

## Possible Mechanism of CCL2-induced Akt Activation in Prostate Cancer Cells

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**Abstract.** *C-C chemokine ligand 2 (CCL2) is a chemokine that has been demonstrated to play a pivotal role in prostate cancer tumorigenesis and metastasis. These effects are mediated by the ligand binding to the G protein-coupled receptor (GPCR) C-C chemokine receptor 2 (CCR2). It has recently been demonstrated that CCL2 increases Akt phosphorylation in prostate cancer cells, and prevents prostate cancer cells from autophagic death through activation of Akt pathway. The purpose of this study was to determine the mechanism by which CCL2 activates Akt in prostate cancer PC-3 cell line. CCL2-induced phosphorylation of Akt was inhibited by pertussis toxin and the adenylyl cyclase inhibitor SQ22536. Akt phosphorylation was promoted by prior treatment with cholera toxin. The results suggest that CCL2-induced Akt phosphorylation is mediated by the Gai complex and adenylyl cyclase. This is the first study that demonstrates a direct involvement of adenylyl cyclase in CCL2-induced Akt phosphorylation.*

Prostate cancer is the most commonly diagnosed malignancy of men in the US (1). Advanced prostate cancer is first treated through chemical or surgical castration because a large percentage of the cancer cells are androgen dependent. The large majority of patients, however, relapse within a few years of treatment because of the emergence of a castration-

resistant clone of cancer cells. Castration-resistant prostate cancer is an incurable disease with little effective therapy and there is a great need for novel therapeutic strategies that target the molecular basis of castration-resistant, chemo-resistant prostate cancer (2).

Several studies have indicated that the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway is up-regulated in various types of human cancer. Akt activation is controlled by phosphorylation of two key residues, threonine 308 (Thr308) and serine (Ser473) (3). Akt phosphorylation plays an important role in prostate cancer cell growth, proliferation, motility and survival (4-6). The tumor suppressor PTEN is often mutated in metastatic prostate cancer, leading to constitutive activation of Akt (4, 6). In this context, the Akt signal transduction pathways are attractive potential therapeutic targets for prostate cancer treatment (3-7).

Akt is activated by several growth factors, and the chemokine C-C ligand 2 (CCL2) increases Akt phosphorylation in human prostate cancer, PC-3 cells (7, 8). CCL2 is a member of the C-C beta chemokine family, and is classically known for activating chemotaxis of monocytes/macrophages and other inflammatory cells *via* its receptor CCR2 (9). CCL2 has been reported to be present in the microenvironment of many types of cancer, and CCR2 has been demonstrated to be up-regulated on cancer cells, including prostate cancer (10, 11). It has been previously demonstrated that CCL2 promotes prostate cancer cell proliferation, migration and survival *via* Akt activation-dependent mechanisms (7, 8, 12). The signals of C-C chemokines are thought to be predominantly transmitted *via* the heterotrimeric Gαβγ receptor complex (13). In some kinds of cells (HEK-293, COS-7, monocytes), intracellular signalling pathways are demonstrated, but not in prostate cancer cells (9).

The purpose of this study was to delineate the signal transduction pathway that links the activation of CCR2 with Akt phosphorylation in prostate cancer cells. Here, the possible G protein-coupled signal transduction pathways for CCR2 receptor were analyzed by using pertussis toxin, cholera toxin and SQ22536, an adenylyl cyclase inhibitor in human prostate cancer PC-3 cells.

**Abbreviations:** AC, Adenylyl cyclase; ERK, extracellular-signal-regulated kinase; MEK, mitogen-activated protein kinase; ROCK, Rho-associated kinase; RTK, receptor tyrosine kinase; p-Akt, phospho Akt; p-MLC, phospho-myosin light chain.

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**Key Words:** CCR2, adenylyl cyclase, G protein, pertussis toxin, ERK and Rho/ROCK.

## Materials and Methods

**Reagents.** All antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Pertussis toxin (Ptx), Y27632 and SQ22536 were obtained from CALBIOCHEM (San Diego, CA, USA). Cholera toxin (Ctx) and lysophosphatidic acid (LPA) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and BIOMOL (Plymouth Meeting, PA, USA), respectively. Recombinant human CCL2 was obtained from APOLLO Cytokine Research (Alexandria, Sydney, Australia).

**Cell culture.** PC-3 cells, an androgen-independent human prostate cancer cell line, were obtained from ATCC (Manassas, VA, USA). Cells were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), 100 units/mL penicillin and 100 µg/mL streptomycin (Invitrogen) under a humidified atmosphere of 5% CO<sub>2</sub> at 37°C.

**Western blotting.** Western blotting was performed as described (7). Briefly, 16 to 18 hours after serum starvation, cells were detached with 0.25 mM EDTA and cells were seeded at 3×10<sup>5</sup> cells per wells in 6-well plates. Six hours after seeding, SQ22536 (100 µM), Ptx (100 ng/mL) and Ctx (10 µg/mL) were added and cells incubated for 1 to 4 hours. CCL2 (100 ng/mL) was then added to cells, and cells were harvested at different time points. Cells were suspended in cell lysis buffer (Cell Signaling Technology) containing a protease inhibitor cocktail (Promega, Madison, WI, USA), and 1 µM okadaic acid, and samples were sonicated briefly. After centrifugation at 13,000 rpm for 10 min at 4°C, the resultant supernatant (10 µg of protein) was subjected to SDS-PAGE followed by Western blotting. The membranes (PVDF) were blocked with 5% non-fat milk in 0.1% Tween in Tris-buffered saline for 30 min at room temperature. They were incubated overnight at 4°C with primary antibodies. The immunoreactive bands were detected with horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology) and the ECL detection system (Millipore, Billerica, MA, USA).

**Rho-GTP assay.** The level of GTP-bound Rho was determined with G-LISA™ RhoA Activation Assay Biochem Kit™ (Cytoskeleton Inc., Denver, CO, USA), according to the protocol supplied by the manufacturer. Briefly, 16 to 18 hours after serum starvation, cells were seeded at 3×10<sup>5</sup> cells per well in 6-well plates. Twenty-four hours after seeding, Y27632 (25 mM) was added and cells were incubated for 6 hours. CCL2 (100 ng/mL) and LPA (10 µM) were then added to cells, and cells were harvested as samples after 15 min stimulation.

## Results and Discussion

Previously, CCL2-induced Akt phosphorylation in PC-3 cells was demonstrated (7, 8). Here, it was confirmed that stimulation with CCL2 leads to Akt phosphorylation (at Ser 473 and Thr 308 sites) as measured at 15 min, 30 min and 1 hour (Figure 1A). To confirm what subtype of Gα is involved in CCL2-related signalling, Ptx, a widely accepted Gαi inhibitor, was used. CCL2-induced phosphorylation of Akt was significantly inhibited by Ptx (Figure 1A), indicating that the CCL2-induced Akt pathway is dependent on the Gαi protein complex. This protein complex is made

up of trimetric molecules, consisting of Gαi and Gβγ. Gαi inhibits adenylyl cyclase, and Gβγ regulates various effectors (e.g. subtypes of adenylyl cyclase, GRK, PI3K, Src family kinase, PLCβ and calcium channels) (5, 14-16). It has been well established that Ptx catalyses the ADP-ribosylation of Gαi proteins, resulting in a loss of adenylyl cyclase inhibition (Figure 2) (15). Previous studies with mammalian cells identified several G-protein α subfamilies (Gαi, Gαs, Gα12 and Gαq) (15, 16). Gαi reduces the concentration of cAMP. As yet, there is no evidence of direct activation of PI3K by Gαi subunits (5). The current proposed mechanisms of G-protein coupled receptor of chemokines suggests the stimulation dissociate Gβγ from Gαβγ complex, and dissociated Gβγ activates PI3Kγ (13). It is also reported that types 2, 4 and 7 of adenylyl cyclase are directly activated by Gβγ dimers (15, 16). Therefore, CCL2 may utilize activated Gαi for releasing Gβγ to activate PI3K directly or *via* cAMP up-regulation through the activation of adenylyl cyclase subtypes. To determine whether CCL2-induced Akt phosphorylation is mediated *via* adenylyl cyclase, the effect of the direct adenylyl cyclase inhibitor, SQ22536, on CCL2-induced Akt phosphorylation was examined. CCL2-induced Akt phosphorylation was inhibited by SQ22536 at 3, 5 and 15 min (Figure 1B). If Gαi was a target molecule of CCL2-induced signalling pathway, SQ22536 should increase Akt phosphorylation, however the opposite was observed, suggesting that Gαi is not involved in CCL2-induced Akt phosphorylation in prostate cancer cells. These results suggest that adenylyl cyclase is involved in CCL2-induced Akt phosphorylation, at least in part, and the established mechanism that adenylyl cyclase is activated by Gαs or Gβγ-dimer, not Gαi, lends support for the involvement of Gβγ-dimers related to adenylyl cyclase activation in CCL2-induced Akt phosphorylation in prostate cancer cells.

To verify the influence of adenylyl cyclase activation upon CCL2-induced Akt phosphorylation, PC-3 cells were treated with Ctx, an activator of adenylyl cyclase through Gαs, for 4 hours prior to CCL2 stimulation. The results demonstrated that treatment with Ctx increased Akt phosphorylation (Figure 3). Ctx stimulated Gαs resulting in activation of adenylyl cyclase, and an increase in cAMP. Ultimately, cAMP activates the PI3K/Akt pathway. Furthermore, the CCL2-mediated responses were sensitive to pretreatment of cells with Ctx. These results may indicate the possibility of involvement of adenylyl cyclase in Akt phosphorylation in response to CCL2. Myers *et al.* reported that cAMP accumulation was inhibited by 10 pM - 100 nM CCL2 stimulation in HEK-293 cells (17). In the present study, it is unclear if CCL2 increases cAMP accumulation, and differences of CCL2-induced cAMP accumulation may be dependent on cell type. Precise mechanisms of CCL2-induced cAMP accumulation in several cell lines, including PC-3 cells, remain to be elucidated.

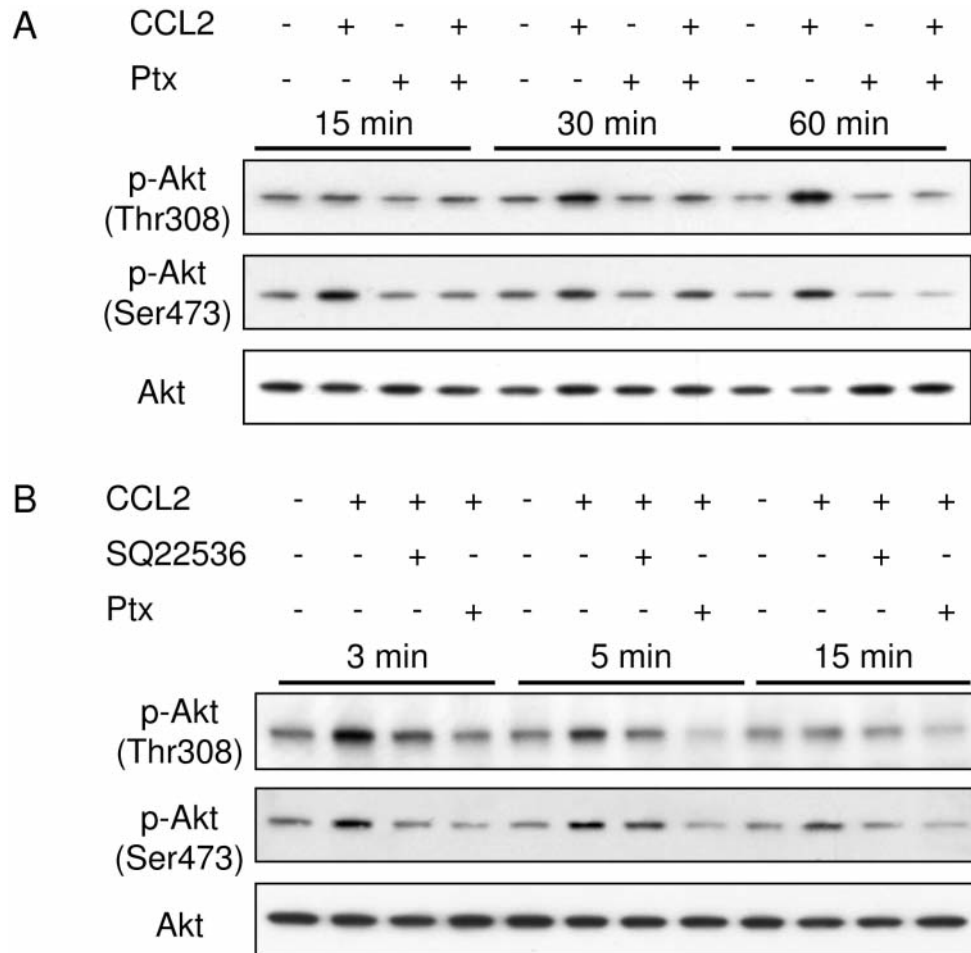


Figure 1. CCL2-induced Akt phosphorylation is dependent on Gai protein complex and adenylyl cyclase. A, Serum-starved PC-3 cells were pre-incubated with Ptx for 4 hours before CCL2 stimulation (100 nM). Top blot, p-Akt (Thr 308); middle, p-Akt (Ser 473); bottom, total Akt. B, Serum-starved PC-3 cells were pre-incubated with SQ22536 and Ptx for 1 and 4 hours respectively before CCL2 stimulation (100 nM). Top blot, p-Akt (Thr 308); middle, p-Akt (Ser 473); bottom, total Akt. The data are representative of two independent experiments.

Other possible signal transduction pathways by CCL2 were also investigated. There are several targets of G $\beta\gamma$  dimer, including the ERK1/2 pathways. To determine whether CCL2-induced Akt phosphorylation pathway crosstalked with other growth factor-stimulated pathways, mitogen-activated protein kinase (MAPK) pathway was investigated. Extracellular signal-regulated kinase 1 (ERK1) activation pathway involves the Ras, Raf MAPKK (MEK) pathway. To investigate CCL2-induced ERK1/2 activation, blots were stripped and re-probed with an anti-phosphorylated ERK1/2 antibody. ERK1/2, however, is not activated by CCL2 stimulation at these time point (3 min to 1 hour) (data not shown). Previous study has indicated that Akt and ERK1/2 were activated in PC-3 cells by 24 hours' stimulation with CCL2 (8). Kleibeulker *et al.* reported both ERK1/2 and Akt are activated by CCL2 in astrocytes at 1-

10 min (18). Studies with human melanoma cells demonstrated that G protein-coupled adenosine A3 receptor stimulation leads to a reduction of phosphorylated ERK1/2 by activation of the PI3K/Akt pathway (19). The crosstalk of these pathways may explain discrepancy of the results at different time point and in different cell lines. Further experiments to determine the precise crosstalk between the PI3K pathway and the ERK pathway are ongoing.

Another potentially important G $\alpha$  subfamily is G $\alpha$ 12 that regulates Rho/ROCK pathway (20). Previous reports suggest that the Rho/ROCK pathway is involved in Akt activation pathway indirectly (Figure 2) (5). In order to examine the effect of CCL2 on Rho/ROCK pathway thorough G $\alpha$ 12, the cells were pre-incubated with 10  $\mu$ M LPA and 25  $\mu$ M Y27632 as a stimulator and a inhibitor of Rho kinase (ROCK), respectively. The activation of the

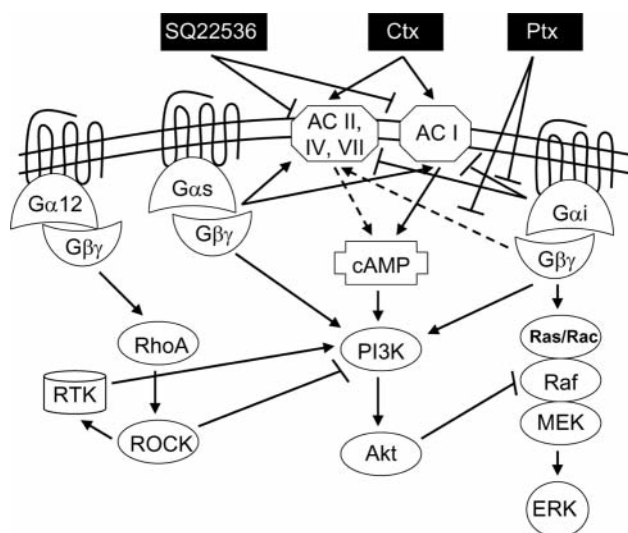


Figure 2. Overview of pathways of possible CCL2-induced Akt activation. Gai inhibits most isoforms of adenylyl cyclase. Subtypes of adenylyl cyclase (II, IV and VII) are activated by Gβγ. Ptx inhibits dissociation of Gβγ from Gaiβγ complex, and may prevent activation of subtypes of adenylyl cyclase (II, IV and VII). Ctx activates adenylyl cyclase via Gas. SQ22536 is an inhibitor of adenylyl cyclase. ROCK pathway inhibits PI3K activation indirectly. The dotted arrows show the possible upstream pathway for the activation of Akt by CCL2 from the results. This figure is drawn from New *et al.* (5).

Pho/ROCK pathway was examined by measuring both Rho-GTP, active GTP-bound Rho and phosphorylated MLC, a downstream ROCK effector. In this experiment, CCL2 stimulation did not increase Rho-GTP and phosphorylation of MLC (Figure 4). These results suggest that CCL2-induced Akt phosphorylation is independent of the Rho/ROCK pathway.

The signalling of the Gαq family including Gα14 and Gα16 by PLC-β is well established (21). Kuang *et al.* reported that CCR2b can couple Gα14 and Gα16, but its splicing variant CCR2a cannot (22). However, there is no report that the Gαq pathway involves adenylyl cyclase; the results presented here indicate that Gα14 and Gα16 are not involved in CCL2-induced Akt phosphorylation in PC-3 cells.

In conclusion, this study is the first to address the elucidation of the GPCR signal transduction pathways associated with the chemokine CCL2 in prostate cancer cells. Adenylyl cyclase appears to have a major role in CCL2-induced Akt activation in PC-3 cells. The results presented here, in combination with previous CCL2 pathway studies, also suggest that the pathway of CCL2-related Akt phosphorylation in PC-3 cells may be different from that in monocytes, transfected HEK-293 cells and COS-7 cells, and the pathway may be an attractive specific target for treatment of prostate cancer.

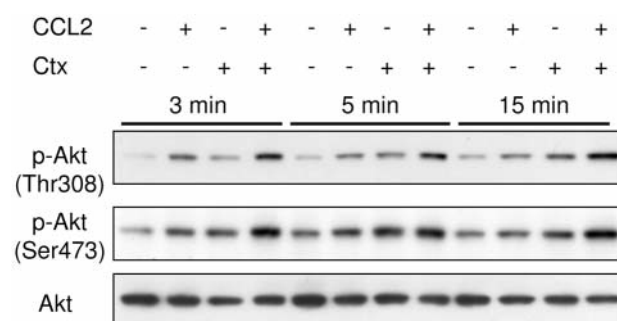


Figure 3. CCL2-induced Akt phosphorylation is accelerated by Ctx. Serum-starved PC-3 cells were pre-incubated with Ctx for 4 hours before CCL2 stimulation (100 μM). Top blot, p-Akt (Thr 308); middle, p-Akt (Ser 473); bottom, total Akt. The data are representative of two independent experiments.

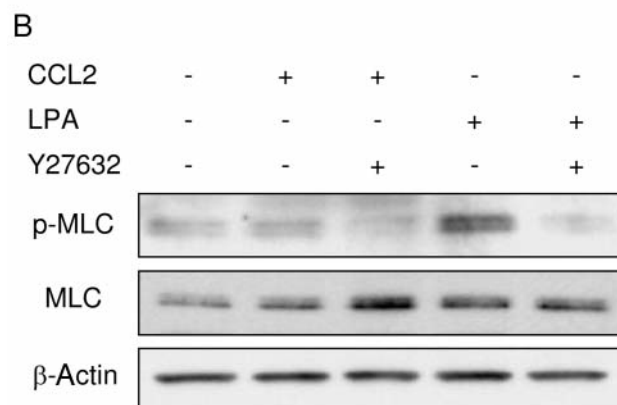
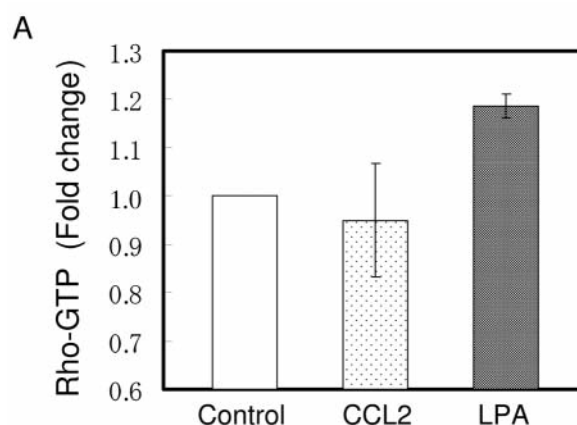


Figure 4. The Rho/ROCK pathway is not involved in CCL2-induced Akt phosphorylation. A, Rho-GTP was quantified as described in Materials and Methods. Fold changes were determined from relative intensity to the control. Values represent means±SD (bars) from three independent experiments. B, Serum-starved PC-3 cells were pre-incubated with Y27632 (25 μM) for 6 hours before stimulation. Top blot, p-MLC; middle, total MLC; bottom, β-actin. The data are representative of two independent experiments.



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## References

- 1 Cancer Facts and Figures. American Cancer Society, 2008 ([http://www.cancer.org/docroot/stt/stt\\_0.asp?from=fast](http://www.cancer.org/docroot/stt/stt_0.asp?from=fast)).
- 2 Isaacs JT: New strategies for the medical treatment of prostate cancer. *BJU Int* 96(Suppl 2): 35-40, 2005.
- 3 Manning BD and Cantley LC: AKT/PKB signaling: navigating downstream. *Cell* 129: 1261-1274, 2007.
- 4 Assinder SJ, Dong Q, Kovacevic Z *et al*: The TGF- $\beta$ , PI3K/Akt and PTEN pathways: established and proposed biochemical integration in prostate cancer. *Biochem J* 417: 411-421, 2009.
- 5 New DC, Wu K, Kwok AW *et al*: G protein-coupled receptor-induced Akt activity in cellular proliferation and apoptosis. *FEBS J* 274: 6025-6036, 2007.
- 6 Majumder PK and Sellers WR: Akt-regulated pathways in prostate cancer. *Oncogene* 24: 7465-7474, 2005.
- 7 Roca H, Varsos Z and Pienta KJ: CCL2 protects prostate cancer PC3 cells from autophagic death *via* phosphatidylinositol 3-kinase/AKT-dependent survivin up-regulation. *J Biol Chem* 283: 25057-25073, 2008.
- 8 Loberg RD, Ying C, Craig M *et al*: Targeting CCL2 with systemic delivery of neutralizing antibodies induces prostate cancer tumor regression *in vivo*. *Cancer Res* 67: 9417-9424, 2007.
- 9 New DC and Wong YH: CC chemokine receptor-coupled signalling pathways. *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao* 35: 779-788, 2003.
- 10 Craig MJ and Loberg RD: CCL2 (monocyte chemoattractant protein-1) in cancer bone metastases. *Cancer Metastasis Rev* 25: 611-619, 2006.
- 11 Lu Y, Cai Z, Xiao G *et al*: CCR2 expression correlates with prostate cancer progression. *J Cell Biochem* 101: 676-685, 2007.
- 12 Loberg RD, Day LL, Harwood J *et al*: CCL2 is a potent regulator of prostate cancer cell migration and proliferation. *Neoplasia* 8: 578-586, 2006.
- 13 Thelen M: Dancing to the tune of chemokines. *Nat Immunol* 2: 129-134, 2001.
- 14 Schwindinger WF and Robishaw JD: Heterotrimeric G-protein  $\beta\gamma$ -dimers in growth and differentiation. *Oncogene* 20: 1653-1660, 2001.
- 15 Fields TA and Casey PJ: Signaling functions and biochemical properties of pertussis toxin-resistant G-proteins. *Biochem J* 321: 561-571, 1997.
- 16 Milligan G and Kostenis E: Heterotrimeric G-proteins: a short history. *Br J Pharmacol* 147: S46-S55, 2006.
- 17 Myers SJ, Wong LM and Charo IF: Signal transduction and ligand specificity of the human monocyte chemoattractant protein-1 receptor in transfected embryonic kidney cells. *J Biol Chem* 270: 5786-5792, 1995.
- 18 Kleibeuker W, Jurado-Pueyo M, Murga C *et al*: Physiological changes in GRK2 regulate CCL2-induced signaling to ERK1/2 and Akt but not to MEK1/2 and calcium. *J Neurochem* 104: 979-992, 2008.
- 19 Merighi S, Benini A, Mirandola P *et al*: Modulation of the Akt/Ras/Raf/MEK/ERK pathway by A3 adenosine receptor. *Purinergic Signal* 2: 627-632, 2006.
- 20 Somlyo AP and Somlyo AV: Signal transduction by G-proteins, rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. *J Physiol* 522: 177-185, 2000.
- 21 Mizuno N and Itoh H: Functions and regulatory mechanisms of Gq-signaling pathways. *Neurosignals* 17: 42-54, 2009.
- 22 Kuang Y, Wu Y, Jiang H and Wu D: Selective G protein coupling by C-C chemokine receptors. *J Biol Chem* 271: 3975-3978, 1996.

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