = y = 0) to the tip of the micropipette (assigned coordinate x = 0, y = 200). As a control, we tested all drugs used in our study for potential effects on cell...
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viability in these experiments. None of these drugs showed any significant effect on cell viability.

Measurement of cAMP—Neutrophils (10^7/ml) were stimulated with fMLP (1 nM) and isobutylmethylxanthine (4 mM) at 37 °C for 5 min or the indicated times, and cAMP was determined with a commercial assay kit (cAMP-Screen® system; Applied Biosystems, Bedford, MA) according to the manufacturer’s instructions.

MAPK Activation—ERK and p38 MAPK activation in response to fMLP stimulation was assessed with Western blotting and phosphospecific antibodies as described previously (11).

Statistical Analyses—Unless otherwise stated, all values are expressed as mean ± S.D., and statistical analyses were performed using unpaired Student’s t tests. Differences between groups were considered statistically significant at p < 0.05.

RESULTS

ATP Release Regulates Gradient Sensing, Cell Shape, and Migration Speed—We have previously shown that chemotactic stimuli induce the release of ATP from human neutrophils and dHL-60 cells and that ATP release via PANX1 channels amplifies chemotactic signals (11). To gain a better understanding of how PANX1 regulates chemotaxis, we blocked it with the mimetic peptide 10panx1, which is a specific inhibitor of PANX1 channels, or CBX, which is a general gap junction inhibitor. Pretreatment of neutrophils with CBX prevented actin polymerization, the formation of polarized cell shapes, and gradient sensing of cells in a chemotactic gradient field generated with a micropipette loaded with 100 nM fMLP (Fig. 1, supplemental Movies S1 and S2). Pretreatment of cells with 10panx1 had similar effects on gradient sensing and cell shape, but 10panx1 was less effective than CBX in reducing the speed of migration in a chemotactic gradient field (Fig. 1; supplemental Movies S1 and S2). Taken together with our previous work, these findings indicate that PANX1 channels have an important role in regulating gradient sensing by providing local excitation via P2Y2 receptors (11). However, these results do not rule out the possibility that PANX1 has additional roles that regulate cell shape, polarity, and migration of cells in a chemotactic gradient field.

PANX1 Induces cAMP Accumulation in Response to Formyl Peptide Receptor (FPR) Stimulation—Recent studies have shown that intracellular cAMP accumulation and downstream activation of the cAMP-dependent protein kinase A (PKA) contribute to the regulation of neutrophil chemotaxis (7, 9, 20). cAMP accumulation was found to involve adenylyl cyclase 9 (AC9), and knockdown of AC9 impaired intracellular cAMP accumulation and chemotaxis (9). Because these findings indicate that chemotactic receptors can induce cAMP signaling, we investigated whether stimulation of FPR elicits cAMP accumulation in purified human neutrophils. Stimulation of cells with 1 nM fMLP, a concentration that causes robust chemotaxis and ATP release, triggered a rapid increase in intracellular cAMP concentrations, which peaked 5 min after fMLP stimulation and remained elevated above base-line levels for >10 min (Fig. 2A). Treatment of neutrophils with the cell-permeable cAMP analog dibutylryl cAMP-AM rapidly blocked the speed of migration of neutrophils in an fMLP gradient field (Fig. 2B). These findings indicate that intracellular cAMP accumulation is an integral aspect of the signaling response of neutrophils to chemotactic stimuli and that cAMP accumulation can inhibit neutrophil migration. To evaluate whether FPR-induced ATP release contributes to cAMP accumulation of fMLP-stimulated neutrophils, we pretreated neutrophils with different concentrations of CBX or 10panx1 to block ATP release. Then we assessed cAMP accumulation in response to fMLP stimulation. CBX and 10panx1, but not a scrambled con-
accumulation, we treated human neutrophils with increasing
mation following FPR stimulation. These findings indicate that
cAMP accumulation in response to fMLP stimulation (Fig. 2
pretreated for 10 min with the indicated concentrations of CBX or 10panx1 or
is required for fMLP-induced cAMP accumulation. Human neutrophils were
indicating that FPR stimulation causes A2A receptor stimula-
tion induces a transient rise in intracellular cAMP. Freshly isolated human
neutrophils were treated with 1 nm fMLP, and intracellular cAMP concentra-
tions were assessed after the indicated times using a CAMP-Scan assay kit. B,
exogenously added cAMP impairs neutrophil migration. Human neutrophils
migrating in an fMLP gradient field generated with a micropipette were
treated with the cell-permeable CAMP analog dibutyryl CAMP-AM (1 μM), and
migration speed was recorded as described in the legend for Fig. 1. C, PANX1
is required for fMLP-induced cAMP accumulation. Human neutrophils were
pretreated for 10 min with the indicated concentrations of CBX or 10panx1 or
with 200 μM control peptide (‘control’). Then cells were stimulated with 1 nm
fMLP, and intracellular CAMP concentrations were measured after 5 min. For-
skolin (100 μM) was used as a positive control; no stim, no stimulation. Values
are expressed as mean ± S.D., and the data shown are representative of three
individual experiments performed on different days; statistical analysis was
done with Student’s t test, * p < 0.05.

trol peptide (‘no fMLP’), dose-dependently inhibited intracellular
cAMP accumulation in response to fMLP stimulation (Fig. 2C).
These findings indicate that PANX1 is involved in cAMP accu-
cumulation following FPR stimulation.

A2A Adenosine Receptors Elicit CAMP Accumulation in
Response to FPR Stimulation—Ectonucleotidases on the cell
surface of neutrophils can hydrolyze released ATP to adeno-
sine. A possible mechanism by which PANX1 might contribute
to cAMP accumulation is through Gαi-coupled A2A adenosine
receptors that are known to induce cAMP accumulation and
are abundantly expressed in human neutrophils (15, 21–24). To
test the involvement of A2A receptors in FPR-induced cAMP
accumulation, we treated human neutrophils with increasing
concentrations of the A2A receptor antagonist CSC or SCH58261 or with the A2A
agonist CGS21680 as a control before assessing fMLP-induced cAMP accumulation as
described above. The A2A receptor antagonists blocked fMLP-
stimulated cAMP accumulation in a dose-dependent fashion,
indicating that FPR stimulation causes A2A receptor stimula-
tion that in turn causes downstream cAMP accumulation (Fig.
3A). Taken together with the findings shown above, these
results suggest that chemoattractant receptors require PANX1
and A2A receptors to induce cAMP accumulation.

A2A Receptors Counterbalance FPR-induced Excitatory Signaling Pathways—Our present and previous findings suggest that FPR stimulation induces two closely associated purinergic signaling mechanisms that involve PANX1 channels: an A2A
receptor-dependent mechanism that elicits cAMP signaling as
well as a P2Y2 receptor-dependent excitatory purinergic mech-
anism that elicits ERK and p38 MAPK signaling (10, 11). Cross-
talk of these two PANX1-dependent mechanisms could explain
the apparent paradox that chemoattractant receptors that
belong to the Gαi-coupled GPCR family can trigger cAMP
accumulation that is a hallmark of Gαi-coupled GPCRs. Because cAMP accumulation is known to block activation sig-
aling pathways downstream of Gαi-coupled GPCRs (25, 26),
we hypothesized that A2A receptors may serve to counterbal-
ance and thus fine-tune the excitatory signaling pathways that are
triggered by chemoattractant receptors.

To test this hypothesis, we investigated whether inhibition of
cAMP signaling with the PKA inhibitor H89 alters FPR-in-
duced ERK and p38 MAPK signaling. H89 dose-dependently
augmented the activation of both MAPKs in response to fMLP
stimulation (Fig. 3B). In addition, we found that inhibition of

FIGURE 2. FPR stimulation induces cAMP signaling via PANX1. A, FPR stim-
ulation induces a transient rise in intracellular cAMP. Freshly isolated human
neutrophils were treated with 1 nM fMLP, and intracellular CAMP concentra-
tions were assessed after the indicated times using a CAMP-Scan assay kit. B,
exogenously added cAMP impairs neutrophil migration. Human neutrophils
migrating in an fMLP gradient field generated with a micropipette were
treated with the cell-permeable CAMP analog dibutyryl CAMP-AM (1 μM), and
migration speed was recorded as described in the legend for Fig. 1. C, PANX1
is required for fMLP-induced cAMP accumulation. Human neutrophils were
pretreated for 10 min with the indicated concentrations of CBX or 10panx1 or
with 200 μM control peptide (‘control’). Then cells were stimulated with 1 nM
fMLP, and intracellular CAMP concentrations were measured after 5 min. For-
skolin (100 μM) was used as a positive control; no stim, no stimulation. Values
are expressed as mean ± S.D., and the data shown are representative of three
individual experiments performed on different days; statistical analysis was
done with Student’s t test, * p < 0.05.

FIGURE 3. A2A receptors regulate FPR signaling via cAMP accumulation. A, FPR-induced cAMP signaling requires A2A receptor stimulation. Human neu-
trophils were pretreated for 10 min with the indicated concentrations of the
A2A receptor antagonists CSC or SCH58261 or the A2A receptor agonist
CGS21680. Then the cells were stimulated with 1 nM fMLP, and intracellular
CAMP concentrations were assessed after 5 min. B and C, A2A receptor signal-
ing fine-tunes MAPK activation in response to FPR stimulation. Human neu-
trophils were treated for 15 min with the indicated concentrations of the PKA
inhibitor H89 (B and C) or the A2A receptor antagonist CSC (C). Then the cells
were stimulated with the indicated concentrations of fMLP and p38, and ERK
MAPK signaling was determined using Western blotting with antibodies that
recognize the phosphorylated and thereby activated forms of these MAPKs.
MAPK activation was estimated by comparing data obtained with these anti-
odies and with corresponding antibodies that recognize the active and inac-
tive forms of these MAPKs. p-p38, phosphorylated p38; p-Erk, phosphorylated
ERK. Results are expressed as mean ± S.D., and the data shown are represent-
itive of at least three individual experiments performed on different days
with cells from different healthy individuals; statistical analysis was done with
Student’s t test, * p < 0.05.
A2A receptors with CSC or inhibition of PKA with H89 potentiated the sensitivity of neutrophils to fMLP, triggering robust MAPK signaling in response to fMLP concentrations that cause strong chemotactic but relatively modest MAPK signaling responses (Fig. 3C). These results indicate that A2A receptor activation and PKA signaling function as compensatory mechanisms that counterbalance the excitatory response of neutrophils to FPR stimulation.

A2A Receptors Translocate toward the Back of Polarized Cells—Taken together with our previous work, these results above suggest that FPR stimulation and ATP release via PANX1 channels induce two distinct purinergic signaling mechanisms and downstream signaling pathways with opposing functional consequences. These closely linked, yet contrasting purinergic signaling mechanisms are reminiscent of the excitatory and inhibitory signaling mechanisms proposed by LEGI models of chemotaxis, which assume interactions of local excitatory mechanisms at the front and complementary inhibitory signaling mechanisms at the back of cells. We investigated whether the distribution of A2A receptors across the cell surface of neutrophils could contribute to their function as a source of global inhibition as suggested by LEGI models. In unstimulated neutrophils, A2A receptors were uniformly distributed across the entire cell surface. However, after stimulation of neutrophils with fMLP, we found that the leading edge of polarized neutrophils was devoid of A2A receptors, whereas A2A receptors remained uniformly distributed across all other regions of the cell surface (Fig. 4A; supplemental Movie S3). This A2A receptor distribution pattern resembles the distribution pattern of intracellular cAMP that is found throughout the cytosol but is absent from extending pseudopods of polarized cells (9).

To study the dynamics of A2A receptor distribution during cell polarization, we used dHL-60 cells expressing a fluorescent A2A receptor fusion protein. A2A receptor redistribution is a highly dynamic process that is associated with cell polarization. Exposure of dHL-60 cells to a chemotactic gradient generated by a micropipette (see asterisks in Fig. 4B) resulted in rapid redistribution of A2A receptors from the membrane region closest to chemotactic source to the back of cells (Fig. 4B; supplemental Movie S4). In contrast to primary neutrophils, A2A receptor translocation in dHL-60 cells resulted in a large region of the leading edge that was devoid of A2A receptors. We also observed that overexpression of A2A receptors affected cell shape, which is consistent with a role for A2A receptors in cell polarization. We further found that the translocation of A2A receptors and elongation of cells during cell polarization was inhibited by CBX, suggesting that A2A receptor redistribution and cell polarization depend on ATP release (Fig. 5C). Taken together, our findings suggest that FPR-induced ATP release via PANX1 is involved in the redistribution of A2A receptors and that endogenous activation of A2A receptors contributes to the polarization of neutrophils in a chemotactic gradient field.

A2A Receptors Regulate Migration Speed—To evaluate the role of A2A receptors in neutrophil chemotaxis, we treated cells with an antagonist (CSC) or an agonist (CGS21680) of A2A receptors or with the PKA inhibitor H89. To study the effect of these treatments on fMLP-induced chemotactic cell behavior, we used the microscope-based micropipette method described above. We found that CSC, CGS21680, and H89 markedly impaired chemotaxis (Fig. 5; supplemental Movie S5). Inhibition of A2A receptors or PKA signaling with CSC and H89 reduced migration speed but did not seem to impair the ability of cells to orient in the chemotactic gradient field. This suggests that the primary function of A2A receptors in neutrophil chemotaxis appears to be to regulate migration speed but not gradient sensing. Pretreatment of cells with the A2A receptor agonist CGS21680 also impaired gradient sensing. Taken together with the other results in Fig. 5, this finding suggests that spatiotemporally appropriate stimulation of A2A receptors via endogenous mechanisms is essential for their proper function and regulation of chemotaxis. Thus, addition of A2A receptor agonist perturbs the endogenous signaling mechanisms that activate A2A receptors in migrating cells, which results in the inhibition of chemotaxis.

DISCUSSION

The formation of a leading and a receding edge is a hallmark of chemotaxis of eukaryotic cells. This process involves the redistribution of specific molecules such as Cdc42, Rac, PI3K, and F-actin to the front and others such as Rho and myosin to the back of cells (3, 6). There, these molecules and their downstream signal transduction pathways determine localized cell responses that correspond to the spatiotemporal differences in external chemoattractant concentrations surrounding cells (4,
Neutrophils possess specific chemoattractant receptors that distinguish among the many different chemoattractant mediators that can be released from inflamed or infected tissues (27, 28). In addition to such specific chemoattractant receptors, however, neutrophils also depend on purinergic receptors that are activated by extracellular ATP and adenosine and play a central role in defining how cells respond to chemotactic agents (10).

For proper chemotactic responses, neutrophils require mechanisms to amplify weak chemotactic signals and to recognize small differences in chemoattractant concentrations at the front and back of cells. In addition, they must be able to maintain their ability to distinguish such small local concentration differences as they move upstream of chemotactic gradient fields and as the absolute chemoattractant concentrations fluctuate dramatically in the different tissues they traverse (8, 29).

LEGI models of chemotaxis propose that excitatory positive feedback mechanisms that facilitate gradient sensing and regulate chemotactic responses at the front of migrating cells (10, 11). In the current study, we found that A2A receptors are translocated from the leading edge toward the back of polarized neutrophils and that inhibitory signaling via A2A receptor-dependent cAMP accumulation inhibits excitatory chemotactic signaling by blocking FPR-dependent ERK and p38 MAPK activation globally with the exception of the leading edge. A2a, alkaline phosphatase; ADO, adenosine; PIP3, phosphatidylinositol (3,4,5)-triphosphate.

Based on these findings, a LEGI model could be envisioned where FPR-induced ATP release via PANX1 controls the excitatory and inhibitory mechanisms of P2Y2, A3, and A2A receptors (Fig. 6). These three purinergic receptor subtypes are GPCRs that can couple to Gαq/11, Gαs, Gα12, and Gα12 proteins, and they thus can trigger downstream signaling pathways such as phosphatidylinositol (3,4,5)-triphosphate and cAMP that are known to define chemotactic responses in polarized cells (8, 9, 12, 14, 35–39). In a proposed “purinergic LEGI model,” PANX1 and P2Y2 receptors would initiate chemotaxis by amplifying an initial FPR signal (11). This initial step triggers cell polarization and the redistribution of PANX1 and A3 receptors to the front, whereas A2A receptors vanish from the leading edge (12, 14, 35–39). Ectonucleoside triphosphate diphosphohydrolase 1 (E-NTPDase1/CD39), an ectonucleotidease anchored to the cell surface of neutrophils, translocates to the leading edge and contributes to the formation of adenosine that stimulates A3
and A$_{2A}$ receptors (40). Although PANX1 channels and E-NTPDase1/CD39 accumulate at the leading edge, sufficient numbers of these molecules may remain globally distributed to provide the adenosine needed for A$_{2A}$ receptor stimulation. Alternatively, adenosine generated at the leading edge may diffuse at a sufficient rate from the leading edge to stimulate A$_{2A}$ receptors that provide global inhibition by blocking adjacent chemotactic receptors.

Interestingly, in addition to the A$_{2A}$ antagonist CSC, interfering with A$_{2A}$ receptor signaling by exogenous stimulation with the A$_{2A}$ receptor agonist CGS21680 also impaired chemotaxis (Fig. 5). This observation indicates that obscuring the endogenous A$_{2A}$ receptor-dependent mechanisms of neutrophils blocks chemotaxis, which has pharmacological implications. Although receptor desensitization mechanisms may be involved in this phenomenon, the fact that a cell-permeable cAMP analog caused similar defects (Fig. 2B) suggests that spatially appropriate A$_{2A}$ receptor stimulation via endogenous purinergic signaling mechanisms is essential for neutrophil chemotaxis.

Recent work has shown that neutrophils share with microglial, macrophages, and other cell types some features of the purinergic signaling systems that regulate cell migration (22, 41, 42). In addition to their role in regulating chemotaxis, these purinergic signaling systems also allow for intercellular communication through paracrine purinergic signaling mechanisms. The dual use of these inside-out signaling mechanisms further increases the flexibility and repertoire of possible responses that make neutrophils capable of carrying out their complex mission in host defense.

Acknowledgments—We thank Dr. Orion Weiner from the University of California San Francisco for providing HL-60 cells expressing YFP-tagged actin and Drs. Oliver Kudlacek, Christian Nanoff, Harald Sitte, and Michael Freimuth from the Medical University Vienna for the A$_{2A}$-EYFP expression vector.

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