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SENSITIZATION ENHANCES THE ADENYLYL CYCLASE RESPONSIVENESS IN ALVEOLAR MACROPHAGES

CHANGES INDUCED AT POST-RECEPTOR LEVEL

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Abstract—Using membrane fractions (MF) from guinea pig alveolar macrophages (AM), we investigated the effects of sensitization and antigen challenge on the stepwise activation of adenylyl cyclase considering receptor binding, G-protein coupling and direct stimulation of the enzyme. Receptor binding studies, using [^{125}I]ICYP as the β-adrenoceptor specific ligand, show that neither receptor number (B_{max}) nor receptor affinity constants (K_d values) were affected by sensitization or antigen challenge. Using forskolin as a direct stimulant of AC, alterations in the enzymatic activity of AC could be excluded. Pretreatment of the different MF with cholera toxin (CT, a toxin which eliminates GTPase activity) and subsequent stimulation of AC with GTP, shows an increased responsiveness in MF from sensitized and antigen challenged AM. In addition, pretreatment of MF from naive AM with increasing doses of CT results in a maximal AC response at the higher concentrations used (50–100 μg/mL), an effect not observed in MF from sensitized and antigen challenged AM. In these MF, the AC response still increases after pretreatment with such doses of CT. These data suggest that the enhanced AC responsiveness in AM, induced by sensitization and antigen challenge, results from alterations in α_s-subunits.

Recently, substantial evidence has been raised supporting a role for alveolar macrophages (AM) in pulmonary inflammatory processes accompanying bronchial hyperreactivity in asthma. Upon exposure to several stimuli, AM from asthmatic patients, compared to AM from control subjects, show enhanced release of reactive oxygen species [1], lysozomal enzymes [2, 3] and different inflammatory mediators like PAF-acether and leukotrienes and IL-1 [4–6].

Macrophages may respond to certain hormones and inflammatory mediators (like prostaglandins) by stimulation of adenylyl cyclase resulting in enhanced cAMP levels which generally induce a down-regulation of cellular activity (e.g. phagocytosis, cytotoxicity, lysozomal enzyme secretion and O_2^- production [7, 8]).

Ovalbumin sensitization of guinea pigs is a commonly used animal model to study allergic bronchial asthma. We previously described [9] that AM obtained from sensitized and antigen challenged guinea pigs showed, compared to AM from naive guinea pigs, a marked enhanced adenylyl cyclase response to various stimuli like β-adrenergic agonists, prostanoids and histamine. As yet, it is unclear which processes induced by ovalbumin sensitization are responsible for the observed enhanced adenylyl cyclase responsiveness. In studies of desensitization mechanisms of adenylyl cyclase, alterations in several components have been considered: receptor density and receptor configuration [10], receptor–G-protein coupling [11] or the modulation of α-subunit quantity [12]. Possibly, sensitization has disregulated such phenomena in an analogous—though opposite to desensitization—way.

The signal transduction system leading to the formation of cAMP is complex as it comprises several interactions of closely related components. As outlined by Gilman [13], receptor occupancy promotes the dissociation of the heterotrimeric G_s-complex yielding free α_s-subunits which become activated through replacement of bound GDP by GTP. In the GTP-activated state, the α_s-subunits interact with adenylyl cyclase resulting in the production of cAMP. Hydrolysis of bound GTP by a GTPase, intrinsic to the α_s-subunit terminates the activating signal. The α_s-subunit reassociates with the $\beta\gamma$-subunit subsequently decreasing the adenylyl cyclase activity and the system is primed for another activation cycle. Changes in the separate steps of this activation cascade will thus result in modified cAMP production and ultimately in altered cellular activity. Indeed, using lung homogenates from ovalbumin sensitized guinea pigs, Gadd and Bhoola [14] have reported an increase in adenylyl cyclase activity whereas the responsiveness to activation by β-adrenergic agonists was reduced. They proposed a selective uncoupling of stimulatory receptors to the guanine nucleotide regulatory protein. In contrast, using tracheal spirals and lung parenchymal strips, Burka and Saad [15] have shown that ovalbumin sensitization of guinea pigs did not induce any change in the adenylyl cyclase activity of the airway tissues.

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To elucidate whether the enhanced adenylyl cyclase responsiveness in AM originates from alterations in this membrane signalling transduction system, we examined the stepwise activation of adenylyl cyclase in alveolar macrophages, considering receptor binding, G-protein coupling and direct activation of the catalytic unit.

MATERIALS AND METHODS

Reagents. IBMX (3-isobutyl-1-methyl-xanthine), a phosphodiesterase-inhibitor, was obtained from Janssen Chimica (Beersse, Belgium) and GTP (guanosine-5'-triphosphate disodium salt), GMP-PCP (guanylyl (β-γ-methylene)-disphosphonate, tetralithium salt) and ATP from Boehringer (Mannheim, F.R.G.). Prostaglandin E_2, (−)-isoprenaline hydrochloride and cholera toxin (CT) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). CT was preactivated shortly before use by incubation at 37° for 20 min with 30 mM dithiothreitol in 0.1 M phosphate buffer (pH 7.4) to release an enzymatically active A_1 fragment [16].

Animals, sensitization and antigen challenge. Male Hartley guinea pigs (weighing 300–500 g) were used throughout the study. Naive animals were anaesthetized by i.p. injection of 70 mg/kg sodium pentobarbitone, the trachea was cannulated and bronchoalveolar lavage was performed by repeated lavages of 8 mL volumes of 0.9% saline (total of 150 mL). Sensitized and antigen challenged alveolar cells were obtained as described before [9]. Briefly, animals were sensitized by i.p. and s.c. injection of an ovalbumin solution (each 50 mg in 0.9% sterile saline). After a 2-week latent period, the animals were either subjected to the normal lavage procedure, rendering sensitized macrophages, or received a booster-injection of ovalbumin after which the lavage was proceeded as normal, rendering antigen challenged macrophages.

Isolation of alveolar macrophages. Bronchoalveolar lavage fluids were filtered through surgical gauze and centrifuged at 400 *g* for 10 min at 4°. After resuspension of the cell pellet in Gey Balanced Salt Solution (GBSS) alveolar macrophages were purified by a Ficoll-Isopaque (Nycomed, Oslo, Norway) gradient centrifugation (400 *g*, 30 min, 4°). More than 95% of the cell suspension obtained by this method consisted of macrophages as judged by May Grünwald Giemsa staining of cytofuge preparations. Viability was tested by Trypan Blue exclusion and always exceeded 95%. The isolated cells were washed thoroughly with GBSS and stored at −70°.

Adenylyl cyclase assay. Membrane fractions (MF) of alveolar macrophages were prepared by disrupting the macrophages in sucrose buffer (0.25 M sucrose, 50 mM Tris–HCl, 25 mM KCl and 5 mM $MgCl_2$, pH 7.4) with a Potter-Elvehjem homogenizer followed by centrifugation at 50,000 *g* for 120 min. The resultant membrane pellet was washed twice in a Tris–HCl buffer (50 mM Tris–HCl, 5 mM $MgSO_4$, 2 mM EGTA and 0.4 mM IBMX) by centrifugation. Protein content was measured according to the method of Lowry *et al.* [17]. Membrane fractions were resuspended at a final protein concentration of 1 mg/mL in the Tris–HCl buffer (pH 7.4). Aliquots of 40 μL membrane suspensions were incubated for 15 min at 30° in 40 μL Tris–HCl buffer containing 1.6 mM ATP and 0.8 mg/mL bovine serum albumin in the presence or absence of forskolin, GTP or GMP-PCP. Pretreatment of MF with CT was performed by incubation of four separate portions of MF (1 mg/mL each) with 0, 25, 50 or 100 μg/mL CT for 16 hr at 4°. The incubation was continued as described above, without previous washing of the treated MF, in the presence of 10^{-4} M GTP. After incubation, samples were boiled for 3 min and centrifuged for 3 min at 12,000 *g*. Content of cAMP was determined by radioimmunoassay using a high affinity binding protein as previously described [18] (cAMP levels are expressed as pmol/mg protein/min).

[^{125}I]*ICYP binding assay.* Binding studies were performed as described before [19]. Briefly, membrane suspensions (10 μg) were incubated with increasing concentrations (5–200 pM) of (−)-3-[^{125}I]iodocyanopindolol ([^{125}I]CYP) in the absence and presence of 0.1 μM timolol to define total and non-specific binding. At a concentration of 80 pM [^{125}I]ICYP, the specific binding was 60% of the total binding. The incubation was performed in a total volume of 200 μL in 50 mM Tris–HCl, containing 10 mM $MgCl_2$ (pH 7.4 at 37°). After 1 hr, samples were rinsed with 50 mM Tris–HCl, containing 10 mM $MgCl_2$ (pH 7.4 at 4°) and filtered through glass fiber filters. This procedure was repeated once, whereafter the filters were washed with cold buffer. Radioactivity was counted using a gamma-counter with an efficiency of 68%.

Data analysis. All data are expressed as the means ± standard error of the mean (SEM). Statistical significance was evaluated by the unpaired Student's *t*-test.

RESULTS

Basal adenylyl cyclase activities of MF from naive and antigen challenged AM are shown in Table 1. In the absence of GTP virtually no cAMP was formed whereas in the presence of 10^{-4} M GTP basal cAMP levels increased 2.6- and 5.6-fold in MF from naive and antigen challenged AM, respectively. Interestingly, prostaglandin E_2 (PGE_2) and isoprenaline (ISO) were remarkably more effective stimulants of cAMP-production in MF from antigen challenged AM compared to naive AM as indicated by the absolute increase (Stim.P). These results are in accordance with our previous findings in whole AM-cell preparations in which we observed a similar 2.5-fold difference in responsiveness between antigen challenged and naive AM [9].

Subsequently, elucidation of which level the stimulatory signal transduction pathway of adenylyl cyclase was modified by sensitization or antigen challenge was attempted. Thus, receptor binding, G-protein coupling and enzymatic activity of the catalytic unit were considered.

Receptor binding studies were performed using the nonselective β-adrenoceptor antagonist (−)-3-[^{125}I]iodocyanopindolol ([^{125}I]ICYP). Analysis of

Table 1. Basal cAMP levels and adenylyl cyclase response to PGE_2 and isoprenaline in membrane fractions from naive, sensitized and antigen challenged alveolar macrophages

Agents	Adenylyl cyclase activity (pmol cAMP/mg protein/min)					
	Naive			Antigen challenged		
	Abs. value	(%)	Stim.P	Abs. value	(%)	Stim.P
−GTP	5.6 ± 0.5	—	—	6.2 ± 0.5	—	—
+GTP (10^{-4} M)	14.7 ± 1.5	—	—	34.2 ± 4.9*	—	—
GTP + PGE_2 (10^{-8} M)	15.5 ± 3.0	(5)	0.8	33.3 ± 2.3*	(0)	−0.7
GTP + PGE_2 (10^{-6} M)	18.8 ± 3.0	(28)	4.1	42.6 ± 4.7*	(25)	8.4
GTP + PGE_2 (10^{-4} M)	21.0 ± 3.3	(43)	6.3	67.3 ± 7.6†	(97)	33.1
GTP + Iso (10^{-8} M)	19.3 ± 3.5	(31)	4.6	41.4 ± 5.9*	(21)	7.2
GTP + Iso (10^{-6} M)	24.4 ± 6.6	(66)	9.7	59.2 ± 6.5*	(73)	25.0
GTP + Iso (10^{-4} M)	27.5 ± 6.8	(87)	12.8	68.8 ± 5.8*	(101)	34.6

In brackets the percentage increase compared to basal cAMP levels in the presence of 10^{-4} M GTP is given. Stim.P denotes cAMP production (pmol/mg protein/min) over basal levels induced by stimulation of adenylyl cyclase with agonist. When PGE_2 and isoprenaline (Iso) were used, 10^{-4} M GTP (GTP) was added to the suspensions. Data are obtained from 22–30 duplicate experiments (without receptor stimulus) from 5–6 duplicate experiments (PGE_2 and Iso) and are expressed in pmol/mg protein/min and as means ± SEM.

* $P < 0.005$; † $P < 0.01$, as compared to the effect of the corresponding concentrations in naive membrane fractions.

Table 2. Receptor density (B_{max}) and equilibrium dissociation constants (K_d) of membrane fractions from naive, sensitized and antigen challenged alveolar macrophages

	B_{max} (fmol/mg protein)	K_d (pM)
Naive	131 ± 14	79 ± 8
Sensitized	127 ± 5	67 ± 5
Antigen challenged	152 ± 29	62 ± 5

[^{125}I]CYP was used as the non-selective β-adrenoceptor ligand. Experimental procedure are described in Materials and Methods. Data shown are obtained from three duplicate experiments and are expressed as means ± SEM.

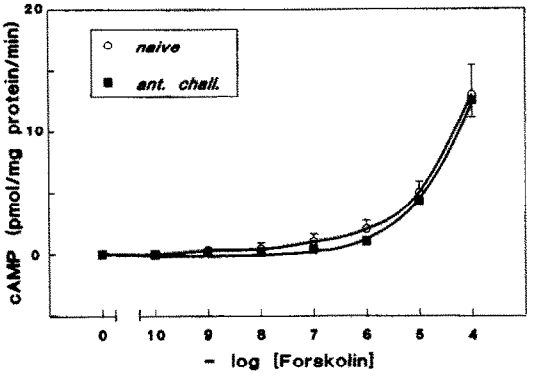

Fig. 1. Effect of forskolin on adenylyl cyclase activity of membrane fractions from naive (○) and antigen challenged (■) AM (in the absence of GTP). Incubation procedures are described in Materials and Methods. Data are expressed as mean absolute increases in pmol/mg protein/min of cAMP levels ± SEM of nine duplicate experiments.

the binding data by nonlinear regression revealed the presence of a single binding site for [^{125}I]ICYP in all three membrane fractions (naive, sensitized and antigen challenged). As shown before [18], this binding site was characteristic for β_2-adrenoceptors. As Table 2 shows, equilibrium dissociation constants (K_d values) were not altered by sensitization or antigen challenge. In addition, no significant differences were found regarding maximal binding (B_{max}, reflecting total receptor number per mg protein) of the radioactive ligand to membranes of naive, sensitized and antigen challenged AM. Apparently, no changes in the conformation or the number of stimulatory β-adrenergic receptors have been induced by sensitization or antigen challenge.

In order to determine whether sensitization or antigen challenge affects the enzymatic activity of the catalytic unit, the effect of direct activation of adenylyl cyclase by forskolin was determined in MF from naive and antigen challenged AM. As depicted in Fig. 1, forskolin induces the same stimulatory effect on adenylyl cyclase in both membrane preparations indicating no alteration in the catalytic properties of the enzyme by sensitization or antigen challenge.

Finally, we determined the effect of sensitization and antigen challenge on the transduction process regulated by G_s-proteins. Exposure of the MF to GTP enhances adenylyl cyclase activity and the subsequent hydrolysis of GTP to GDP by GTPase activity intrinsic to the α_s-subunit, terminates this signal. Hence, it seemed that the enhanced basal adenylyl cyclase activity in sensitized and antigen challenged AM (cf. Table 1) could be due to a

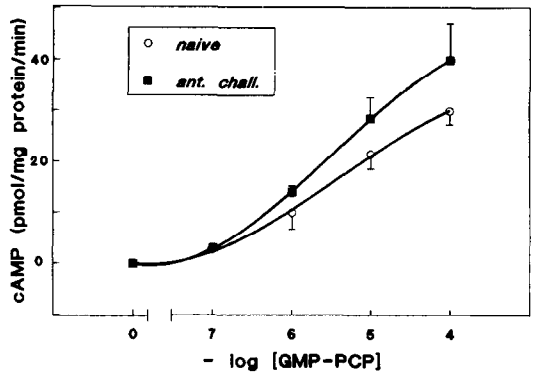

Fig. 2. Effect of GMP-PCP and on adenylyl cyclase activity of membrane fractions from naive (○) and antigen challenged (■) AM. Incubation procedures are described in Materials and Methods. Data are expressed as mean absolute increases in pmol/mg protein/min of cAMP levels ± SEM of nine duplicate experiments.

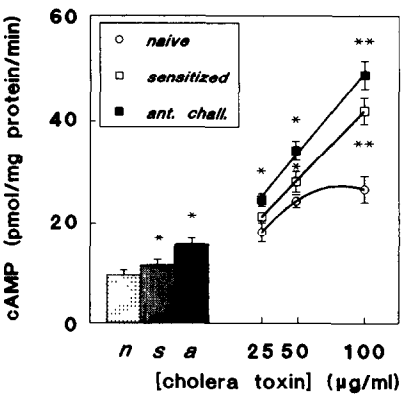

Fig. 3. Effect of cholera toxin pretreatment of membrane fractions on the effect of 10^{-4} M GTP on adenylyl cyclase activity of membrane fractions from naive (○), sensitized (□) and antigen challenged (■) AM. Inset: n, s and a refer to basal cAMP levels in MF from naive, sensitized and antigen challenged AM. Incubation procedures are described in Materials and Methods. Data are expressed as mean cAMP levels (pmol/mg protein/min) ± SEM of five (naive), three (sensitized) and five (antigen challenged) duplicate experiments. * $P < 0.05$; ** $P < 0.005$ as compared to naive MF.

decreased GTPase activity of the α_s-subunit prolonging adenylyl cyclase activation. Since GTP is also able to stimulate G_i, an enhanced GTPase on the G_i-proteins in MF from antigen challenged AM would also explain the effects of GTP on basal cAMP levels. Both theories imply that GTP analogues, not susceptible to hydrolysis by GTPase, would show, in contrast to GTP, a similar stimulatory response in all three MF. However, Fig. 2 shows that the non-hydrolysable GTP analogue GMP-PCP stimulates adenylyl cyclase slightly more effectively in MF from antigen challenged AM as compared to naive MF, though the difference is not significant. This minor effect is reflected by a divergement of the two dose–response curves which tend to result in different maximal values for adenylyl cyclase stimulation at higher ($>10^{-4}$ M) GMP-PCP concentrations. For practical reasons (solubility) it was not possible to establish a significant difference in maximal effect.

The GTP-hydrolysing enzymes residing on the α-subunit of G_s-proteins can be specifically inactivated with cholera toxin (CT) which will likewise result in a prolonged activation of adenylyl cyclase due to the accumulation of α_s in a GTP-liganded state. Pretreatment of MF of AM with increasing doses of CT and the subsequent challenge of adenylyl cyclase with a standard (maximal) dose of 10^{-4} M GTP thus provides other means to determine differences in GTPase activities among the different MF.

Figure 3 shows that the stimulatory activity of adenylyl cyclase in naive MF by 10^{-4} M GTP clearly depends on the dose of CT used in the pretreatment period, as at low doses CT (<50 µg/mL) GTP is still inactivated by some GTPase activity. Beyond 50 µg/mL of CT, a maximum is reached in the capability of 10^{-4} M GTP to stimulate adenylyl cyclase, suggesting complete elimination of GTPase activity after pretreatment of MF with CT at doses ≥50 µg/mL. However, in CT-pretreated MF from sensitized and antigen challenged AM, such maximal stimulation by 10^{-4} M GTP is not attained. Interestingly, 10^{-4} M GTP challenge of these pretreated MF results in cAMP levels exceeding the maximal level observed in CT-pretreated naive MF. Secondly, irrespective of the doses of CT used in the pretreatment period, 10^{-4} M GTP stimulates adenylyl cyclase in MF from antigen challenged AM more effectively as compared to naive AM. Under the same conditions the stimulatory action of 10^{-4} M GTP in MF from sensitized AM is comparable to the results obtained in MF from antigen challenged AM and corresponds with intermediate values.

DISCUSSION

Using ovalbumin sensitization of guinea pigs, we previously observed an enhanced adenylyl cyclase responsiveness in alveolar macrophages (AM) to different stimuli like β-adrenergic agonists and inflammatory mediators [9]. We presently confirm this using membrane fractions (MF) of macrophages from naive, sensitized and antigen challenged guinea pigs. Regarding the difference in basal cAMP levels in the presence of 10^{-4} M GTP (cf. Table 1) an imbalance in G_s/G_i-status in favour of G_s-stimulation has apparently been induced by antigen challenge. Whether G_i-pathways are impaired or G_s-pathways are enhanced cannot be determined merely on the basis of these data on basal cAMP levels.

If impairment of G_i-pathways was responsible for the difference of 19.5 pmol/mg protein/min in basal cAMP levels (in the presence of 10^{-4} M GTP), one should—upon stimulation with G_s-activating agents like isoprenaline and PGE_2—observe the same difference in their response when MF from antigen challenged and naive AM are compared. This is, however, not observed. Using these two agonists, the differences exceed the value of 19.5 pmol/mg

protein/min from which can be concluded that the extra-stimulatory capacity of isoprenaline and PGE_2 in MF from antigen challenged AM is due to enhanced G_s-coupled receptor signal transduction.

Sensitization and antigen challenge do not affect the configuration or number of β-adrenoceptors (no change in K_d or B_{max} respectively) and therefore exclude the possibility that such changes are, in addition, responsible for the enhanced adenylyl cyclase response in sensitized and antigen challenged AM.

No differences between the various MF are observed, considering direct stimulation of adenylyl cyclase by forskolin. Therefore, sensitization and/or antigen challenge apparently have not altered the catalytic site of the enzyme itself. Similar results were obtained by others [15, 20] who showed that adenylyl cyclase activity of guinea pig lung smooth muscle homogenates was unaffected after a similar ovalbumin sensitization procedure.

Gadd and Bhoola [14] have recently suggested a reduction in GTPase activity of the α_s-subunits to explain a more effective adenylyl cyclase activation in guinea pig lung homogenates. GTPase promotes the hydrolysis of GTP to GDP which results in the deactivation of the GTP-liganded α-subunit and ultimately in the termination of the transduction signal. Differences in basal cAMP levels determined in the presence of GTP (cf. Table 1) might well be ascribed to a decrease in GTPase activity on α_s or an increase in its activity on α_i in MF from antigen challenged AM. Such interference of GTPase activity can be determined using a non-hydrolysable GTP analogue to promote the irreversible dissociation of the α-subunit from the $\beta\gamma$-subunit. Only minor differences in the adenylyl cyclase stimulatory response to GMP-PCP were observed comparing MF from naive and antigen challenged AM. Therefore, these results do not allow whether sensitization and/or antigen challenge has altered GTPase activity in either α_i- or α_s-subunits to be firmly established.

In analogy with studies which showed an increase in the number of α_i-subunits by desensitization [12], we considered whether sensitization would have induced an increase in the number of α_s-subunits. Such an increase would not only explain the enhanced basal cAMP levels in MF from sensitized and antigen challenged AM, but also the supposed difference in maximal values of adenylyl cyclase stimulation by GMP-PCP. To elucidate the mechanisms of α_s-subunit activation and the subsequent coupling to adenylyl cyclase in further detail, we studied in the different MF (naive, sensitized and antigen challenged) the effect of pretreatment with cholera toxin on the GTP-induced adenylyl cyclase response. This toxin ADP-ribosylates specific amino acids of the α_s-subunits resulting in the inhibition of α_s-GTPase activity and constitutive activation of adenylyl cyclase due to the accumulation of α_s in a GTP-liganded state [21]. CT-pretreatment of MF and subsequent challenge with GTP thus enables elucidation of whether sensitization or antigen challenge reduce the GTPase activity. Obviously, at a CT concentration sufficient to completely eliminate GTPase activity in naive MF, where the highest GTPase activity is retained, a similar stimulatory effect of 10^{-4} M GTP would be obtained in all three populations of MF. Moreover, the absolute value of the maxima, in terms of cAMP production, would be the same for all preparations. The data, however, show that pretreatment with CT in a dose of 50 μg/mL indeed appears to eliminate GTPase activity in naive MF but not in MF from sensitized and antigen challenged AM. Pretreating these MF with this dose of CT would, as delineated above, result in the same or even decreased stimulatory effect of 10^{-4} M GTP. Remarkably, such CT-pretreatment of MF from sensitized and antigen challenged AM results in cAMP values exceeding the maximal value obtained with MF from naive AM. This effect is even more pronounced when MF are pretreated with a two-fold higher dose of CT (100 μg/mL). Apparently, pretreatment of MF from sensitized and antigen challenged AM with 50 μg/mL of CT is not sufficient to completely eliminate GTPase activity. It should be emphasized that elimination of GTPase activity would not result in an enhancement of the maximal stimulatory effect of GTP, but merely to a leftward shift of the GTP dose–response curve. Assuming an increment in the number of α_s-subunits by sensitization or antigen challenge could well explain the observed increase in maximal stimulation. Consequently, as such an increase in the number of α_s-subunits coincides with an increase in GTPase activity (residing on these subunits), more CT is necessary to fully inactivate the (enhanced) GTPase activity in antigen challenged compared to naive MF (cf. Fig. 3). Despite the simultaneous increase in both α_s-subunits and GTPase activity by antigen challenge, an improved signal transduction is the net result suggesting that the enhanced GTPase activity is of less importance for the activation of adenylyl cyclase. Using other approaches like immunoblotting will enable us to establish to what extent the number of α_s- subunits are affected by sensitization and/or antigen challenge.

As yet, we conclude that the enhanced responsiveness of adenylyl cyclase observed in sensitized and antigen challenged AM results from alterations in α_s-subunits of the signal transduction pathway induced by sensitization, ultimately leading to an enhanced cAMP-production and a subsequent decrease in cellular activity.

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