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A role for N_i in the hormonal stimulation of adenylate cyclase

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The best understood system for transduction of extracellular messages into intracellular signals is the hormone receptor-coupled adenylate cyclase. In such systems receptors are functionally coupled to the enzyme by two special proteins, termed the stimulatory and inhibitory guanine nucleotide regulatory proteins (N_s and N_i, respectively). These proteins, thought to mediate, respectively, stimulatory and inhibitory influences on the adenylate cyclase, are members of a larger class of heterotrimeric guanine nucleotide regulatory proteins involved in membrane signal transduction[1-5]. We have studied the interactions of the various components of the adenylate cyclase system by co-reconstituting pure β-adrenergic receptors, pure N_s and N_i, and functionally resolved preparations of the catalyst in phospholipid vesicles. In the absence of N_i, β-adrenergic receptor/N_s-mediated catecholamine stimulation of the enzyme is relatively modest (~1.3-fold). Surprisingly, however, when N_i is also present, stimulation increases dramatically (up to 7–8-fold) because of a greater suppression of basal relative to agonist-stimulated enzyme activity. Thus, N_i may actually be required for maximal agonist stimulation as well as for inhibition of the adenylate cyclase.

It is believed that hormonal stimulation of the adenylate cyclase (C) system is a simple vectorial signal transduction process whereby the receptor first facilitates the activation of N_s by GTP and the GTP/N_s complex then activates adenylate cyclase. The stimulation process is terminated by the GTPase activity of N_s. Recently, we reported that the co-insertion into phospholipid vesicles of the pure guinea pig lung β-adrenergic receptor (βAR), with the pure human erythrocyte N_s protein and a resolved bovine caudate adenylate cyclase preparation (that is, resolved from βAR and N_s by Sepharose-6B chromatography) indeed results in the establishment of a modest responsiveness of adenylate cyclase activity to isoprenaline[6]. The results of Fig. 1 confirm this observation. For the conditions shown in Fig. 1 the maximal stimulation by isoprenaline never exceeds two fold and in some cases is nearly non-existent. The average (fold) stimulation by isoprenaline in 11 such experiments is 1.32 ± 0.07 (mean ±s.e.m.). It seems likely that the modest effect of isoprenaline in these three component systems reflects, at least in part, the effective activation of N_s molecules by GTP alone, thus resulting in a significant basal level of cyclic AMP production.

The construction of a vesicle system comprised of pure βAR, pure N_s, resolved adenylate cyclase and pure human erthrocyte N_i results in three striking effects (Fig. 1): (1) the absolute levels of adenylate cyclase activity are reduced (relative to βAR/N_s/C vesicles) both when assayed in the absence and in the presence of isoprenaline; (2) the reduction of activity in the absence of isoprenaline is much more marked than in its presence; and due to this, (3) the relative stimulation of adenylate cyclase activity by isoprenaline ('fold stimulation') is increased. In the case shown in Fig. 1, the stimulation of adenylate cyclase activity by isoprenaline is 1.8-fold in the βAR/N_s/C vesicles and increases to 7-fold in the βAR/N_s/N_i/C vesicles. In a series of 11 experiments the 'fold' stimulation of the enzyme by isoprenaline was 1.32 ± 0.07 in the absence and 3.61 ± 0.6 in the presence of N_i. We have documented that the effects of N_i in the reconstituted system were not the result of preferential reduction of the basal (GTP) activities due to a reduced incorporation of adenylate cyclase or N_s or of any change in the efficiency of βAR reconstitution (data not shown).

Figure 2*a* demonstrates clearly that the ability of N_i to increase the relative stimulation of adenylate cyclase activity by isoprenaline is a dose-dependent phenomenon. At the highest level of N_i incorporated, the adenylate cyclase activity assayed in the absence of isoprenaline was inhibited by ~85%, while activity measured in the presence of isoprenaline was inhibited by ~42% (Fig. 2*b*); this results in an increase in the stimulation of adenylate cyclase activity by isoprenaline from ~1.1-fold for control

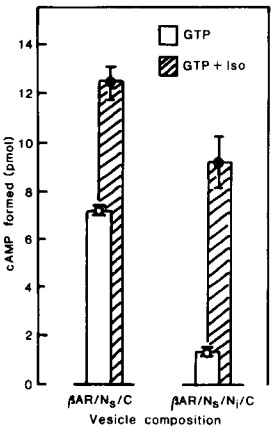

Fig. 1 The effect of N_i on β-adrenergic-mediated adenylate cyclase activity in phospholipid vesicles. The data are presented as the means of triplicate determinations, with the ranges denoted by error bars.

Methods. Resolved bovine caudate adenylate cyclase (C) preparations (150 μl; see below), 1.14 μg of human erythrocyte N_s (≃12 pmol), 12 pmol of guinea pig lung β-adrenergeic receptor (βAR) and 1.8 μg of human erythrocyte N_i (≃19 mol) were inserted into phospholipid vesicles as follows. Soybean phosphatidylcholine (0.1 ml of a 17 mg ml^{-1} sonicated solution; Sigma) was incubated with the adenylate cyclase preparation (0.15 ml), 0.08–0.12 ml of 100 mM NaCl, 10 mM Tris-HCl *p*H 7.4 and 25 μl of octyl glucoside (17%) for 20–30 min on ice. At this point the nucleotide regulatory proteins (30 μl of N_s, 36 μl of N_i) and the receptor [40 μl of βAR which had been preincubated with 10 μl of alprenolol (5 μM) and 10 μl of bovine serum albumin (BSA, 50 mg ml^{-1})] were added to the lipid solutions, which had been incubated for ~1–2 min at 4 °C. Rhodopsin (195 pmol) was also included in the incubations containing N_i as we had found previously that rhodopsin promotes the maximal activation of this regulatory protein[11]. However, under the conditions described for these experiments, N_i alone is effectively activated and essentially identical results are obtained in the absence of rhodopsin. These incubation mixtures were then immediately applied (at 4 °C) to Extracti-gel columns (1 ml of gel) pretreated with 4 vol, of 100 mM NaCl, 10 mM Tris-HCl *p*H 7.4 containing 2 mg ml^{-1} of BSA, then equilibrated with 100 mM NaCl, 10 mM Tris-HCl *p*H 7.4. the protein-vesicles, in the column eluate, were then pelleted using polyethylene glycol essentially as described previously[11]. The resultant protein-lipid pellets were resuspended in 75 mM Tris-HCl *p*H 7.8, 1 mM dithiothreitol (DTT, final volume 0.35 ml) and 20 μl of the vesicles were assayed for adenylate cyclase activity as described below. The β-adrenergic receptor was purified (≥95%) from guinea pig lung membranes as described previously[16]. The pure receptor preparations were concentrated to ~300 pmol ml^{-1} using an Amicon concentration cell with a YM 30 membrane, and then stored at −90 °C in 50 mM Tris-HCl, 50 mM NaCl, 0.2% digitonin *p*H 7.4. N_s and N_i were purified (>90%) as described by Codina *et al.*[12] and stored at −90 °C in 10 mM HEPES *p*H 8.0, 1 mM EDTA, 20 mM β-mercaptoethanol, 30% ethylene glycol, 150 mM NaCl, 50 μg ml^{-1} BSA and in 1.65–10% Lubrol PX. The catalytic moiety (C) of adenylate cyclase was solubilized from bovine caudate nucleus with sodium cholate and isolated from the other components of the system by Sepharose-6B chromatography as described elsewhere[6,17]. These preparations were stored in 50 mM Tris-HCl, 200 mM sucrose, 1 mM DTT, 15 mM $MgCl_2$, 3.5 mg ml^{-1} crude soybean phosphatidylcholine and 0.6% sodium cholate, *p*H 7.6. The concentrations of BAR are expressed in terms of ^{125}I-iodocyanopindolol binding activity. The amounts of N_s and N_i are expressed in terms of total protein as described previously[12]; the molar concentrations of these proteins were determined assuming a relative molecular mass of 95,000 (ref. 12). The adenylate cyclase assays were performed in a total volume of 0.1 ml containing 50 mM Na-HEPES *p*H 8.0, 1 mM EDTA, 0.25 mM ATP, 3 mM phosphoenolpyruvate, 10 μg ml^{-1} pyruvate kinase, 0.1 mg ml^{-1} BSA, 0.1 mM cyclic AMP (cAMP), 10 μg ml^{-1} myokinase, 2 μCi of [α-^{32}P]ATP and 2 mM $MgCl_2$. Isoprenaline (Iso) = 0.1 mM; GTP = 10 μM.

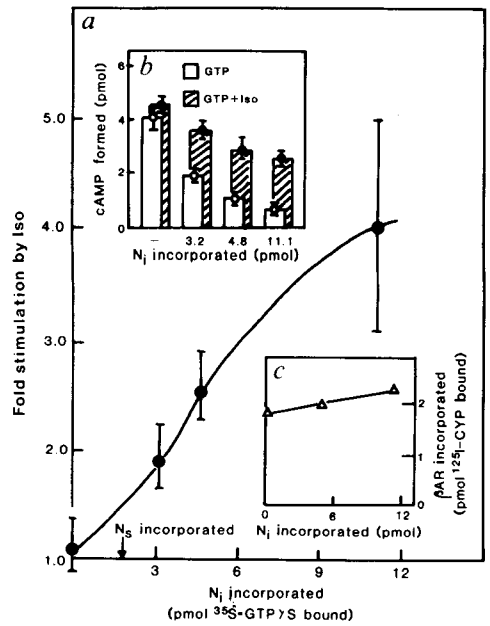

Fig. 2 *a*, Effect of varying N_i on the stimulation (fold) by isoprenaline of the adenylate cyclase activity in phospholipid vesicles. Resolved bovine caudate adenylate cyclase (C) preparations (150 μl), human erythrocyte N_s (1.16 μg ≃ 12 pmol), human erythrocyte N_i (0.63 μg, 1.27 μg, 3.82 μg ≃ 6.6 pmol, 13.4 pmol, 40.2 pmol, respectively) and 12 pmol guinea pig lung βAR were added to reconstitution incubations and protein-lipid vesicles were then formed and isolated as described in Fig. 1 legend. The isolated vesicles were resuspended in 0.38 ml of 75 mM Tris-HCl *p*H 7.8, 1 mM DTT and assayed (20 μl) for adenylate cyclase activity (30 min at 30 °C) as described in Fig. 1 legend. Isoprenaline (Iso) = 20 μM, GTP = 0.1 mM. The amount of N_i incorporated into the lipid vesicles was determined by assaying ^{35}S-GTPγS binding as described previously[11]. *b*, Effect of varying N_i on the total adenylate cyclase activity in phospholipid vesicles. The experimental conditions were as described for *a*. *c*, Effect of varying N_i on the incorporation of the β-adrenergic receptor into phospholipid vesicles. Experimental conditions as for *a*. The amount of βAR incorporated into the lipid vesicles was determined by ^{125}I-iodocyanopindolol (^{125}I-CYP) binding as described previously[18].

vesicles, to 4.1-fold in vesicles containing N_i. The mean inhibitions of basal and isoprenaline-stimulated activities by N_i were, respectively, 76 ± 3% and 43 ± 4% ($n = 11$). In a separate series of experiments using the resolved subunits of N_i or transducin, we have found that the activities of N_i described here are essentially properties of the βγ- rather than the α-subunit (manuscript in preparation).

Our results indicate a dual role for the inhibitory nucleotide binding regulatory protein (N_i) of the adenylate cyclase system, one being the mediation of inhibition of adenylate cyclase activity, the other being a tightening of the coupling between adenylate cyclase activity and stimulatory hormones by the marked attenuation of the basal (N_s-mediated) adenylate cyclase activity.

Moreover, not only do our results suggest a dual role for N_i, they also imply two distinct functions for the stimulatory hormone/receptor complex. The first is to accelerate the actual activation of N_s. The second is to suppress the inhibitory action of N_i on GTP-activated N_s. Evidence for the first function derives from studies in which it has been shown that β-agonists promote the interaction between the pure βAR and N_s in phospholipid vesicles, leading to increases in the rate of GTPγS binding to N_s and the turnover number for the GTPase activity[7-11]. Evidence for the second function of the hormone/receptor complex is provided by the present work, which indicates that addition of agonists to vesicles containing βAR/N_s/N_i/C markedly relieves the N_i inhibition, leading to a significant increase in hormonal stimulation.

Of further interest is the fact that purification studies have indicated that in native membranes there is 4–10 times more N_i than N_s (ref. 12), this agrees well with the relative ratios of N_i/N_s (4–5 times) required in our reconstitution systems to both inhibit GTP-activated N_s and enhance the relative stimulation by hormone.

The specific mechanisms by which N_s and N_i are activated under physiological conditions (that is, in the presence of stimulatory and inhibitory receptors, GTP and in a lipid milieu) still remain to be delineated. Both N_s and N_i are heterotrimeric in structure (αβγ) and in detergent solution their activation by non-hydrolysable analogues of GTP involves subunit dissociation into α_s (ref. 13) or α_i (ref. 14), and an intact βγ species J.C., J. D. Hildebrandt & L.B., unpublished results. It has been suggested that it is the α_s/guanine nucleotide complex which stimulates adenylate cyclase activity. However, the mechanism by which inhibition occurs has been a more controversial subject. It has been suggested that it is the generation of βγ following N_i activation (and dissociation) which effectively complexes activated α_s (refs 1, 14), while other studies in N_s-deficient S49 *cyc*$^-$ cells suggest a direct inhibitory action of α_i on adenylate cyclase[15]. Recent studies using phospholipid vesicle systems similar to those described here indicate that bovine brain N_i or bovine retinal transducin inhibit the N_s-stimulated adenylate cyclase rather than the intrinsic activity of the enzyme itself[19]. Moreover, experiments using the isolated subunits of these proteins indicate that the βγ subunit complex is primarily involved in mediating the inhibition of GTP- (or isoprenaline plus GTP-)stimulated adenylate cyclase (manuscript in preparation). This would then suggest that a hormone/receptor-mediated release of the system from inhibition by N_i, as documented here, would be due to the complex between hormone/receptor and activated α_s/GTP becoming insensitive to the inhibitory action of βγ.

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