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Research article

Simultaneous stimulation of GABA and *beta* adrenergic receptors stabilizes isotypes of activated adenylyl cyclase heterocomplex

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Abstract

Background: We investigated how the synthesis of cAMP, stimulated by *isoproterenol* acting through β-adrenoreceptors and *Gs*, is strongly amplified by simultaneous incubation with *baclofen*. *Baclofen* is an agonist of δ-aminobutyric acid type B receptors [GABA$_B$], known to inhibit adenylyl cyclase via *Gi*. Because these agents have opposite effects on cAMP levels, the unexpected increase in cAMP synthesis when they are applied simultaneously has been intensively investigated. From previous reports, it appears that cyclase type II contributes most significantly to this phenomenon.

Results: We found that simultaneous application of *isoproterenol* and *baclofen* specifically influences the association/dissociation of molecules involved in the induction and termination of cyclase activity. *Beta/gamma* from [GABA]B receptor-coupled *Gi* has a higher affinity for adenylyl cyclase isoform(s) when these isoforms are co-associated with *Gs*. Our data also suggest that, when *beta/gamma* and *Gαs* are associated with adenylyl cyclase isoform(s), *beta/gamma* from [GABA]B receptor-coupled *Gi* retards the GTPase activity of *Gαs* from adrenergic receptor. These reciprocal regulations of subunits of the adenylyl cyclase complex might be responsible for the drastic increase of cAMP synthesis in response to the simultaneous signals.

Conclusions: Simultaneous signals arriving at a particular synapse converge on molecular detectors of coincidence and trigger specific biochemical events. We hypothesize that this phenomenon comes from the complex molecular architectures involved, including scaffolding proteins that make reciprocal interactions between associated molecules possible. The biochemistry of simultaneous signaling is addressed as a key to synaptic function.

Background

The defensive withdrawal reflex of *Aplysia* affords a model of classical conditioning. This involves a conditioned stimulus (touching the siphon) and an unconditioned stimulus (shocking the tail), and has been interpreted in terms of the integration of two separate simultaneous signals in the same motor neuron: Ca++ and *Gαs*-GTP [1,2]. Genetic analysis in *Drosophila* showed that a mutant of learning and memory, *rutabaga*, has a defect in the gene coding for an isoform of adenylyl cyclase (type II), which is regulated by both Ca++ and *Gαs* [3,4]. This mutant shows low and unregulated levels of cAMP. Paradoxically, elevation of cAMP levels in *dnc* mutants (defect in the cAMP phosphodiesterase gene) causes an identical learning and memory deficit. The strongest phenotype comes from an allele that increases the cAMP level to as much as eight times normal [4-6]. Moreover, the double mutant *dnc/rut* has a normal cAMP level but still shows a learning

and memory deficit, which suggests that these behavioral defects depend on failure to regulate cAMP levels [7]. Studies of these molecules in mutants and/or transgenic animals have led to an accumulation of interesting data in behavioral analysis of memory, learning and associative competence [4]. Moreover and surprisingly, it has been demonstrated that a high level of cAMP also follows the simultaneous activation of *Gi* by GABA and *Gs* by *isoproterenol* in rat neurons, whose combined (opposing) effects were expected to restore the basal cAMP level [2,8-10]. Biochemical analysis has revealed the role of adenylyl cyclase type II in this phenomenon, which is highly active when complexed with both *beta/gamma* and *Gαs*. Interestingly, *beta/gamma* in such complexes comes from the *Gi* component [2,11,12]. Six *beta* and 13 *gamma* isoforms and a large family of cyclase isoforms have been cloned [13-18], which suggests that only specific combinations might be involved in coincidental signaling. Molecules that act as detectors of coincidental signals allow us to understand how the topology of the synaptic network influences its biochemistry. Our analysis of the molecular components involved in simultaneous GABA/*isoproterenol* signaling has been facilitated by previously reported data on the role of *beta4/gamma2* and *beta1/gamma2* in the activation of cyclase type II [19,20]. In the present study we investigated whether *beta/gamma* might influence the GTPase activity of *Gαs* when *beta/gamma*, *Gαs* and cyclase are associated in a complex form and conversely, whether *Gαs* pre-associated with a cyclase, might increase the affinity of *beta/gamma* for this complex.

Results

Simultaneous signaling determines the scale of cAMP synthesis

We used isolated neuronal plasma membranes instead of membranes of transfected cells in order to preserve the molecular topology and protein localisation. We thought that the complex scaffolding proteins, which link molecules attached to plasma membranes, might be absent and/or different in cell transfection systems. The isolated membranes were treated with *isoproterenol* (a *beta* adrenergic receptor agonist) and/or *baclofen* (a [GABA]B receptor agonist). Simultaneous binding produced 340 +/-55 pmol/ min/mg of cAMP, compared to 105 +/-30 pmol/ min/mg and 15 +/-10 pmol/min/mg respectively for *isoproterenol* or *baclofen* alone (Fig. 1). We also observed that when the membranes were preincubated with antagonists of [GABA]b (*saclofen*) or *beta* adrenergic (*propanolol*) receptors before the simultaneous incubation of agonists (*iso+bac*), the levels of cAMP generated by *isoproterenol* or *baclofen* respectively were restored (Fig. 1).
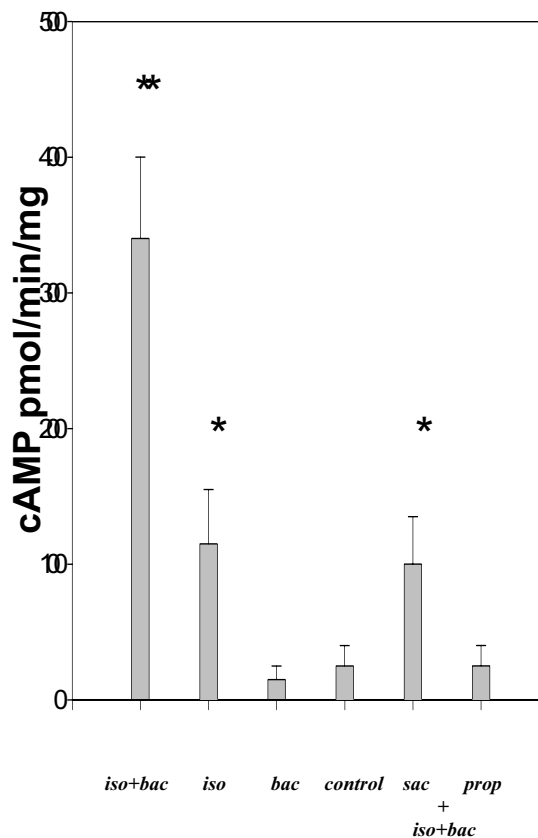

Figure 1
Determination of adenylyl cyclase activity from isolated rat brain plasma membrane: measure of cAMP synthesis by isoprenolol *plus* baclofen Membranes were assayed in presence of the following drugs (50 nM): *isoproterenol + baclofen* (*iso+bac*), *isoproterenol* (*iso*), *baclofen* (*bac*). A control membrane is included. Membranes were also incubated first with *saclofen* (*sac*) or *propanolol* (*prop*) (100 nM) for 10 min, then with *iso + bac* as above (50 nM). cAMP levels were analyzed using a RIA procedure (NEN kit). The bars show the averages of three determinations +/-SE. Asterisks represent values statistically different from the control: ** P < 0.005 * P < 0.01

The affinity of **beta/gamma** subunits for cyclase isoform(s) depends on co-association with **Gαs**

After solubilizing the membranes with nonionic detergent, we performed a 'pull down' experiment with Forskolin-agarose to isolate the cyclase complex. Lubrol is known to inhibit G protein GTPase activity, which allowed us to 'freeze' the G protein activated state while we isolated the cyclase complex. ADP-ribosylation by *cholera* toxin inhibits *Gαs* GTPase. ADP-ribosylation by *pertussis* toxin uncouples receptors and *Gi* [36,37]. We

measured the amount of radioactive ADP-ribosylated *Gαs* in the cyclase complex isolated with Forskolin-agarose.

Isotopic ADP ribosylation was not performed quantitatively (in conditions that eliminate "back ribosylations"). We observed that these optimal conditions saturated the signal.*Gαs* from inactivated receptors represents a huge amount compared to *Gαs* activated by *isoproterenol*. This interference might overwhelm the specific signal induced by *isoproterenol* if a probable artefactual slow exchange GDP/GTP occurs at silent *Gαs*. We carried out therefore the radioactive ADP ribosylation for a short time and a limited concentration of reagent in order to label preferentially the *isoproterenol* activated *Gαs*. This experimental design allowed us to compare relative levels of labeling as an index when membranes were treated with different drugs. The quantification of radioactivity was normalized with the quantity of protein present in each individual 'pull down'.

Simultaneous incubation with *baclofen + isoproterenol* significantly increased the amount of *Gαs* bound to isolated cyclase heterocomplex(s), compared to the amount bound after *isoproterenol* treatment alone (Fig. 2a). We analyzed also the kinetics of *Gαs*/cyclase association induced by drugs at low concentration on a time scale of 10 min, by measuring the amount of ADP radiolabel in the isolated complex. *Beta/gamma* subunits released upon activation of the [GABA]b receptors by *baclofen* increased the amount of *Gαs* bound to cyclase (300 cpm +/- 80 and 470 cpm +/- 50 at 1 and 5 min respectively for *iso + bac*, compared to 180 cpm +/- 50 and 270 cpm +/- 70 at 1 and 5 min for *iso* alone) (fig. 2b).

We tried also to quantify the ratio of molar amounts *Gαs/ beta* attached to affinity chromatography when membranes were incubated with drugs. Analysis by densitometry of immunostained bands was not carried out because the relation quantity/staining was not linear. We proceeded also as follows: proteins isolated with forskolin-agarose were dissociated with 1 M NaCl and derivatized with the reagent: N-hydroxysuccinimido biotin. The biotinyl proteins were then precipitated with avidin-agarose. These complex were incubated with an antibody against *Gαs* or *Gbeta* then a rabbit antibody-alkaline phosphatase in order to quantify these molecules. Precise quantity of *Gαs* and *Gbeta* (few µg of immunoprecipitated material) were submitted to the same protocol of biotinylation (data not shown). Unfortunately this method gave us variable data to some extent. We decide therefore to shift to the *Bolton Hunter* iodination method that has the advantage to be faster and more accurate. The labeled material attached to affinity chromatography was submitted to gel electrophoresis, then we measured the radioactivity in 35 and 45 Kda bands of dried electrophoresis gels although

these iodinated bands might include unrelated proteins and don't discriminate between isoforms of *Gα*. We obtained the following ratio: *isoproterenol + baclofen, Gα/ Gbeta* = 1.8+/-0.5, *isoproterenol, Gα/Gbeta*= 5+/-2.2, *baclofen,Gα /Gbeta* = 1.1+/-0.2, *isoproterenol + pertussis* toxin, *Gα/Gbeta*= 15+/-4). Again, this method don't discriminate *Gαs* from *Gαi*. The amount of cyclase(s) was >10 fold to the amount of *Ga* and/or *Gbeta*. The quantification of *Bolton Hunter* labeled bands were normalized with the quantity of protein present in each individual 'pull down'.

A representative colloidal blue stained gel of attached material to affinity chromatography is shown (fig. 2c). We see two bands at 45 and 35 kDa, corresponding to the *Gαs* and *Gbeta* subunits respectively. The 35 kDa band vanished when membranes were treated with *pertussis* toxin and a strong doublet appeared when membranes were incubated with *iso+bac*. Moreover, the specificity of the 'pull down' procedure was assessed with membranes incubated with GDP *versus* GTPyS+*iso+bac* (fig. 2c). We see clearly the absence of the doublet with the GDP treated membranes and the non specific proteins attached to agarose. We did also competition experiment with samples pre-incubated with free Forskolin at 10 nM and 10 µM. We observed that two bands (45 and 35 kDa) disappeared while the non specific binding was unchanged (data non shown).

Western blot analysis using an anti-*beta* antibody showed that [GABA]b receptor activation induced the formation of the cyclase-*beta/gamma* complex, detected in the 'pull down' material attached to Forskolin affinity beads (fig. 3a). Treatment of the membranes by *pertussis* toxin largely eliminated the formation of this complex. This suggests that *beta/gamma* bound to the cyclase(s) complex mostly comes from the *Gi* component (fig. 3a). Kinetic studies on a time scale of 10 min show that *baclofen* is more efficient than *isoproterenol* in generating *beta/gamma*-cyclase(s) association (fig. 3b). This was confirmed by quantifying the radioactivity incorporated in the 35 kDa band using *Bolton Hunter* reagent (1000 cpm+/-250 and 750 cpm+/-150 for *bac* and *iso* respectively) (fig. 3c). We observed again that membranes treated with *pertussis* toxin and then incubated with the drugs gave a basal level, similar to control membranes (fig. 3c).

We carried out a time course analysis under different experimental conditions to determine how *beta* faded out in the 'pull down' material at 4°C after the samples were treated with an excess of GDP and Mg. The complex was more abundant and more durable when *baclofen* and *isoproterenol* were used simultaneously than when *baclofen* alone was used (Fig. 4a and 4b). To analyze further how activated *Gαs* might influence the affinity of *beta/gamma*
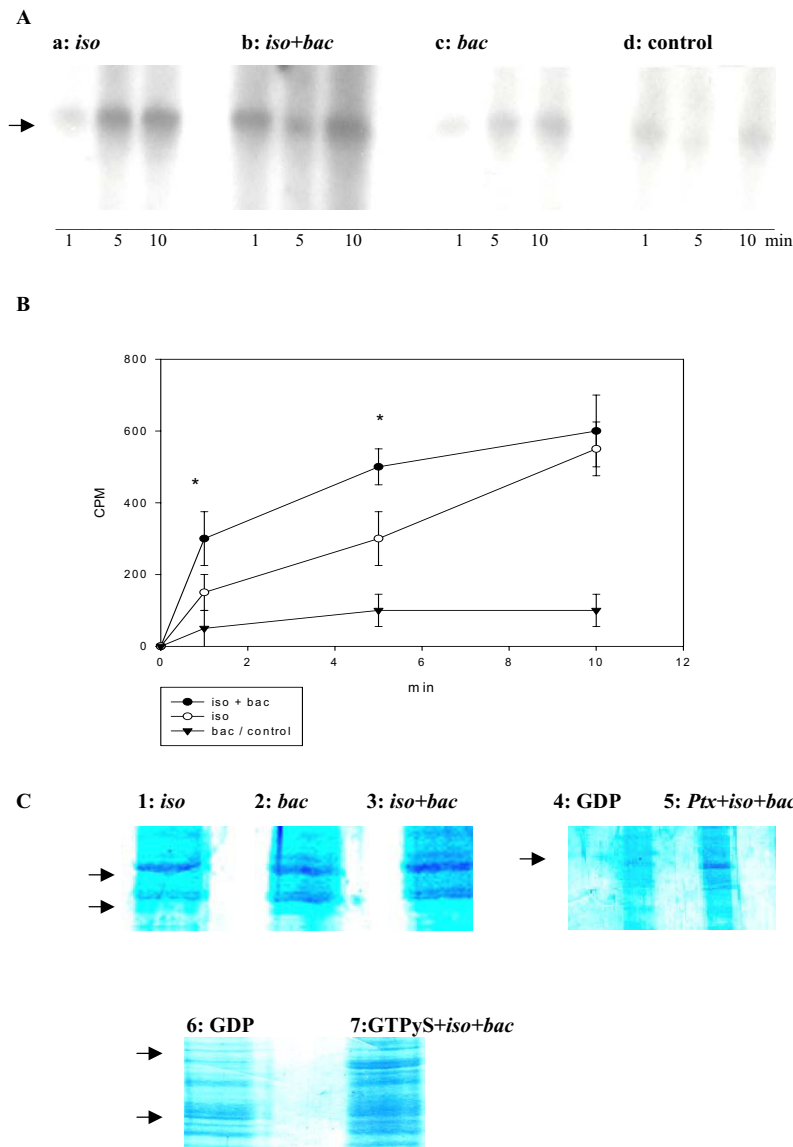
Figure 2
Analysis of *cholera* toxin [$^{32}\gamma$P]-ADP-ribosylated *Gαs* bound to cyclase A: ADP ribosylation of *Gás* in Forskolin agarose 'pull down' Plasma membrane (50 μg protein) was incubated with *isoproterenol* (10 nM) (**a**), *isoproterenol +baclofen* (10 nM each) (**b**), or *baclofen* (50 nM) (**c**), GTP (10 μM), MgCl$_2$ (1 mM) and *cholera* toxin *plus* [^{32}P] NAD (see 'materials and methods'). A control membrane (without drugs) is shown (**d**). At the indicated time membranes were placed in ice and immediately solubilized in nonionic detergent, and a 'pull down' experiment was performed using Forskolin-agarose (see 'materials and methods'). Numbers represent the timing of incubation prior to solubilization. The arrow corresponds to MW = 45 kDa. **B: Quantification of ADP ribosylated *Gás* in Forskolin agarose 'pull down'** Same experiment as in A except that the 45 kDa band was excised and its radioactivity counted. Values are means +/- SE of 3 determinations. * corresponds to values *iso+bac (isoproterenol + baclofen)* statistically different from *iso (isoproterenol)*, at P < 0.01. **C: electrophoresis analysis of the 'pull down' material** Electrophoretic analysis of the 'pull down' material using Forskolin–agarose was carried out with solubilized membranes previously incubated with *isoproterenol* (lane1), *baclofen* (lane 2) or *baclofen + isoproterenol* (lane 3) at 100 nM for each. Control experiments were carried out with membranes incubated with an excess of GDP (lane 4), and also with membranes treated with *pertussis* toxin (see 'materials and methods') then incubated with *baclofen + isoproterenol* at 100 nM (lane 5). The upper band corresponds to MW = 45 kDa, the lower band to MW = 35 kDa. Controls of chromatography procedure was carried out as follows: membranes were incubated with GDP (1 mM) (lane 6) and GTPγS (50 μM) *plus bac+iso* (100 nM each) (lane 7) then solubilized and proteins isolated with Forskolin-agarose were directly analyzed (without washing) in acrylamide gel electrophoresis.
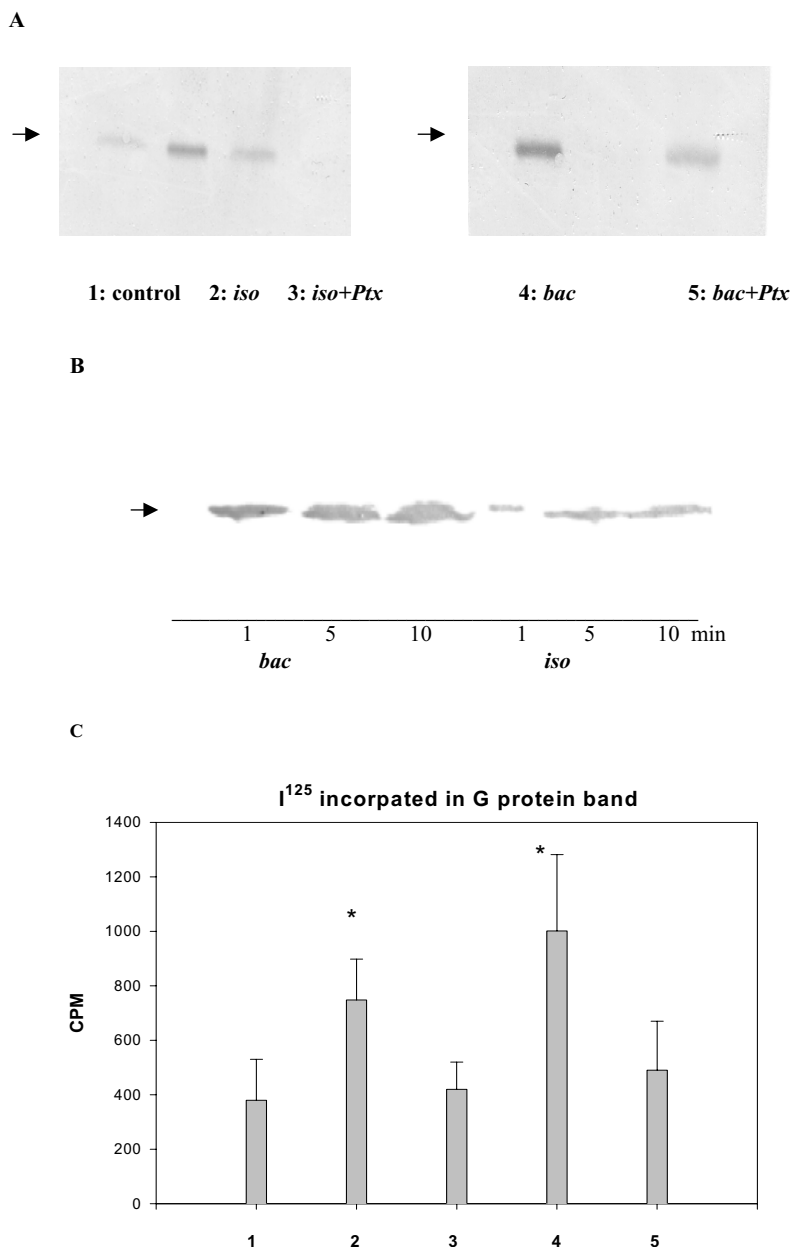
Figure 3
***Gbeta/gamma* binding to cyclase(s): Western analysis and quantification A: Analysis of *Gbeta* bound to cyclase**
Western blot analysis of *beta/gamma* bound to cyclase(s) complex. Plasma membrane (50 μg protein) was incubated with *iso-proterenol* or *baclofen* (200 nM) plus Mg (1 mM) and GTP (10 μM) for 10 min at 20°C. A 'pull down' experiment was performed in ice with Forskolin-agarose and solubilized membranes as described in 'materials and methods'. The material bound to the affinity beads was submitted to gel electrophoresis followed by Western blot analysis using anti-*beta* antibody. Membrane control (*lane 1*), isoproterenol (*lane 2*), isoproterenol + *pertussis* toxin (*PTx*) treatment (*lane 3*), baclofen (*lane 4*) and *baclofen + pertussis* toxin (*PTx*) treatment (*lane 5*). The arrow corresponds to MW = 35 kDa. **B: Time course of association *Gbeta*/cyclase** Same protocol as in A except that membranes were incubated with *baclofen* (*left*) and *isoproterenol* (*right*) at 200 nM. for 1.5 and 10 min before the cyclase complex was isolated by affinity chromatography. **C: Quantification of *Gbeta* bound to cyclase (in A)** To quantify the *beta* subunit bound to affinity beads, we used *Bolton Hunter* labeling of the affinity isolated material obtained under the conditions described in A, then carried out gel electrophoresis. The band about MW = 35 kDa was excised and counted. The bars represent 3 determinations +/-SE. * corresponds to values statistically different from control, P < 0.05. *Lane 1*: membrane control, *lane 2: isoproterenol, lane 3: isoproterenol + pertussis* toxin (PTx), *lane 4: baclofen, lane 5: baclofen + pertussis* toxin (PTx).

for the cyclase(s) molecule(s), we incubated the membranes with both *baclofen* and *isoproterenol* in the presence of GTPγS. The results showed that the *beta* subunit was present in the 'pull down' material for longer (Fig. 4b). *Baclofen/isoproterenol* was also used simultaneously to stimulate *cholera* toxin ADP-ribosylated membranes. Again we observed that the presence of *beta* in the 'pull down' lasts for longer, compared to the experiment without *cholera* toxin treatment (fig. 4b).

Cross-linking of proteins in the 'pull down' material gave similar results (fig. 5a). The molecular weight of the bands correspond to cyclase/*beta*/Gαs, *beta*/Gαs and *beta*. We observed that GTPγS re-enforces the effect of *iso+bac*. These data were confirmed by quantifying the radioactivity incorporated in the 35 kDa band by *Bolton Hunter* reagent at 1 and 10 min after a large excess of GDP and Mg were added (fig. 5b). We obtained for *iso + bac*: 620 cpm+/- 250 and 220 cpm+/- 170 respectively at 1 and 10 min; *iso + bac + GTPγS*: 830 cpm+/- 180 and 520+/- 45 respectively at 1 and 10 min; *iso + bac + CTx* gave similar results; *GTPγS* alone: 380 cpm+/-190 and 330 cpm+/- 50 respectively at 1 and 10 min. Membranes treated with *cholera* toxin gave higher counts at 1 or 10 min compared to membranes incubated with *isoproterenol* (50 +/- 30 and 250 +/- 50 at 10 min for *iso* and *CTx* respectively). Altogether, these results suggest that *beta*/*gamma* from *Gi* coupled to [GABA]B receptors has an affinity for adenylyl cyclase(s) that depends on co-association of *Gαs* with the complex.

The GTPase activity of Gαs bound to the cyclase(s) complex is decreased by co-association with beta/gamma subunits

Conversely, we checked whether co-association of *beta/gamma* in the cyclase(s) complex isolated in the 'pull down' material, might influence the *Gαs* GTPase activity. We determined the fractional occupancy of GTP in the catalytic site of *Gαs* and the rate of GTP hydrolysis. We used [$^{32}\gamma$P]-GTP bound to ADP-ribosylated *Gαs* (*cholera* toxin treated) as an internal control. Simultaneous treatment by *GABA* and *isopreterenol* decreased the rate of the cleavage of the γ-phosphate compared to *isopreterenol* or *GABA* alone after 10 min at 0°C (0.3+/-0,1 compared to 0.05+/-0,05 for *isopreterenol* and 0.05 +/-0.1 for *GABA*) (Fig. 6a). Moreover, the decrease of [$^{32}\gamma$P]-GTP bound to the cyclase complex in a single turnover experiment at 0°C was significantly less marked when the membranes were incubated with both agents (fig. 6b). At 5 min, *iso + bac* gave 0.25 +/- 0.15 compared to 0.05 for *iso* or *bac* alone. To further characterize the effect of simultaneous *iso + bac* effect on GTPase activity, we examined the amount of [$^{32}\gamma$P]-GTP in the 'pull down' material, 5 min at 0°C after the samples were complemented with Mg (fig. 7a). This amount was compared with the corresponding amount at

t = 0 without Mg. Simultaneous incubation resulted in a level of radioactivity (1300 cpm +/-300) considerably greater than the sum of the counts obtained with *iso* and *bac* alone (150 cpm+/-105 and 200 cpm+/-120 respectively). As expected, the remaining amount was greater when the membranes were treated with *cholera* toxin (3500 cpm+/-550 for *iso+bac+CTx* and 2600 cpm+/-250 for *CTx* alone).

Discussion

Adenylyl cyclases contain two conserved homologous cytoplasmic domains (C1 and C2) that form the catalytic core of the enzyme [21]. Forskolin appears to induce the assembly of these two domains by interacting with the catalytic cleft [21-23]. The affinity between C1 and C2 is also facilitated by Gαs binding. These data have been confirmed by the finding that Forskolin and Gαs stimulate synergistically the cyclase activity [22]. In the presence of *Gαs* the affinity of Forskolin for the dimer C1/C2 is high (K_d = 0.1 μM), which suggests a stronger affinity for the complete cyclase molecule [23]. The site of interaction of the cyclase (type I or II) for *beta/gamma* has been located in the C1 b region. This site is independent of the *Gαs* and Forskolin interaction domains[24]. These findings argue in favor of isolating the cyclase(s) and its associated proteins using Forskolin-agarose affinity chromatography although this procedure enriches indistinctly the different isoforms of the cyclase family. The authors who described this method [25] reported that the complex could be dissociated with high salt concentrations in order to purify the cyclase(s). Our 'pull down' material is heterogeneous because this isolation procedure does not discriminate between the different isotypes of cyclase. Furthermore, Gαs recognizes all the isoforms of adenylate cyclase whereas the association *Gbeta*/cyclase is limited to two isotypes (type I and II) which share similar features. Consequently, this mismatch makes the comparison of Gαs/ *Gbeta* precipitated in the cyclase 'pull down' difficult. Inconstant data were observed in experiments of kinetics where both molecules were quantified on the same gel. On the other hand, Gαs-GTP in the Gαs/cyclase(s) complex might have a *Kcat* for the hydrolysis of bound GTP different from one isoform of cyclase to another. The heterogeneity of cyclases in the 'pull down' makes the analysis of co-associated Gαs uncertain, specially if we aim to parallel the dynamics of association/dissociation of G*beta* and Gαs. Furthermore, our analysis might be hampered by variable elution/retention of components from the affinity column and the yield of their isolation from crude membranes. These limitations are difficult to assess.

On the other hand, the drastic increase of cAMP synthesis by simultaneous *Gi/Gs* activation finely analyzed in rat hippocampus [26] is known to be attributable to the type II isoform [2]. Type I is also regulated by *beta/gamma* and
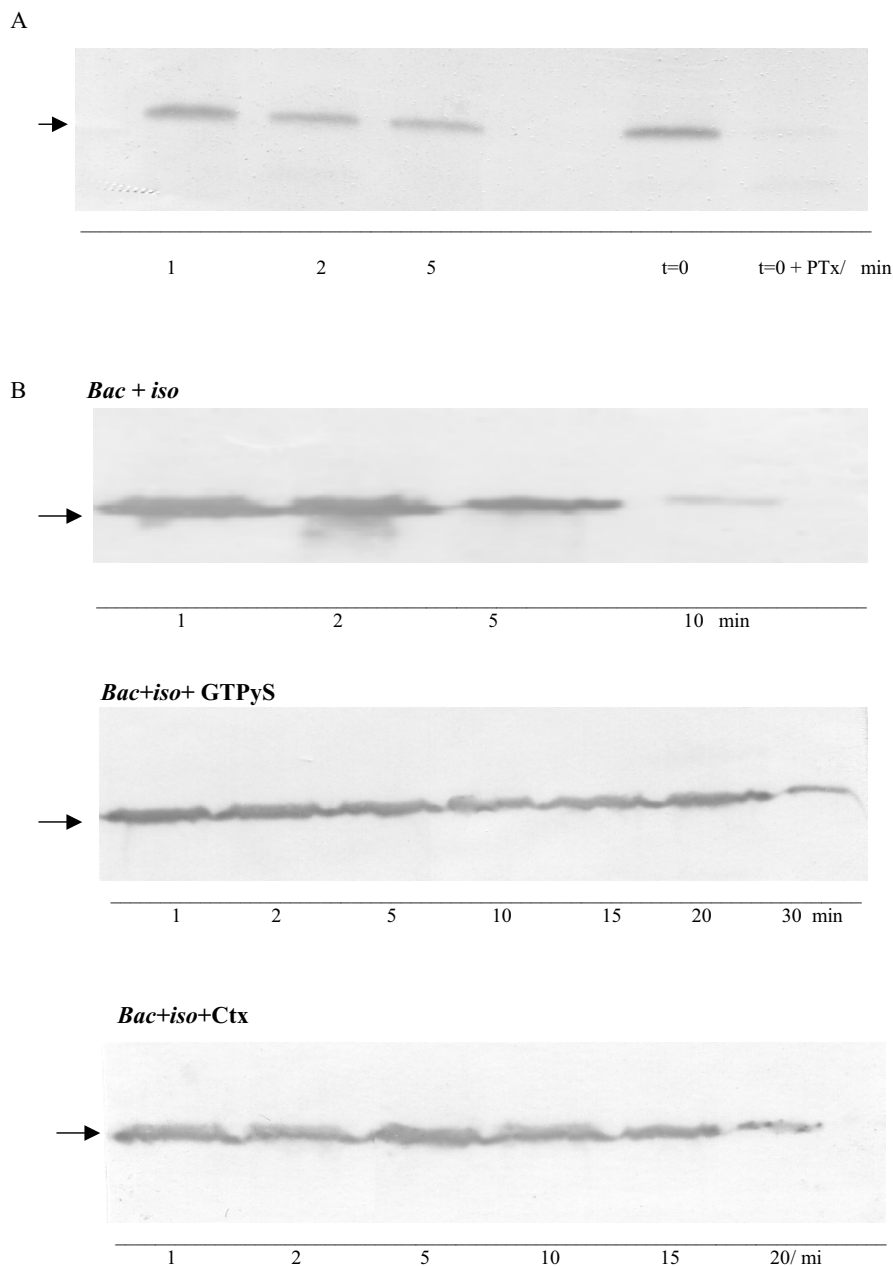
A

B *Bac + iso*

Bac+iso+ GTPγS

*Bac+iso+*Ctx

Figure 4
Kinetics of dissociation of *beta/gamma* from cyclase(s) complex formed during simultaneous activation of [GABA]b and β-adrenergic receptors (A, B) Plasma membranes (50 μg protein) were incubated with drugs (200 nM) and GTP (10 μM) + EDTA (1 mM) at 20°C for 20 min then placed in ice with Mg (5 mM) and an excess of GDP (1 mM) for 1, 2 or 5 min. Membranes were immediately solubilized (see 'materials and methods'). A 'pull down' experiment using Forskolin-agarose was performed in ice followed by gel electrophoresis, and Western blot analysis with anti-*beta*, of the material bound to the affinity beads. **A: dissociation of G*beta*/cyclase complex: analysis of baclofen** *baclofen*: at 1, 2, 5 min. A control t = 0, and a control t = 0 using membrane treated with *pertussis* toxin (*PTx*), are shown. **B: dissociation of G*beta*/cyclase complex: analysis of the coincidence *iso + bac*** Top: *baclofen + isoproterenol*: at 1, 2, 5, 10 min. *Middle*: *baclofen + isoproterenol* + GTPγS: membranes were incubated in presence of GTPγS (10 μM) and Mg (5 mM) for 20 min at 20°C, then placed in ice with an excess of GDP (1 mM) (t = 0). Membranes were then solubilized at the indicated times and a 'pull down' experiment was carried out as indicated above. *Bottom*: *baclofen + isoproterenol + cholera* toxin (*CTx*) treatment. Same experiment as above except membranes were incubated with GTP (10 μM) + *cholera* toxin (*CTx*) instead of GTPγS. The arrow corresponds to MW = 35 kDa.
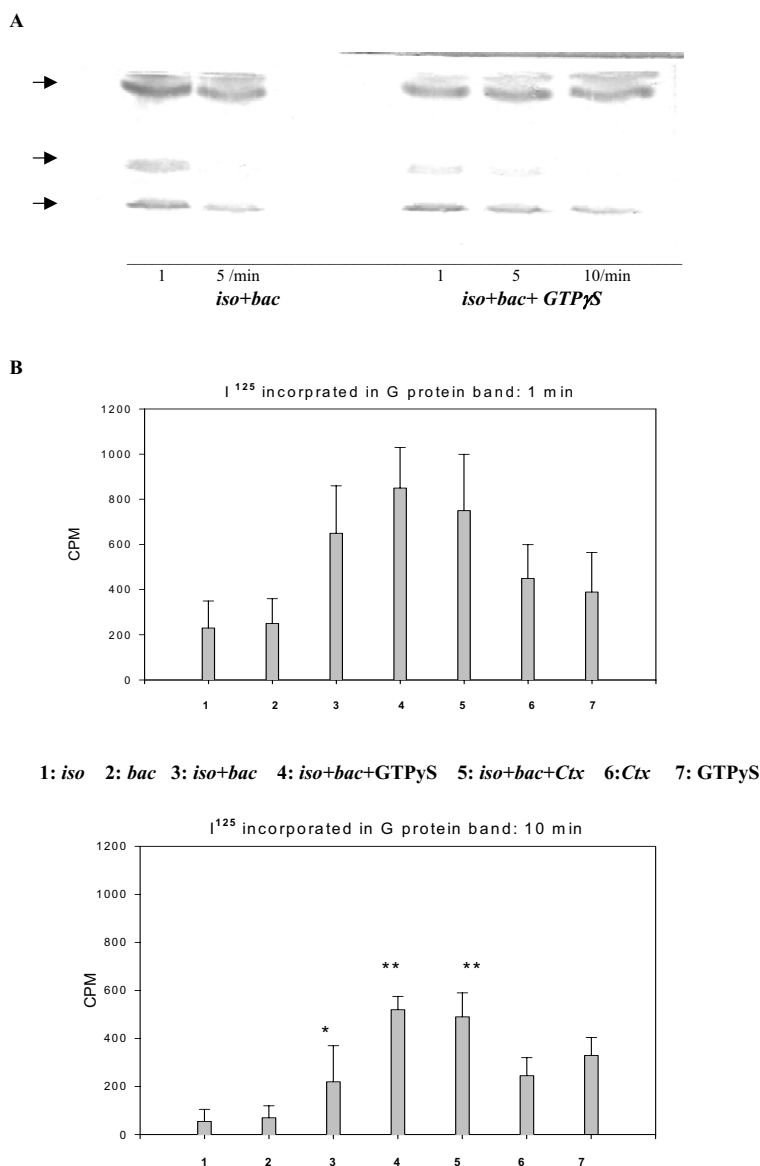
A

1   5 /min   1   5   10/min
iso+bac   *iso+bac+ GTPγS*

B

I ^{125} incorporated in G protein band: 1 min

1: *iso*   2: *bac*   3: *iso+bac*   4: *iso+bac*+GTPyS   5: *iso+bac*+Ctx   6:*Ctx*   7: **GTPyS**

I ^{125} incorporated in G protein band: 10 min

Figure 5

Covalent crosslinking of cyclase complex and quantification of G*beta* in the 'pull down' material. **A: cross linking of cyclase complex** Membranes were incubated with *baclofen* (200 nM) + *isoproterenol* (200 nM) in the presence of GTP (10 μM) + EDTA (1 mM) with or without GTPγS (10 μM) *plus* Mg (5 mM). Membranes were then placed in ice with an excess of GDP (1 mM) and Mg (5 mM) (t = 0), then were solubilized at different times (1, 5 and 15 min). A 'pull down' of the cyclase(s) complex was carried out with Forskolin-agarose beads and the material bound to the affinity beads was cross-linked with linkers (see 'materials and methods'). The molecular complexes were separated by gel electrophoresis and analyzed by Western blot using anti-*beta* antibody. *left*: bac + iso at 1, 5 min. *Right*: bac + iso + GTPγS at 1, 5 and 15 min respectively. Arrows correspond to MW > 200, 80 and 35 kDa respectively. **B: time course dissociation *Gbeta*/cyclase: quantification of *Gbeta* at 1 and 10 min** Quantification of *beta*/gamma in cyclase(s) complex, 1 min and/or 10 min after a large excess of GDP was added. Membranes were incubated with *isoproterenol* (200 nM) and/or *baclofen* (200 nM) for 20 min at 20°C, according to the same protocol as in figure 4 then placed in ice. Large excesses of GDP (1 mM) and Mg (5 mM) were added. The membranes were solubilized after 1 min and/or 10 min and the cyclase(s) complex was isolated by Forskolin-agarose. To quantify the *beta* subunit bound to the affinity beads, we carried out a *Bolton Hunter* labeling of the affinity isolated material followed by gel electrophoresis. Instead of Western blot analysis, gels were dried and the band about MW = 35 kDa was excised and counted. The bars represent 3 determinations +/-SE. * and ** correspond to P < 0.1 and P < 0.05 respectively compared to *iso* and/or *bac*. *Lane 1*:*isoproterenol*, *lane 2*: baclofen, *lane 3*: isoproterenol + baclofen, *lane 4*: isoproterenol + baclofen + GTPyS, *lane 5*: isoproterenol + baclofen + CTx, *lane 6*: CTx, *lane 7*: GTPyS
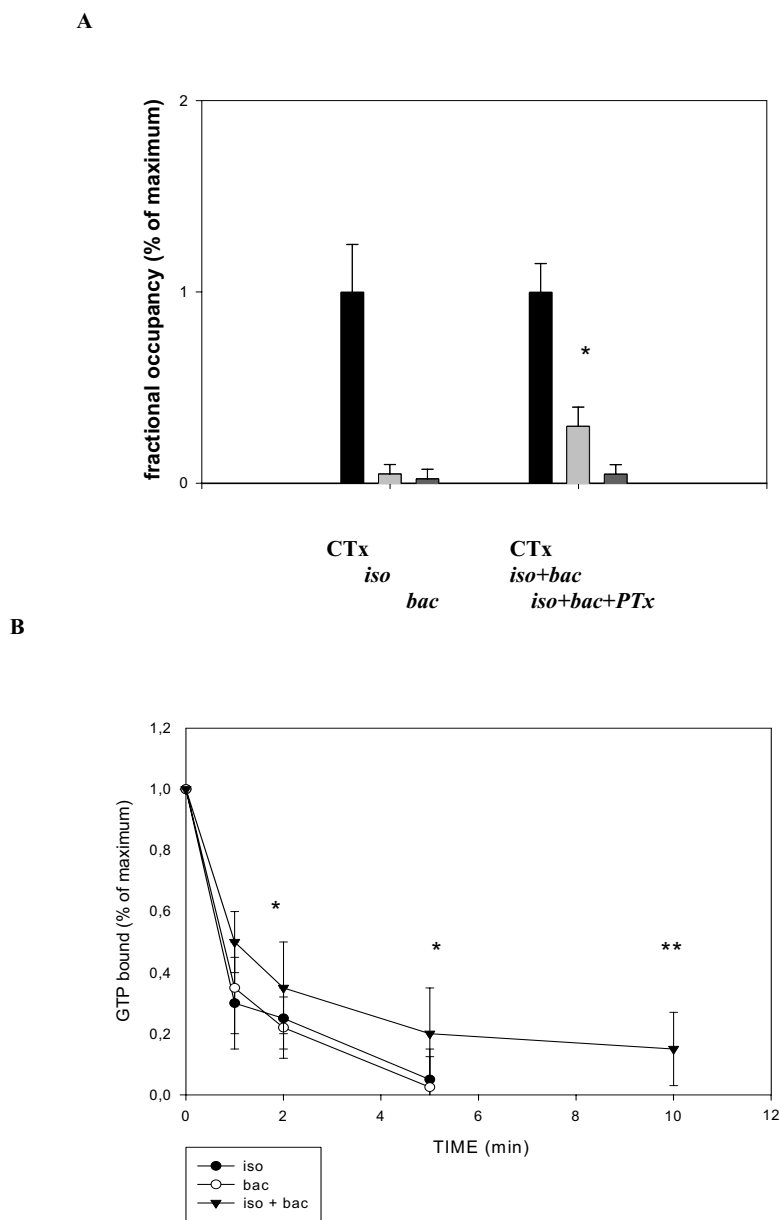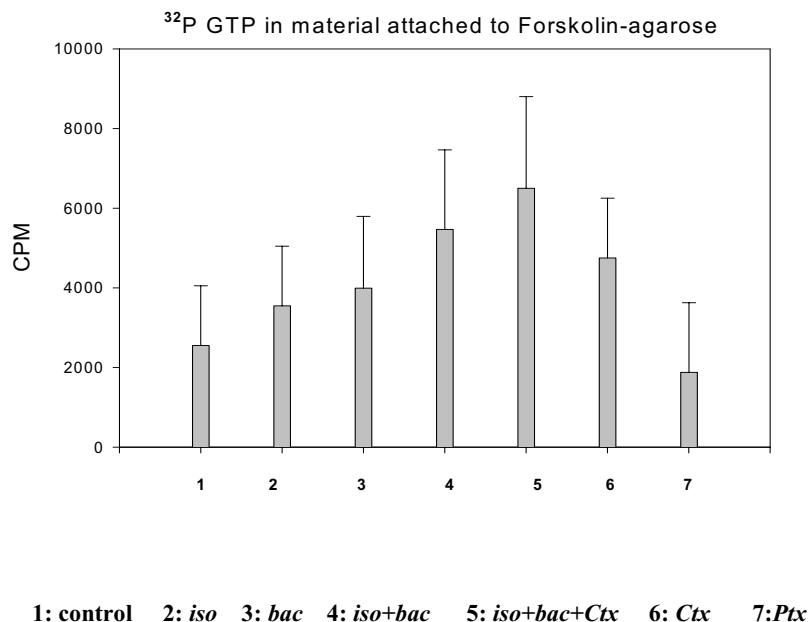
Figure 6
Determination of the fractional occupancy and hydrolysis of GTP in the catalytic site of *Gαs* complexed with adenylyl cyclase(s) A: Fractional occupancy of GTP in cyclase(s) associated *Gs*. Comparative kinetics of GTP hydrolysis at 0°C. Membranes (50 μg protein per assay) were incubated with [γ32-P] GTP (1 μM, 10 000 cpm/pmol) and *isoproterenol, isoproterenol + baclofen or baclofen* (each 200 nM) in the presence of EDTA (1 mM) for 20 min at 20°C. The samples were placed on ice and complemented with Mg (5 mM) and an excess of cold GTP (1 mM) for 10 min, then the membranes were solubilized in presence of NaF (100 μM) and a 'pull down' experiment using Forskolin-agarose was carried out as above. The radioactivity in the isolated 'pull down' was measured by filtration on nitrocellulose membranes and recorded as a percentage (+/-SE) of the radioactivity measured in parallel experiment using membranes treated with *cholera* toxin (*CTx*). Bars represent the mean +/-SE of 5 experiments. * represents values of *iso+bac* significantly different from *iso* or *bac* (P < 0.01) **B: Single turnover GTP hydrolysis of cyclase(s) associated *Gs* at 0°C.** Membranes (50 μg protein per assay) were incubated with *iso, iso+bac* or *bac* (each 200 nM) and with [γ32-P] GTP (1 μM, 10 000 cpm/pmol) and EDTA (1 mM) for 20 min at 20°C. The GTPase reaction was initiated by the addition of MgCl2 (5 mM) and an excess of cold GTP (1 mM) in ice. At various time points, NaF (100 μM) was added and membranes were immediately solubilized and incubated with Forskolin-agarose. Radioactivity in the 'pull down' complex attached to the Forskolin-agarose was counted as above and reported as percentage of the radioactivity at t = 0* P < 0.05 and ** P < 0.01
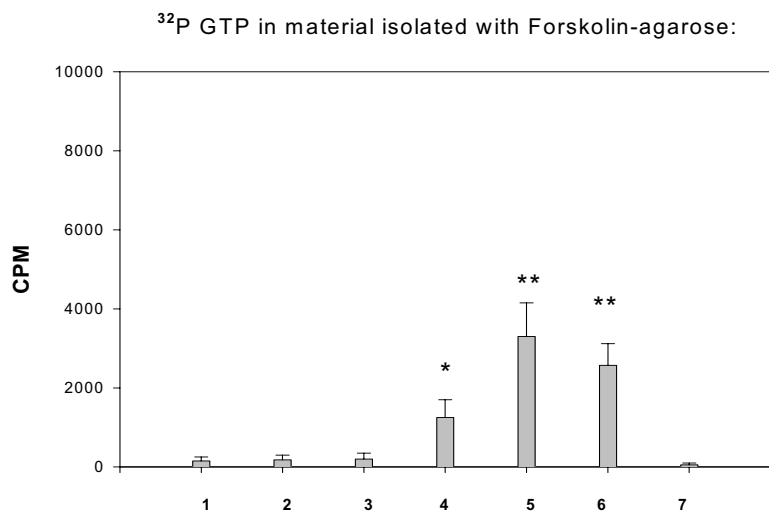
A

1: control   2: *iso*   3: *bac*   4: *iso+bac*   5: *iso+bac+Ctx*   6: *Ctx*   7:*Ptx*

B

Figure 7
Quantification of [γ32-P] GTP bound to cyclase complex A: Quantification [^{32}yP- GTP] in cyclase(s) complex at t = 0. Membranes (50 μg protein) were incubated with [γ32-P] GTP and *isoproterenol, isoproterenol + baclofen* or *baclofen* (each 200 nM) in presence of EDTA (1 mM) for 20 min at 20°C. The membranes were then placed in ice and solubilized, and the cyclase(s) complex was isolated by Forskolin-agarose. The radioactivity in the isolated 'pull down' was measured by filtration on nitrocellulose membrane. Bars represent the mean +/-SE of 3 experiments. Control experiments with only membrane, *CTx* or *PTx* treated membrane are also shown. *Lane 1*: control membrane, *lane 2*: *iso*, *lane 3*: *bac*, *lane 4*: *iso + bac*, *lane 5*: *iso + bac + CTx*, *lane 6*: *CTx*, lane 7: *PTx* **B: Quantification [^{32}yP-GTP] in cyclase(s) complex at t = 5 min.** [γ^{32}-P] GTP bound to cyclase(s) complex was measured 5 min after a large excess of GTP was added. Membranes were incubated as indicated above, placed in ice and complemented with Mg (5 mM) for 5 min, then solubilized in the presence of NaF (100 μM). The cyclase(s) complex was isolated as above. The radioactivity in the isolated 'pull down' was measured by filtration on nitrocellulose membrane. Bars represent the mean +/-SE of 3 experiments. * p < 0.01 and ** p < 0.005 *Lane 1*: control membrane, *lane 2*: *iso*, *lane 3*: *bac*, *lane 4*: *iso + bac*, *lane 5*: *iso + bac + CTx*, *lane 6*: *CTx*, lane 7: *PTx*

Gαs but here *beta/gamma* is inhibitory. High concentrations of *beta/gamma* do not eliminate the cyclase type I activity induced by *Gαs*, but stabilize it at intermediate levels, which suggests that the conformation of cyclase type I is rearranged by association with *beta/gamma* [11]. The two isoforms show similar affinities for *beta/gamma* (half-maximal effect about 5 nM) [11]. We postulate therefore that the molecular events described in this report mostly highlight the relevance of cyclase type II, despite isotype I contamination. We might also speculate that the intra-complex regulatory mechanisms described here have the same characteristics for both isotypes, although they induce opposite effects on cyclase activity.

Furthermore, we carried out our experiments with intact membranes, in order to preserve the architecture of the multiple associated proteins that might be relevant to the biochemistry involved in coincidental phenomenon. For example, regulators of G proteins (RGS) interact mostly with the *Gα* subunit of *Gi/o/q* by accelerating their intrinsic GTPase activity, which alters the amplitude of the effect of a stimulated receptor [27]. Each member of this large family of genes (16 isotypes have been described) displays differential selectivity for these G proteins [27]. Interestingly, RGS 14 and 12 are unique in that they inhibit the guanine nucleotide exchange of *Gi* in addition to their GAP (GTPase activating protein) activity. A recent report shows that PKA phosphorylation of RGS 14 increases the inhibition of nucleotide exchange 3-fold, thereby increasing the binding *Gi*/GDP and consequently limiting the interactions of *Gi* with effectors [28]. RGS 14 is expressed in brain [29], which might be of interest in relation to our work. By increasing PKA activity, high cAMP concentrations should inhibit more *Gi* molecules enhancing the effect of the simultaneous stimulation (*Gαs* and *Gbeta*) of cyclase type II. After a while *beta/gamma* would then be captured by *Gi*/GDP, ending the simultaneous stimulation of cyclase type II by a retro-control loop.

In this report, we have investigated whether *beta/gamma* bound to cyclase(s) might influence the rate of GTP hydrolysis of *Gαs*, or conversely whether *Gαs* bound to cyclase(s) might modify its affinity for *beta/gamma*. We found that the GTPase activity of *Gαs* coupled to the cyclase(s) complex decreases when *beta* adrenergic and *GABA* receptors are simultaneously stimulated. We also demonstrated that the affinity of *beta/gamma* (from *Gi*) for the cyclase(s) is increased when the latter is associated with *GTP-Gαs*. Our data strongly suggest that simultaneous application of stimuli, which individually exert opposing effects on the levels of second messengers, triggers specific kinetics of protein association and enzyme catalysis. This highlights the paradoxically high level of cAMP obtained by simultaneous *GABA/beta* adrenergic receptor activation. Adenylate cyclase was reported to

function as a GTPase activating protein for *Gαs* [30]. Our data support the idea that *beta/gamma* from [GABA]b receptor-coupled *Gi* retards the GTPase activity of adrenergic receptor-coupled *Gαs* when these molecules are complexed with adenylyl cyclase isoforms.

Conclusions

Cyclases are not the only molecules to be regulated '*à la carte*' by simultaneous signaling. Various molecules in different tissues correspond to the definition of 'detectors of simultaneity': for example, muscle phosphorylase kinase, a tetramer constituted of a catalytic subunit, two cAMP dependent regulatory subunits and calmodulin. Two stimuli (Ca++ and cAMP) synergistically stimulate phosphorylase kinase activity [31]. Furthermore, some molecules fine-tune their activities, depending on the local context, in 'reading' the temporal order of their regulatory signals. For example, phosphatase I is associated with a potent protein inhibitor: this inhibitor has a site that is phosphorylated by caseine kinase I. This phosphorylation protects an adjacent phosphorylated site from calcineurin and consequently these two phospho sites in the inhibitor controls the activity of the phosphatase [32]. Another example is the endothelial nitric oxide synthase (eNOS). This enzyme has two major phosphorylation sites, which have opposite functions. One, phosphorylated by PKA, stimulates the enzyme activity, the other, phosphorylated by PKC, inhibits it. Interestingly, PKA and/or PKC phosphorylation of one site induces dephosphorylation of the other. Two phosphatases associated with eNOS have one or the other phosphorylated site as specific substrate. When one phosphatase is active the other is inactive [33]. All these coordinated controls display different modes used by key molecules acting as sensors of multiple converging second messengers. Specific molecular events induced by simultaneous signals and/or sequentially ordered signals imply interactions of components in a large complex. This seems the essential paradigm of synaptic function.

Methods
Reagents
Western blotting was carried out with a polyclonal anti-*Beta* antibody (Santa Cruz/Biotechnology, 2161 Delaware Avenue, Santa Cruz Ca 95060), which recognizes all the isoforms of *beta*. *Cholera* toxin (activated subunit), *pertussis* toxin and NAD+ were purchased from *Sigma* (St. Louis Missouri 63178-9916 P.O. Box 14508). Radioactive [γ-^{32}P] GTP (*NEG004Z*, 6000 Ci/mmol) and [^{32}P] NAD+ (NEG023X, 800 Ci/mmol) were purchased from *NEN Life Sciences Products* (PerkinElmer life Sciences, 549 Albany Street, Boston MA 02118–2512). Cyclic AMP was determined using a radioactive kit purchased from *NEN* (RIA kit using [^{125}I] *NEK033*) with membranes in experimental conditions described in figure legends. *Bolton Hunter* rea-

gent (diiodinated), $[^{125}I]$ (*NEX120H*) was purchased from *NEN*. *Saclofen*, *propanolol*, *isoproterenol* and *baclofen* were purchased from *Sigma (cell signaling and neuroscience)*. Forskolin-agarose was purchased from *Sigma*.

Preparation of plasma membrane of isolated nerve terminals (synaptosomes)

Synaptosomes were prepared by homogenization of rat brain (Sprague Dawley, 150 g) in 310 mM sucrose, 10 mM Hepes (pH 7.4), 1 mM EDTA. The homogenate was centrifuged for 5 min at 900 g_{max} at 4°C. The supernatants were combined and centrifuged at 11 000 g_{max} for 15 min. Pellets were re-suspended and this synaptosome-enriched fraction was layered on to each of two discontinuous gradients (12% (w/v), 9% and 6% Ficoll) and centrifuged for 30 min at 75,000 g_{max} according to the protocol described elsewhere [34]. The intermediate band (within the 9% layer) was resuspended in HEPES 5 mM (pH 7.4), EDTA 1 mM for 1 h in ice and the plasma membrane fraction released by osmotic shock was re-centrifuged for 5 min at 10,000 g_{max}. The isolated plasma membranes were incubated at 30°C for 15 min with anti proteases (PMSF: 1 μg/ml, leupeptin: 1 μg/ml and aprotinin: 1 μg/ml) in order to wash out endogenous neuromediators, then aliquoted and frozen at -70°C.

Isolation of the cyclase complex

Membranes (50 μg protein) were assayed as described in the figure legends, then solubilized in Triton X-100 1%, 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA. Samples were briefly centrifuged and the supernatant was diluted five times (final concentration of Triton X-100 = 0.2%) in 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA *plus* 0.5 mM Mg. Lubrol at these concentrations potently inhibits GTPase activity by preventing the release of hydrolysis products, as reported elsewhere [35]. We observed that Triton X-100 or Tween 20 (Sigma) gave similar effects. Forskolin-agarose (25 μl) was added to the extract and incubated for 10 min at 4°C. Samples were constantly re-suspended by tube rotation. Samples were then centrifuged briefly, the supernatants discarded and the pellets washed once with the same buffer. Molecules attached to affinity beads were separated by SDS-PAGE alongside pre-stained molecular weight markers (Sigma). The electrophoresis gels were stained with colloidal blue (Sigma).

ADP-ribosylation of Gs and Gi by Pertussis toxin and cholera toxin

ADP-ribosylation using *pertussis* toxin (*Sigma*) was carried out as indicated in the figure legends, in the presence of 1 mM EDTA, 2 mM DTT, 5 mM thymidine, 10 mM HEPES (pH 7.4), 25 μg/ml *pertussis* toxin and 10 μM NAD$^+$, according to the protocol described elsewhere [36]. ADP-ribosylation was also carried out with 10 μg/ml *cholera*

toxin as described elsewhere [37] with a slight modification: the endogenous cofactor for *cholera* toxin (ARF) was activated with only GTP, as indicated in the figures. For radioactive labeling by *cholera* toxin (10 μg/ml toxin), 10 μM [^{32}P] NAD$^+$ (50 000 cpm/pmol) were incubated with membranes in a buffer with 10 mM thymidine and 200 mM Nacl as indicated in figure legends. Radioactivity attached to the isolated cyclase complex was counted and normalized with the amount of proteins precipitated by the affinity chromatography as follows: an aliquot of each sample was incubated with 5 μl of trypsin-agarose (Sigma) for 30 min in 10 mM phosphate buffer pH = 8 and the released peptides were measured at 220 nm in 150 μl of the same buffer.

Cold ADP-ribosylation by *cholera* toxin was carried out with membranes 20 min at 30°C with 30 μg/ml toxin, 50 μM NAD$^+$ and the concentration of GTP indicated in figure legends. Cold ADP-ribosylation by *pertussis* toxin was carried out with membranes with 50 μg/ml toxin and 50 μM NAD$^+$ for 20 min at 30°C.

Analytical methods

The material bound to Forskolin-agarose was submitted to SDS acrylamide gel electrophoresis (12%) using pre-stained standards (*Sigma*). To monitor the accumulation of *beta/gamma*, the gels were blotted on to nitrocellulose for Western analysis using antibodies at the dilution indicated by *Santa Cruz Biotech*. The anti-*beta* used (T-20) is broadly reactive with *beta* 1, 2, 3 and 4. The blots were developed by alkaline phosphatase conjugated anti rabbit IgG (*Sigma*). For cross-linking experiment, the precipitate was incubated with glutaraldehyde 1% in 0.2 M Na carbonate pH = 8 for few minutes then the reaction was stopped with 10 μl of lysine solution (1 mM). Experimental conditions for the kinetic analysis are given in the figure legends. To quantify the *beta* subunit, the material bound to Forskolin-agarose was labeled with *Bolton Hunter* reagent (*NEN*) (20 000 cpm/samples) in 150 μl phosphate buffer 10 mM (pH 7.4) for few minutes, then molecules were separated by SDS polyacrylamide gel electrophoresis. Gels were dried and the bands around 35 kDa were excised and counted. Radiolabeled ADP-ribosylated *Gαs* was counted according to the same method. Counts were normalized with the amount of protein in each individual 'pull down'.

To quantify the molar amount of precipitated Gα and *beta*, the material attached to affinity chromatography was labeled with *Bolton Hunter* reagent as above. This material was first submitted to high salt treatment in 50 μl of 500 mM Nacl and 10 mM phosphate pH = 8 for 1 hour to dissociate proteins, then samples were diluted 3 times before the labeling. This material was precipitated by trichloracetic acid (5%), the pellet was neutralized with Tris buffer

and loaded on electrophoresis gel (10% acrylamide). The respective bands were excised from the dried gel and counted. We assumed that the intensity of I^{125} labeling is proportional to the size of the protein and we divided the counts by an index of molecular weight: 1 for *beta*, 1.28 for $G\alpha$ and *3.15* for cyclase (*beta*/*beta* = 1, $G\alpha$/*beta* = 1.28 and Cyclase/*beta* = 3.15) in order to compare the numbers. Counts were normalized with the amount of protein as described above and the ratio of normalized counts are reported as the ratio of molar amount of molecules. Results represent the average of three experiments +/-SE.

Determination of cAMP levels

Adenylate cyclase activity was estimated in membranes in a medium consisting of 20 mM MOPS (pH 7.4), 10 mM creatine phosphate, 50 μg per ml of creatine kinase, 5 mM $MgCl_2$ and 500 μM radioactive ATP (200 cpm/pmol) using a RIA kit (*NEN*).

GTPase assay

GTPase activity was measured by the release of $[^{32}P_i]$ from $[\gamma^{32}P]$ GTP previously bound to $G\alpha s$. GTP hydrolytic activity was detected by pre-equilibrating the G proteins with $[\gamma^{32}P]$ GTP in the absence of Mg, then the GTPase reaction was started by adding $MgCl_2$ at 0°C (5 mM final concentration). The decrease of the radiolabeled GTP/G protein complex was quantified by nitrocellulose filtration. This method, described elsewhere [38], was used with slight modifications in the experimental conditions described in the figure legends.

Single turnover GTPase assay

This assay, described elsewhere [39], was slightly modified. Membranes (50 μg protein) in 250 μl final volume were incubated with 1 μM $[\gamma^{32}P]$ GTP (10,000 cpm/pmol) in 50 mM Hepes (pH 8.0), 1 mM DTT, 1 mM EDTA, at 20°C for 20 min. Samples were placed in ice for 5 min. The GTPase reaction was initiated at 0°C (in ice) by the addition of $MgCl_2$ and GTP to final concentrations of 5 mM and 1 mM respectively. At the indicated time points, samples were solubilized and the cyclase complex was isolated with 25 μl of Forskolin-agarose in presence of NaF (100 μM). The attached material was re-suspended in 150 μl of the initial buffer at 0°C (without Mg) and submitted to immediate filtration on nitrocellulose. Filters were counted in Beckman *beta* counter.

Determination of fractional occupancy by GTP

This assay was adapted from the procedure described elsewhere [40,41]. Membranes (50 μg protein) were incubated at 20°C for 20 min with 1 μM $[\gamma^{32}P]$ GTP (10,000 cpm/pmol) in 50 mM Hepes (pH 8.0), 1 mM DTT, 1 mM EDTA and drugs. Control membranes were treated with *cholera* toxin (*CTx*) as indicated above. The membranes (incubated with drugs or *CTx* according to the figure leg-

ends) were placed in ice, then the GTPase reaction was initiated by 5 mM $MgCl_2$. At the indicated times, the cyclase complex was isolated in presence of NaF (100 μM) and radioactivity was counted as above.

Abbreviatons

Beta, *gamma*: subunits of G protein, *Gs*: stimulatory G protein, *Gi*: inhibitory G protein, *G*α: alpha subunit of G protein, *CTx*: *cholera* toxin, *PTx*: *pertussis* toxin, *G*α*s*: stimulatory alpha subunit of G protein, *iso*: isoproterenol, *bac*: baclofen

Authors' contributions

A.R. drafted the manuscript and supervised the overall conduct of the research. S.T. participated in the team work and provided continuous help with the experimental work. F.P. and C.C. carried out part of the experimental work (dosage, electrophoresis analysis and affinity isolation of proteins). All authors approved the manuscript.

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