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

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Published: 09 June 2004
Received: 29 July 2003
Accepted: 09 June 2004

This article is available from: http://www.biomedcentral.com/1471-2121/5/25

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Abstract

Background: We investigated how the synthesis of cAMP, stimulated by isoproterenol acting through β-adrenoceptors and Gs, is strongly amplified by simultaneous incubation with baclofen. Baclofen is an agonist of δ-aminobutyric acid type B receptors [GABAB], 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 adenyl cyclase type II in this phenomenon, which is highly active when complexed with both beta/gamma and Gs. 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 Gs when beta/gamma, Gs and cyclase are associated in a complex form and conversely, whether Gs 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 adenyl 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 Gs
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 Gs GTPase. ADP-ribosylation by pertussis toxin uncouples receptors and Gi [36,37]. We
measured the amount of radioactive ADP-ribosylated Ga 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. Ga from inactivated receptors represents a huge amount compared to Ga activated by isoproterenol. This interference might overwhelm the specific signal induced by isoproterenol if a probable artefactual slow exchange GDP/GTP occurs at silent Ga. 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 Ga. 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 Ga bound to isolated cyclase heterocomplex(s), compared to the amount bound after isoproterenol treatment alone (Fig. 2a). We analyzed also the kinetics of Ga/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 Ga 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 Gα/Gβ 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. The biotinylagarose were dissociated with 1 M NaCl and derivatized with the reagent: N-hydroxysuccinimido biotin. The biotinylated material was then a rabbit antibody-alkaline phosphatase. We observed that two bands (45 and 35 kDa) disappeared when membranes were incubated with isoproterenol. Moreover, the specificity of the 'pull down' procedure was assessed with membranes incubated with GDP versus GTPγS+iso+bac. 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 not 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 Ga might influence the affinity of beta/gamma
Analysis of cholera toxin [\(^{32}\gamma P\)]-ADP-ribosylated G\(\alpha_s\) bound to cyclase

A: ADP ribosylation of G\(\alpha_s\) in Forskolin agarose 'pull down'

Plasma membrane (50 \(\mu\)g protein) was incubated with isoproterenol (10 nM) (a), isoproterenol + baclofen (10 nM each) (b), or baclofen (50 nM) (c), GTP (10 \(\mu\)M), Mg\(\text{Cl}_2\) (1 mM) and cholera toxin plus [\(^{32}\text{P}\)] 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 G\(\alpha_s\) 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 isoproterenol + baclofen statistically different from 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 (lane 1), baclofen (lane 2) or isoproterenol + baclofen (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 GTP\(\gamma S\) (50 \(\mu\)M) plus baclofen + isoproterenol (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 Gbeta/gamma bound to cyclase(s) complex. Plasma membrane (50 µg protein) was incubated with isoproterenol 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 Gbeta 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 [GABAB] 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 [32P]-GTP bound to ADP-ribosylated Gαs (cholera toxin treated) as an internal control. Simultaneous treatment by GABA and isopreteteneol decreased the rate of cleavage of the γ-phosphate compared to isopreteteneol or GABA alone after 10 min at 0°C (0.3 +/-0.1 compared to 0.05 +/- -0.05 for isopreteteneol and 0.05 +/-0.1 for GABA) (Fig. 6a). Moreover, the decrease of [32P]-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 [32P]-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 (Kd = 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 isoforms of cyclase. Furthermore, Gαs recognizes all the isoforms of adenylyl cyclase whereas the association Gbeta/cyclase is limited to two isoforms (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 Gbeta 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/Gαs 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
Kinetics of dissociation of beta/gamma from cyclase(s) complex formed during simultaneous activation of [GABA]b and beta-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 Gbeta/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 Gbeta/cyclase complex: analysis of the coincidence iso + bac: 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.
Figure 5  
Covalent crosslinking of cyclase complex and quantification of Gβ 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-β 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 Gβ/cyclase: quantification of Gβ at 1 and 10 min**  
Quantification of β/γ 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 β 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 + GTPγS, lane 5: isoproterenol + baclofen + CTx, lane 6: CTx, lane 7: GTPγS.
**Figure 6**

**Determination of the fractional occupancy and hydrolysis of GTP in the catalytic site of G\(\alpha_S\) complexed with adenylyl cyclase(s)**

**A:** Fractional occupancy of GTP in cyclase(s) associated G\(\alpha_S\).

Comparative kinetics of GTP hydrolysis at 0°C. Membranes (50 µg protein per assay) were incubated with \(^{\gamma^{32-P}}\) 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 G\(\alpha_S\) at 0°C. Membranes (50 µg protein per assay) were incubated with iso, iso + bac or bac (each 200 nM) and with \(^{\gamma^{32-P}}\) 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 MgCl\(_2\) (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
Quantification of $[\gamma^32P]$ GTP bound to cyclase complex

**A**

Quantification $[\gamma^32P]$-GTP in cyclase(s) complex at $t = 0$.

Membranes (50 µg protein) were incubated with $[\gamma^32P]$ 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, lane 2: iso, lane 3: bac, lane 4: iso+bac, lane 5: iso+bac+Ctx, lane 6: Ctx, lane 7: PtX

**B**

Quantification $[\gamma^32P]$-GTP in cyclase(s) complex at $t = 5$ min. $[\gamma^32P]$ 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
Gas but here beta/gamma is inhibitory. High concentrations of beta/gamma do not eliminate the cyclase type I activity induced by Gas, 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 Gas 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 isoforms 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 (Gas 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 Gas, or conversely whether Gas bound to cyclase(s) might modify its affinity for beta/gamma. We found that the GTPase activity of Gas 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-Gas. 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 Gas [30]. Our data support the idea that beta/gamma from [GABA] receptor-coupled Gi retards the GTPase activity of adrenergic receptor-coupled Gas 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 [y-32P] GTP (NEG004Z, 6000 Ci/mmol) and [32P] 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[32P] NEK033) with membranes in experimental conditions described in figure legends. Bolton Hunter rea-
gent (diiodinated), $[^{125}I]$ (NEX120H) was purchased from NEN. Saxofen, 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 Heps (pH 7.4), 1 mM EDTA. The homogenate was centrifuged for 5 min at 900 g<sub>max</sub> at 4°C. The supernatants were combined and centrifuged at 11 000 g<sub>max</sub> 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<sub>max</sub>. The isolated plasma membranes were incubated for 10 min at 4°C. Samples were extracted and incubated for 10 min at 4°C. Samples were 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 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<sup>+</sup>, 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 [32P] NAD<sup>+</sup> (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<sup>+</sup> 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<sup>+</sup> 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 G<sub>α</sub> 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 G<sub>s</sub> 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 125I 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 Ga and 3.15 for cyclase (beta/beta = 1, Ga/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 MgCl₂ and 500 µM radioactive ATP (200 cpm/pmol) using a RIA kit (NEN).

**GTPase assay**
GTPase activity was measured by the release of [32P] from [γ32P] GTP previously bound to Gas. GTP hydrolytic activity was detected by pre-equilibrating the G proteins with [γ32P] GTP in the absence of Mg, then the GTPase reaction was started by adding MgCl₂ at 0°C (5 mM final concentration). The decrease of the radiolabeled GTP/G concentration (activity was detected by pre-equilibrating the G proteins with [γ32P] GTP) in a medium consisting of 20 mM MOPS (pH 7.4), 10 mM creatine phosphate, 50 µg per ml of creatine kinase, 5 mM MgCl₂ and 500 µM radioactive ATP (200 cpm/pmol) using a RIA kit (NEN).

**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 [γ32P] 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 MgCl₂ 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 [γ32P] 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 legends) were placed in ice, then the GTPase reaction was initiated by 5 mM MgCl₂. At the indicated times, the cyclase complex was isolated in presence of NaF (100 µM) and radioactivity was counted as above.

**Abbreviations**
Beta, gamma: subunits of G protein, Ga: stimulatory G protein, Gi: inhibitory G protein, Gα: alpha subunit of G protein, CTx: cholera toxin, PTx: pertussis toxin, Gas: 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.

**Acknowledgments**
This work was supported by CNRS and a grant from the local government of Burgundy. We are grateful to Pierre Hericourt and Amélie Garnier for technical assistance and discussions. We thank Sue Broughton, Ralph Greenspan and Paul Salin for the many discussions that initiated this work.

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