Enhanced fMLP-stimulated chemotaxis in human neutrophils from individuals carrying the G protein β3 subunit 825 T-allele

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Abstract We have recently described a C825T polymorphism in the gene encoding for the Gβ3 subunit of heterotrimeric G proteins. The 825T allele is associated with a novel splice variant (Gβ3-s) and enhanced signal transduction via pertussis toxin (PTX)-sensitive G proteins. fMLP-induced chemotaxis, but not O^{2-} generation, was increased in neutrophils with the TC/TT (EC_{50} = 1.5 ± 1.3 nM) genotypes compared to the CC genotype (EC_{50} = 5.9 ± 1.5 nM). Maximal fMLP-induced increase in $[Ca^{2+}]_i$ was significantly reduced in neutrophils from individuals with TC/TT genotype vs. CC genotype (212.9 ± 10.1 nM vs. 146.4 ± 24.2 nM). Gβ3-s appears to be associated with enhanced immune cell function in humans.

Key words: Hypertension; Superoxide generation; Signal transduction

1. Introduction

We recently described a C825T polymorphism in the gene GNB3 encoding for the ubiquitously expressed β3 subunit of heterotrimeric G proteins [1]. The 825T allele is associated with a novel splice variant of Gβ3 (Gβ3-s) which, although being 41 amino acids shorter than the wild-type Gβ3, is biologically active. Predominant expression of Gβ3-s in lymphoblasts and platelets from individuals carrying at least one 825T allele was confirmed by Western blot analysis. Moreover, the 825T allele is associated with enhanced signalling via receptors coupled to PTX-sensitive G proteins [2]. Since previous studies have shown that fMLP-stimulated chemotaxis, Ca^{2+} signals and O^{2-} generation are mediated by PTX-sensitive G proteins [3–6], we quantified signal transduction and cellular functions of fMLP-stimulated neutrophils from individuals homozygous for the GNB3 C825 allele, or homo- or heterozygous for the GNB3 825T allele, respectively. Our data show enhanced fMLP-induced chemotaxis in neutrophils from individuals carrying the GNB3 825T allele, whereas fMLP-stimulated O^{2-} generation is similar in GNB3 C825 and 825T allele carriers.

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Abbreviations: BSA, bovine serum albumin; $[Ca^{2+}]_i$, intracellular free calcium concentration; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; HBSS, Hanks' balanced salt solution; HPF, high power field; PMA, phorbol 12-myristate 13-acetate; PTX, pertussis toxin

2. Materials and methods

2.1. Materials

RPMI 1640 medium, BSA, fMLP, collagen type I, PMA, and lucigenin (methylacridinium nitrate) were from Sigma (Deisenhofen, Germany). Fura-2/AM was from Molecular Probes (Eugene, OR, USA), and PTX from List Biological Laboratories (Campbell, CA, USA). Ficoll-Paque was from Pharmacia (Uppsala, Sweden). Polyvinylpyrrolidine-free polycarbonate filter membranes were from Nucleopore (Tübingen, Germany), and staining solution Diff-Quick from Dade (Duedingen, Switzerland).

2.2. Isolation of human neutrophils

Venous blood was obtained from healthy, male, non-smoking individuals after informed consent. Human neutrophils were isolated through sedimentation of erythrocytes with dextran, followed by centrifugation through Ficoll-Paque gradient and hypotonic lysis of residual erythrocytes [7]. Thereafter, neutrophils were resuspended into RPMI 1640 or HBSS medium, containing 135 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM D-glucose, buffered at pH 7.4 with 20 mM HEPES. These final preparations consisted of at least 95% neutrophils as determined by immediate staining of samples with Diff-Quick according to the manufacturer's instructions. In some experiments cells were treated for 2 h with PTX (100 ng/ml) prior to the experiments.

2.3. Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined using the fluorescent calcium indicator dye Fura-2 in a Perkin-Elmer LS 5B spectrofluorometer at 37°C as described [8]. Briefly, neutrophils were loaded with 2.5 μM Fura-2/AM at 37°C in RPMI 1640 containing 0.1% BSA. After 45 min incubation, cells were pelleted and resuspended in HBSS. Calibration of fluorescence data in terms of $[Ca^{2+}]_i$ was performed as described [8]. Data represent maximal increases (peak values) of $[Ca^{2+}]_i$ after stimulation.

2.4. Chemotaxis assay

The bottom wells of a 48-well microchemotaxis chamber (NeuroProbe, Cabin John, MD, USA) were filled with 25 μl RPMI 1640 containing the receptor agonists and were carefully covered with a polyvinylpyrrolidone-free polycarbonate filter membrane with a pore size of 3 μm coated with collagen type I (100 μg/ml for 2 h). After adjustment of the top plate, 50 μl of the cell suspension with a density of 0.5×10^6 cells/ml were applied to the upper wells. After 1 h incubation in a humidified atmosphere of 5% CO_2 at 37°C, the filter membrane was removed from the chemotaxis chamber, and non-migrated cells were wiped off by drawing the filter membrane carefully over a wiper blade. The filter membrane was air-dried, stained in Diff-Quick-staining solution according to the manufacturer's instructions and finally mounted on a microscope slide with the cell side up and embedded with Entelan. Migrated cells were quantified on the lower side of the filter by counting three random areas of each well under a microscope with a magnification of 400-fold (HPF), and mean ± S.D. for each well was calculated [9,10]. In some experiments, fMLP was also added to the cells in the upper wells of the microchemotaxis chamber.

2.5. Measurement of O^{2-} generation

O^{2-} generation was measured with the lucigenin assay in a lumin-

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ometer as described previously [11]. Briefly, neutrophils resuspended in HBSS at a density of 1×10^6 cells/ml were transferred to luminometer cuvettes in 360-μl aliquots. During continuous stirring lucigenin was added to a final concentration of 0.1 mg/ml and equilibration was allowed for 2 min. Finally, cells were stimulated with receptor agonists or PMA in a total volume of 400 μl, and responses were recorded continuously. Stimulation with 100 nM PMA served as positive control for all cells and represent maximal, G protein independent O^{2-} generation. The chemoluminescence peak in response to 100 nM PMA was defined as 100% value.

2.6. DNA genotyping

DNA extraction from blood and genotyping was performed as recently described [2].

2.7. RT-PCR

RNA was prepared with the RNeasy Kit obtained from Qiagen according to the manufacturer's instructions. The reverse transcription was performed with Superscript from Gibco-BRL using oligo-dT-primers followed by PCR with the Gβ3-specific primers 5′-CGGGAGCTTTCTGCTCACAC-3′ (sense) and 5′-TGTTCACTGCCTTCCACTTCC-3′ (anti-sense). PCR reactions began with denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C (1 min), annealing at 60°C (45 s), extension at 72°C (1 min) and a final extension at 72°C (7 min). PCR products were size-fractionated on 2.5% agarose gels containing ethidium bromide and visualized under UV illumination. RT-PCR products encoding Gβ3-s with a size of 528 bp are 123 bp smaller than those encoding wild-type Gβ3 with a size of 651 bp [2]. Neutrophils from each three homozygous C825 allele carriers and heterozygous 825T allele carriers were investigated. Results were confirmed using samples from independent reverse transcription reactions.

2.8. Data presentation and analysis

For final analysis, CC genotypes were compared with combined TC/TT genotypes, since the 825T allele is associated with alternative splicing [2]. All experiments were performed in a blinded fashion, i.e. investigators were unaware of the individuals' genotypes. Data represent means ± S.E.M. from independent experiments as indicated in the figure legends. Concentration response curves were calculated using iterative, non-linear regression analysis (Prism2, GraphPAD Software, San Diego, CA, USA). S.D. of EC_{50} values represent the larger difference calculated from the asymmetric, logarithmic S.D. given by Prism. Differences were regarded significant at $P<0.05$ (Student's *t*-test).

3. Results

3.1. Detection of mRNA encoding Gβ3 in human neutrophils

Performing RT-PCR, we were able to detect mRNA encoding Gβ3 and Gβ3-s in human neutrophils. In neutrophils from individuals homozygous for the 825C allele, only one RT-PCR product with the predicted size of 651 bp could be detected. An additional, smaller band of approximately 528 bp was exclusively found in RNA preparations from neutrophils of heterozygous 825T allele carriers suggesting the presence of an alternatively spliced, shorter variant of Gβ3, i.e. Gβ3-s (Fig. 1).

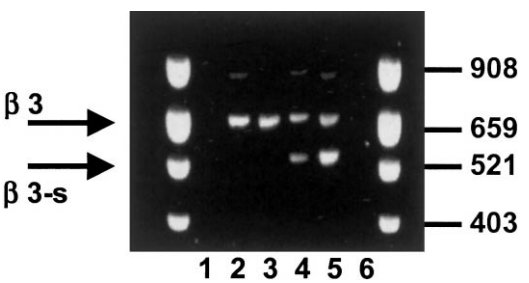

Fig. 1. Detection of Gβ3- and Gβ3-s-specific PCR products in neutrophils from individuals with *GNB3* CC or TC genotype. Displayed is a representative result. Lanes 2, 3: CC genotype; lanes 4, 5: TC genotype; lanes 1 and 6: blank (negative control). Size standard: *Alu*I-digested pBR322 DNA. For details see Section 2.

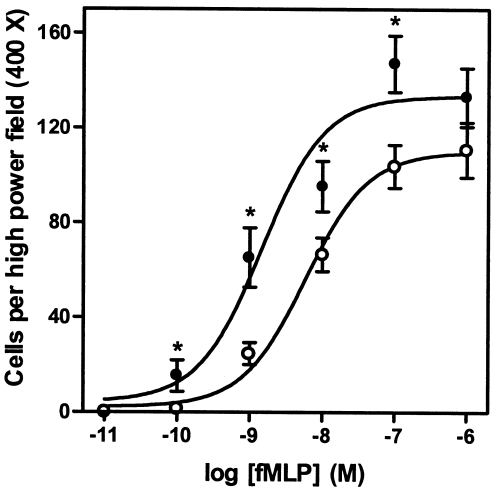

Fig. 2. fMLP-stimulated chemotaxis in human neutrophils. Data shown are means ± S.E.M. of migrated neutrophils per high power field. Experiments were performed in quadruplicate for each individual. ○, CC genotype ($n=12$); ●, TC ($n=8$) and TT ($n=2$) genotypes. $^*P<0.05$.

3.2. fMLP-stimulated chemotaxis of human neutrophils

Treatment with PTX completely inhibited fMLP-stimulated chemotaxis in neutrophils regardless of GNB3 genotype (not shown). Chemotaxis was enhanced in neutrophils from individuals with the GNB3 TC or TT genotypes upon stimulation with fMLP concentrations ranging from 0.1 to 100 nM (Fig. 2). The potency of fMLP was significantly increased in neutrophils from individuals with TC/TT-genotype ($EC_{50}=1.5\pm1.3$ nM) compared to neutrophils from individuals with CC-genotype ($EC_{50}=5.9\pm1.5$ nM). Maximum chemotactic response after stimulation with 1 μM fMLP was similar in all genotypes and averaged 133.0 ± 12.4 (TC/TT) vs. 110.7 ± 11.7 (CC) cells per HPF. To ascertain that the fMLP-stimulated response was chemotactic, fMLP was also added to the cells in the upper wells. Compared to basal migration of 30.2 ± 6.1 (CC, $n=10$) vs. 37.8 ± 3.9 (TC, $n=7$; TT, $n=3$) cells per HPF in the absence of fMLP, random migration was not significantly induced. If fMLP was present in equal concentrations in the lower and upper wells, stimulation with 1 nM fMLP led to 35.9 ± 3.8 (CC) vs. 45.2 ± 4.0 (TC/TT) cells per HPF, stimulation with 30 nM fMLP led to 46.2 ± 4.0 (CC) vs. 42.6 ± 8.8 (TC/TT) cells per HPF, and the response to 1 μM fMLP present in the upper and lower wells was 33.6 ± 4.6 (CC) vs. 37.7 ± 5.3 (TC/TT) cells per HPF. Furthermore, if the concentration of fMLP was higher in the upper than in the lower wells, migration of neutrophils was slightly reduced compared to basal migration with no significance between genotypes (not shown). These results indicate that the enhanced neutrophil migration in response to fMLP stimulation was chemotactic (directed) rather than chemokinetic (random).

3.3. Effect of fMLP on $[Ca^{2+}]_i$ in human neutrophils

Baseline values of $[Ca^{2+}]_i$ were not significantly different in neutrophils from individuals with CC or TC genotype (58 ± 4.5 nM and 88 ± 15 nM, respectively). fMLP-evoked

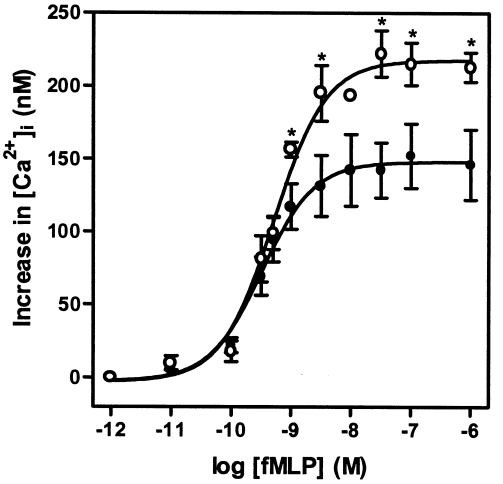

Fig. 3. fMLP-stimulated increase in $[Ca^{2+}]_i$ in human neutrophils. Values represent peak increases in $[Ca^{2+}]_i$. ○, CC genotype ($n=7$); ●, TC genotype ($n=7$). *$P<0.05$.

Ca^{2+} signals were completely blocked after pre-treatment with 100 ng/ml PTX for 2 h regardless of GNB3 genotype (data not shown). The potency of fMLP to increase $[Ca^{2+}]_i$ (Fig. 3) was slightly higher in neutrophils from individuals with the TC/TT genotype ($EC_{50} = 0.33 \pm 0.04$ nM) compared to the CC genotype ($EC_{50} = 0.52 \pm 0.05$ nM). Surprisingly, the increase in $[Ca^{2+}]_i$ above baseline evoked by fMLP at concentrations ranging from 1 nM to 1 μM was significantly smaller in individuals carrying a 825T allele, and averaged to 212.9 ± 10.1 nM (CC genotype) and 146.4 ± 24.2 nM (TC genotype) at 1 μM fMLP. The plateau phase of elevated $[Ca^{2+}]_i$ following the initial peak, which most likely represents continuous trans-membrane Ca^{2+} influx, was not significantly different in individuals with CC and TC genotype (Fig. 4A,B; 130 ± 11.1 nM vs. 109.3 ± 8.1 nM).

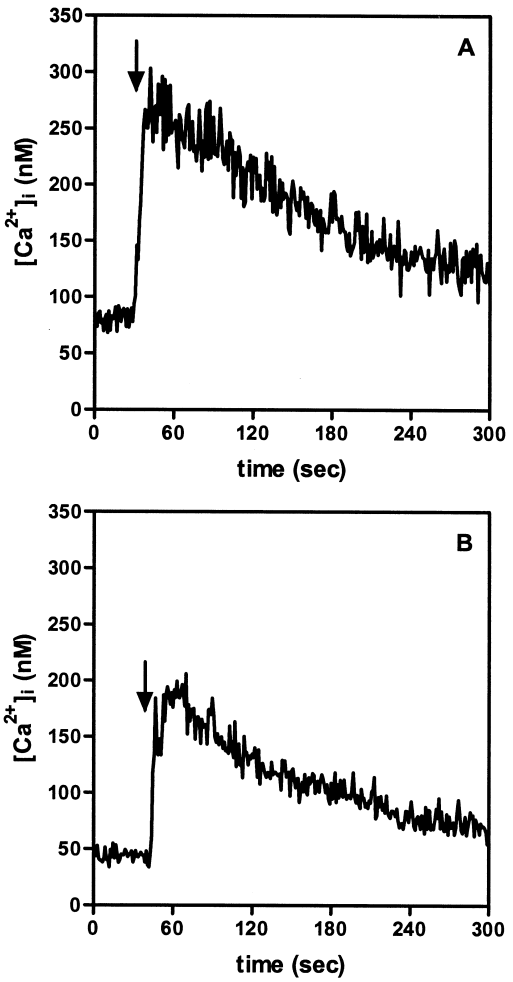

Fig. 4. Changes in $[Ca^{2+}]_i$ after the addition of 1 μM fMLP in human neutrophils with CC genotype (A) and TC genotype (B). Time courses are representative for several independent experiments ($n=7$). Arrow indicates addition of fMLP.

3.4. *fMLP-stimulated O^{2-} generation*

Pre-treatment with 100 ng/ml PTX for 2 h completely inhibited fMLP-, but not PMA-stimulated O^{2-} generation (data not shown). Values observed upon stimulation with 100 nM PMA were defined as control (100% values). O^{2-} generation in response to increasing concentrations of fMLP was virtually independent of GNB3 genotype (Fig. 5) and averaged 68.9 ± 15.8% (CC) and 69.6 ± 21.8% (TC/TT) of that evoked by 100 nM PMA. Moreover, the calculated EC_{50} values for fMLP-stimulated O^{2-} generation were rather similar (58.4 ± 16.1 vs. 54.6 ± 26.1 nM; CC vs. TC/TT genotypes, respectively).

4. Discussion

Recently, a C825T polymorphism in exon 10 of the GNB3 gene was described which is associated with alternative splicing of the gene and the generation of a functionally active splice variant (Gβ3-s) in which 41 amino acids are lacking. The finding that all alternatively spliced gene products carried the corresponding T→C substitution suggested, but did not finally prove a functional role of the 825T allele in alternative splicing. Alternatively, a functionally relevant, but hitherto undetected gene alteration could be in linkage disequilibrium with the 825T allele. Gβ3-s appears predominantly expressed

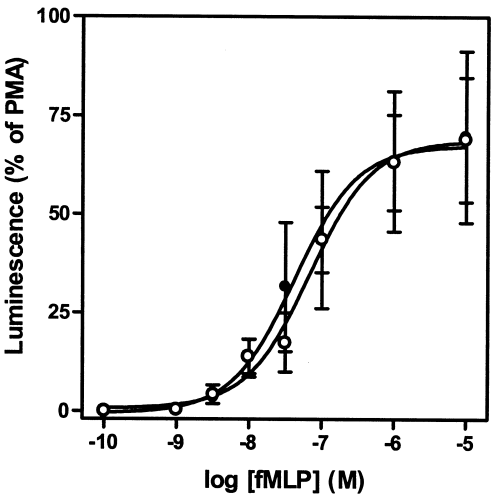

Fig. 5. Superoxide anion generation in fMLP-stimulated human neutrophils. Peak increases in chemoluminescence were determined using the lucigenin assay as described in Section 2. Data are expressed as percent of values measured upon stimulation with 100 nM PMA (100% value). ○, CC genotype ($n=4$); ●, TC genotype ($n=4$). *$P<0.05$.

in cells from individuals carrying the 825T allele, suggesting that this allele enhances the recognition of a cryptic splice site contained in exon 9 of the GNB3 gene [2].

fMLP is a potent chemoattractant for neutrophils representative of a variety of bacterial-derived peptides, and the fMLP receptor is known to activate PTX-sensitive G proteins [3–6,12]. Given the strong association between the GNB3 825T allele and enhanced PTX-sensitive cell responses in lymphoblasts [2,8] and fibroblasts [13], we determined PTX-sensitive, fMLP-stimulated cell responses in neutrophils from individuals with defined genotype at the GNB3 locus. The genotype-related expression of Gβ3-s in human neutrophils could not be confirmed by immunoblots, presumably because the commercially available anti Gβ3 antibody is rather insensitive [2]. This is also illustrated by other studies. Although a cDNA encoding for Gβ3 could be cloned from rat heart [14], the same group of investigators failed to detect Gβ3 protein in neonatal and adult rat cardiac tissue [15]. However, we were able to detect Gβ3-specific RT-PCR products in human neutrophils and a PCR product of the predicted reduced size, most likely representing Gβ3-s, exclusively in neutrophils from individuals heterozygous for the 825T allele. Material from homozygous 825T allele carriers was not available for these investigations. We speculate that Gβ3 and Gβ3-s are expressed at rather low concentrations, but nevertheless may play a decisive role in intracellular signal transduction.

The finding of an enhanced fMLP-stimulated chemotaxis of neutrophils from individuals carrying a 825T allele stays in line with the pivotal role of G protein βγ subunits in chemotaxis [16,17] and may suggest that expression of Gβ3-s could enhance this cell function. Preliminary results from our laboratory actually suggest that COS-7 cells display enhanced chemotaxis upon transfection with Gβγ heterodimers containing Gβ3-s compared to wild-type Gβ3 (Virchow, unpublished). In contrast, the significantly reduced Ca^{2+} signal at higher concentrations of fMLP in neutrophils from individuals carrying the GNB3 825T allele was unexpected, especially because PTX-sensitive Ca^{2+} signals were previously found to be enhanced in cell lines from which the cDNA encoding for Gβ3-s was originally cloned [8]. Additional studies will have to clarify the underlying mechanisms. Potentially, expression of Gβ3-s could result in differential activation of phospholipase C isoforms [12,18,19], alter cellular Ca^{2+} transport mechanisms, or could influence receptor activation via altered interaction with G protein-coupled receptor kinases. [20]. It should be noted that the observation of enhanced chemotaxis despite reduced Ca^{2+} signals in neutrophils from individuals with the TC/TT genotypes is not in contradiction. The chemotactic response does not solely depend on a rise of $[Ca^{2+}]_i$ and, conversely, stimulation of a variety of G protein-coupled receptors does not result in a chemotactic response although $[Ca^{2+}]_i$ is significantly increased [9,16,17].

Efficacy of fMLP-stimulated O^{2-} generation was independent from GNB3 genotype. Others reported that O^{2-} generation of neutrophils is independent from $[Ca^{2+}]_i$ [21]. This may explain why reduced $[Ca^{2+}]_i$ signals in 825T allele carriers at higher fMLP concentrations does not result in correspondingly decreased O^{2-} generation.

The present report is the first to show an enhanced chemotactic response associated with the GNB3 825T allele. Future studies will have to demonstrate whether this also holds true for other compounds inducing chemotaxis in neutrophils, e.g. interleukin 8. Furthermore, it will be interesting to see whether enhanced cell responsiveness in carriers of the 825T allele is restricted to neutrophils or can be also observed in other cells of the immune system, e.g. lymphocytes.

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References

[1] Ansari-Lari, M.A., Muzny, D.M., Lu, J., Lu, F., Lilley, C.E., Spanos, S., Malley, T. and Gibbs, R.A. (1996) Genome Res. 6, 314–326.
[2] Siffert, W., Rosskopf, D., Siffert, G., Busch, S., Moritz, A., Erbel, R., Sharma, A.M., Ritz, E., Wichmann, H.E., Jakobs, K.H. and Horsthemke, B. (1998) Nat. Genet. 18, 45–48.
[3] Spangrude, G.J., Sacchi, F., Hill, H.R., van Epps, D.E. and Daynes, R.A. (1985) J. Immunol. 135, 4135–4143.
[4] Feister, A.J., Browder, B., Willis, H.E., Mohanakumar, T. and Ruddy, S. (1988) J. Immunol. 141, 228–233.
[5] Iiri, T., Ohoka, Y., Ui, M. and Katada, T. (1992) J. Biol. Chem. 267, 1020–1026.
[6] Ruotsalainen, M. and Savolainen, K.M. (1995) Toxicology 99, 67–76.
[7] Boyum, A. (1968) Scand. J. Clin. Lab. Invest. 21, 77–89.
[8] Siffert, W., Rosskopf, D., Moritz, A., Wieland, T., Kaldenberg-Stasch, S., Kettler, N., Hartung, K., Beckmann, S. and Jakobs, K.H. (1995) J. Clin. Invest. 96, 759–766.
[9] Lümmen, G., Virchow, S., Rümenapp, U., Schmidt, M., Wieland, T., Otto, T., Rübben, H. and Jakobs, K.H. (1997) Naunyn-Schmiedeberg's Arch. Pharmacol. 356, 769–776.
[10] Allport, J.R., Donnelly, L.E., Hayes, B.P., Murray, S., Rendell, N.B., Ray, K.P. and MacDermot, J. (1996) Br. J. Pharmacol. 118, 1111–1118.
[11] Bhakdi, S. and Martin, E. (1991) Infect. Immun. 59, 2955–2962.
[12] Jiang, H., Kuang, Y., Wu, Y., Smrcka, A., Simon, M.I. and Wu, D. (1996) J. Biol. Chem. 271, 13430–13434.
[13] Pietruck, F., Moritz, A., Montemurro, M., Sell, A., Busch, S., Rosskopf, D., Virchow, S., Esche, H., Brockmeyer, N., Jakobs, K.H. and Siffert, W. (1996) Circ. Res. 79, 974–983.
[14] Ray, K. and Robishaw, J.D. (1994) Gene 149, 337–340.
[15] Hansen, C.A., Schroering, A.G. and Robishaw (1995) J. Mol. Cell. Cardiol. 27, 471–484.
[16] Neptune, E.R. and Bourne, H.R. (1997) Proc. Natl. Acad. Sci. USA 94, 14489–14494.
[17] Arai, H., Tsou, C.L. and Charo, I.F. (1997) Proc. Natl. Acad. Sci. USA 94, 14495–14499.
[18] Sternweis, P.C. (1994) Curr. Opin. Cell Biol. 6, 198–203.
[19] Camps, M., Hou, C., Sidiropoulos, D., Stock, J.B., Jakobs, K.H. and Gierschik, P. (1992) Int. J. Biochem. 206, 821–831.
[20] Daaka, Y., Pitcher, J.A., Richardson, M., Stoffel, R.H., Robishaw, J.D. and Lefkowitz, R.J. (1997) Proc. Natl. Acad. Sci. USA 94, 2180–2185.
[21] Liang, S.L., Woodlock, T.J., Whitin, J.C., Lichtman, M.A. and Segel, G.B. (1990) J. Cell. Physiol. 145, 295–302.