

α_2 -Adrenergic agonists stimulate DNA synthesis in Chinese hamster lung fibroblasts transfected with a human α_2 -adrenergic receptor gene

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To test the hypothesis that agents activating receptors negatively coupled to adenylyl cyclase (AC) can stimulate cell proliferation, we have expressed a human α_2 -adrenergic receptor (α_2 -C10) in CCL39 cells and studied the effects of α_2 -agonists on reinitiation of DNA synthesis in quiescent cells. We report that the α_2 -agonists epinephrine and clonidine stimulate [3 H]-thymidine incorporation in synergy with fibroblast growth factor and that the α_2 -antagonist yohimbine efficiently inhibits this response. Epinephrine- and clonidine-stimulated DNA synthesis is completely blocked by pertussis toxin and correlates well with the inhibition of prostaglandin E_1 -stimulated AC. Thus, their action closely resembles the action of serotonin in the same cell system, which is mediated through 5-HT_{1b} receptors. In fact, serotonin- and epinephrine-stimulated DNA synthesis reinitiation is not additive, suggesting that both agents act through a common pathway. Interestingly, α_2 -agonists also induced a moderate release of inositol phosphates, indicating that α_2 -adrenergic receptors can interact both with the AC and phospholipase C messenger system. Activation of phosphoinositide (PI) turnover by epinephrine leads to a significant stimulation of Na⁺/H⁺ exchange but is insufficient to trigger a mitogenic response in CCL39 cells, as will be discussed. We found no evidence for epinephrine-induced activation of Na⁺/H⁺ exchange by a mechanism independent of PI breakdown. Our data show that α_2 -adrenergic receptors can play a role in the regulation of cell proliferation in an appropriate context;

also, the data support the hypothesis that receptors negatively coupled to AC must be taken into account as mediators of growth factor action in fibroblasts, in particular when activated in parallel with receptor tyrosine kinases.

Introduction

In Chinese hamster lung fibroblasts (CCL39 cell line), the mitogenic action of α -thrombin and serotonin is mediated via one or more pertussis toxin (PTX)-sensitive G proteins, whereas other growth factors, which activate receptor tyrosine kinases, do not act through this pathway (Chambard *et al.*, 1987; Pouyssegur *et al.*, 1988; Seuwen *et al.*, 1988a). Similar results have been reported for NIH-3T3 cells, where bombesin action is inhibited by PTX treatment of cells and cell proliferation stimulated by platelet-derived growth factor (PDGF) is unaffected (Letterio *et al.*, 1986). The mitogenic action of growth factors acting through G-protein-coupled receptors has generally been attributed to their activation of phospholipase C (PLC), whereas other G-protein-mediated processes often observed to occur in parallel (like the inhibition of adenylyl cyclase [AC]) have received less attention. However, our work on the mitogenic action of serotonin (Seuwen *et al.*, 1988a) as well as other studies (Zachary *et al.*, 1987; Kavanaugh *et al.*, 1988; van Corven *et al.*, 1989) suggested that the signaling event relevant for the stimulation of DNA synthesis and sensitive to the toxin was different from activation of phosphoinositide breakdown. Instead, we have formulated the hypothesis that inhibition of AC or the activation of another, so far undefined, effector system by G_i proteins is at the origin of the PTX-sensitive component of the growth response (Seuwen *et al.*, 1988a). We therefore predicted that activation of any receptor negatively coupled to AC should produce a mitogenic signal comparable with the one elicited by serotonin in CCL39 hamster fibroblasts; consequently, we decided to express receptors of this kind by DNA transfection in this cell system to study

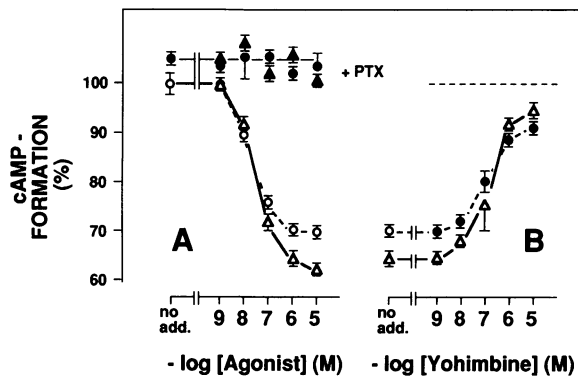


Figure 1. Inhibition of adenylyl cyclase. Experiments were performed as described in the methods section. (A) $39\alpha_2$ -21 cells pretreated (●, ▲) or not (○, Δ) with PTX (50 ng/ml) were stimulated with PGE₁ and exposed to increasing concentrations of epinephrine (Δ, ▲) or clonidine (○, ●), as indicated on the abscissa. (B) Reversal of the epinephrine (1 μM, Δ)- and clonidine (1 μM, ○)-induced inhibition of AC by increasing concentrations of the α_2 -adrenergic antagonist yohimbine, which was added 30 min before the agonists. Data are expressed as the cAMP accumulation measured relative to that observed with PGE₁ alone (100%). Error bars indicate SEM for duplicate or triplicate determinations.

the effects of appropriate agonists on growth control.

The α_2 -adrenergic receptors mediate a variety of biological responses to the agonist epinephrine. Activation of the receptor evokes PTX-sensitive inhibition of AC (Jakobs, 1979; Katada and Ui, 1981) and therefore seemed ideal for the studies we aimed to carry out. Two different genes coding for human α_2 -adrenergic receptors—designated α_2 -C4 and α_2 -C10 because of their localization on chromosomes 4 and 10, respectively—have recently been isolated by molecular cloning (Kobilka *et al.*, 1987; Regan *et al.*, 1988). We used a vector containing the α_2 -C10 gene, normally expressed in human platelets, to stably transfect CCL39 hamster fibroblasts, which show no endogenous responses to α -adrenergic stimulation. We achieved functional expression and report results in support of our hypothesis presented above: agonists activating α_2 -adrenergic receptors stimulate DNA synthesis in the transfected cells.

Results and discussion

Stable expression of α_2 -adrenergic receptors in CCL39 cells

CCL39 cells expressing high numbers of α_2 -adrenergic receptors (>100 000 per cell) were obtained as described in Methods. In these cells, α_2 -agonists efficiently inhibited AC, whereas

they were without effect in untransfected CCL39 cells. The inhibitory effect of the α_2 -agonists epinephrine and clonidine on prostaglandin E₁ (PGE₁)-stimulated AC in clone $39\alpha_2$ -21 is shown in Figure 1A. Both agents induce a dose-dependent reduction of cyclic adenosine monophosphate (cAMP) formation, which is completely abolished by PTX pretreatment of cells. Epinephrine was found to be slightly more potent than clonidine. The α_2 -receptor antagonist yohimbine reverses the effect with the expected IC₅₀-value in the submicromolar range (Figure 1B). However, the reversal is not complete, because yohimbine alone weakly inhibits cAMP formation at concentrations > 1 μM (not shown) because of a partial agonist effect on 5-HT_{1b}-receptors (Seuwen and Pouyssegur, in preparation). Yohimbine has already been described to activate 5-HT_{1d} receptors (Hoyer and Midlemis, 1989).

The results shown demonstrate that α_2 -adrenergic receptors can be expressed in CCL39 cells and that they function as in their natural environment, the platelet, by inhibiting stimulated AC.

Epinephrine and clonidine stimulate DNA synthesis

Figure 2 shows the results of DNA synthesis reinitiation experiments carried out on quies-

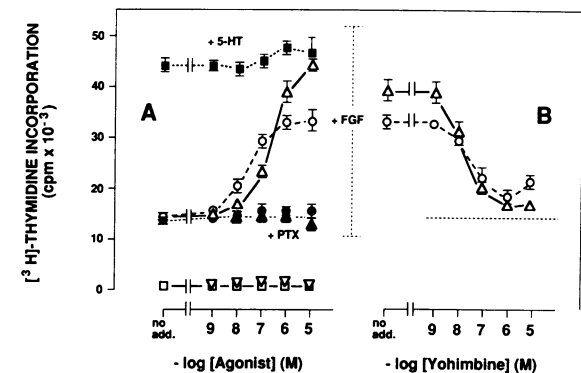


Figure 2. Stimulation of DNA synthesis reinitiation. Experiments were performed as described in the methods section. (A) $39\alpha_2$ -21 cells pretreated (●, ▲) or not (○, Δ, □, ▽) with PTX (50 ng/ml) were stimulated with recombinant FGF (25 ng/ml) and increasing concentrations of epinephrine (Δ, ▲) or clonidine (○, ●), as indicated on the abscissa. ▽, □: Cells stimulated with epinephrine and clonidine alone, respectively; ■: cells stimulated with FGF (50 ng/ml), serotonin (10 μM), and increasing concentrations of epinephrine. (B) Reversal of the mitogenic response caused by epinephrine (1 μM, Δ) and clonidine (1 μM, ○) by increasing concentrations of the α_2 -adrenergic antagonist yohimbine, which was added 30 min before the agonists. The ordinate shows the amount of incorporated [³H]-thymidine. Error bars indicate SEM for duplicate determinations.

cent cells. As previously observed for serotonin (Seuwen *et al.*, 1988a), epinephrine and clonidine do not stimulate cell proliferation on their own, but act in synergy with fibroblast growth factor (FGF) as well as insulin and epidermal growth factor (EGF) (not shown) in the 39 α_2 -21 clone. No response is observed in untransfected CCL39 cells. The effects shown are significant: the [³H]-thymidine incorporation signal obtained with FGF + epinephrine reaches >80% of the one obtained with 10% fetal calf serum (FCS). The response to clonidine and epinephrine is almost completely abolished by pretreatment of cells with PTX and efficiently antagonized by yohimbine (Figure 2B). Again, yohimbine inhibition was not complete. At concentrations > 1 μ M, the agent stimulated DNA synthesis, reflecting activation of 5-HT_{1b} receptors.

We tested the effect of epinephrine in the presence of a saturating concentration of serotonin to see whether the two agents produced additive responses. As is shown in Figure 2A, this is not the case, suggesting that both factors act through a common pathway. Similar results were obtained with the use of clonidine instead of epinephrine (not shown).

The results shown are not specific for clone 39 α_2 -21. We found stimulation of DNA synthesis by α_2 -adrenergic agonists in all cell clones tested that expressed functional receptors, as judged by clonidine-induced inhibition of AC.

α_2 -Adrenergic receptors can activate PLC

Recent reports suggested that α_2 -adrenergic receptors can activate the PLC signaling pathway (Michel *et al.*, 1989; Cotecchia *et al.*, 1990). We measured the rate of inositol phosphate (IP) formation after stimulation of cells by α_2 -agonists and compared them with other well-known PLC-stimulating agents active in CCL39 cells. As is shown in Figure 3, clonidine and epinephrine indeed stimulate the release of IPs, epinephrine showing a considerably stronger agonist activity than clonidine. In both cases, however, the rate of IP production is much lower than the one observed after addition of a mitogenic concentration of thrombin (10 nM); epinephrine, in fact, roughly equals the potency of a 100-fold lower thrombin concentration (Figure 4). The PLC response induced by both α_2 -agonists is inhibited by yohimbine (Figure 3B) and PTX (Figure 4). The α_1 -antagonist prazosin had no effect (not shown). Interestingly, inhibition by PTX of the clonidine and epinephrine response is almost complete, whereas IP release triggered by α -thrombin and serotonin is dimin-

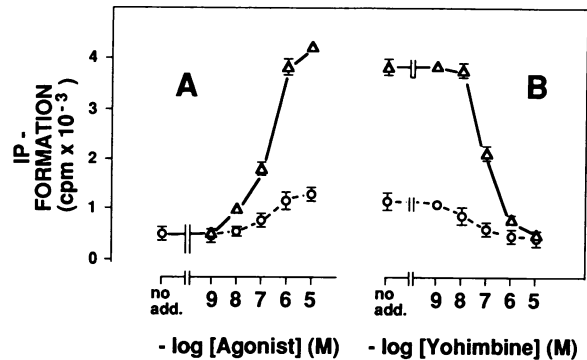


Figure 3. Stimulation of phospholipase C. Experiments were performed as described in the methods section. (A) 39 α_2 -21 cells were stimulated with increasing concentrations of epinephrine (Δ) or clonidine (\circ), as indicated on the abscissa. (B) Inhibition of the epinephrine (1 μ M, Δ)- and clonidine (1 μ M, \circ)-induced inositol phosphate formation by increasing concentrations of the α_2 -adrenergic antagonist yohimbine, which was added 30 min before the agonists. The ordinate shows the amount of labeled inositol phosphates released during 10 min of incubation. Error bars indicate SEM for duplicate determinations.

ished by only 40%–50% and the response to the AIF₄-complex by ~75%, as observed before with untransfected CCL39 cells (Paris and Pouyssegur, 1986; Seuwen *et al.*, 1988a). The basis of this differential PTX sensitivity remains unclear, but it probably reflects the implication of different G proteins, PTX sensitive and insensitive, in the control of PLC activity. The various G proteins seem to couple preferentially to different receptors. For instance, PLC activation through M1 muscarinic receptors is completely PTX insensitive in CCL39 cells (Figure 4; Seuwen *et al.*, in preparation) as well as in other systems (Masters *et al.*, 1985). Therefore, different degrees of PTX sensitivity of PLC activation can be observed within the same cell system depending on the receptors triggering the response.

The observation that α_2 -adrenergic receptors not only inhibit AC but also stimulate phosphoinositide (PI) turnover parallels reports on the M2 muscarinic receptor (Ashkenazi *et al.*, 1987; Ashkenazi *et al.*, 1989a) and on the 5-HT_{1a} receptor (Fargin *et al.*, 1989). These receptors are preferentially coupled to AC, where they exert inhibitory action but also weakly stimulate PLC. Two possible explanations may account for these observations: First, it is possible that the G_i proteins inhibiting AC have a weak but detectable potential to stimulate PLC; alternatively, different species of PTX-sensitive G proteins may be involved in the negative control of AC activity and in PLC activation, respectively.

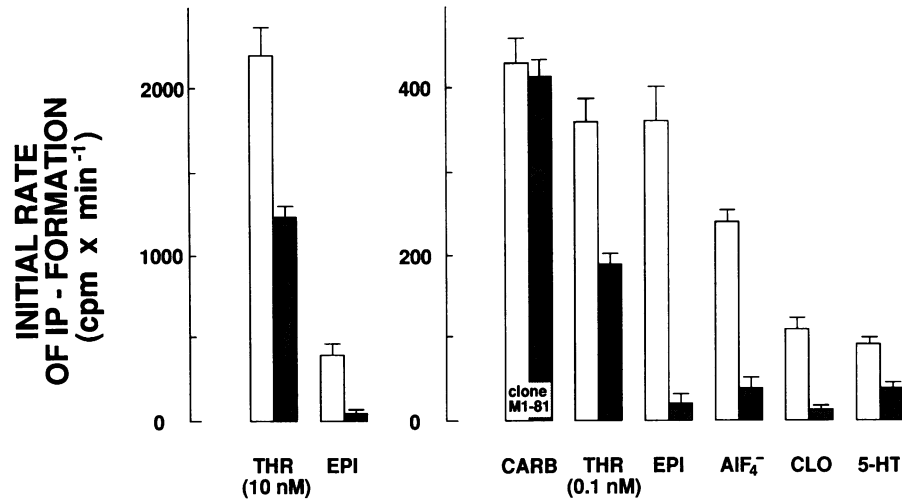


Figure 4. Effect of pertussis toxin on phospholipase C activity stimulated by different agonists. Experiments were performed as described in the methods section. $39\alpha_2-21$ cells pretreated (□) or not (■) with PTX (50 ng/ml) were stimulated with α -thrombin (THR, concentrations indicated), epinephrine (EPI, 1 μ M), clonidine (CLO, 1 μ M), serotonin (5-HT, 1 μ M), and the AIF₄⁻-complex (5 μ M AlCl₃ + 10 mM NaF). The effect of PTX on PLC stimulation by carbachol (CCH, 10 μ M) in a CCL39 cell clone (39M1-81) expressing human muscarinic M1-receptors is shown for comparison. The ordinate shows the amount of labeled inositol phosphates released per minute of incubation. All incubation times were 10 min, except for 10 nM of thrombin, where it was reduced to 1.5 min to allow the initial rate measurement. Error bars indicate SEM for duplicate or triplicate determinations.

As CCL39 cells contain at least two immunologically different PTX substrates (G_{i2} and G_{i3} , Pouyssegur, 1990), it is not possible at the moment to eliminate either of the two hypotheses. Interestingly, no release of IPs can be observed on activation of 5-HT_{1b} receptors, which mediate inhibition of AC by serotonin in CCL39 cells (Seuwen *et al.*, 1988a).

It is also interesting to note the different agonist activity of clonidine when measuring AC inhibition versus PLC activation. Whereas clonidine has almost the same potency as epinephrine in the AC assay, it behaves like a partial agonist in PLC measurements. In accordance with our data, Michel *et al.* (1989) observed Ca^{2+} release from erythroleukemia cells in response to epinephrine but not in response to clonidine. These results seem to indicate that, on binding, the two agonists induce activated forms of the receptor with different characteristics, regarding either their specificity or efficiency of interaction with G proteins.

Activation of Na^+/H^+ exchange by α_2 -adrenergic agonists correlates with the stimulation of PLC

The plasma membrane Na^+/H^+ exchanger is an ubiquitous protein that becomes activated in response to agents stimulating receptor tyrosine kinases or receptors coupled to PLC (Sar-

det *et al.*, 1990). In addition, some reports described activation of the enzyme by α_2 -adrenergic receptors and other receptors coupled negatively to AC (Isom *et al.*, 1987), but this notion remained a matter of controversy. In an attempt to elucidate whether G_i -coupled receptors can activate the Na^+/H^+ exchanger, we studied its activity in $39\alpha_2-21$ cells, measuring intracellular alkalinization in response to different stimuli (Figure 5). Indeed, epinephrine induced a relatively strong intracellular pH change (0.14 pH units) comparable with the effects of FGF or 0.1 nM thrombin. A much weaker activation was observed for clonidine and serotonin (0.05 pH units), and no significant alkalinization was detected with serotonin in the presence of the 5-HT₂-receptor antagonist ketanserin, which completely inhibits serotonin-induced PLC activation in CCL39 cells, leaving intact the inhibition of AC by 5-HT_{1b} receptors (Seuwen *et al.*, 1988a). These results are consistent with the notion that the observed activation of Na^+/H^+ exchange by clonidine, epinephrine, and serotonin is a consequence of the stimulation of PI breakdown (compare Figure 4) and probably cannot be attributed to an independent signaling mechanism. No synergistic effect was observed when FGF and epinephrine were added together.

Because we had no means to uncouple selectively either PLC activation or AC inhibition

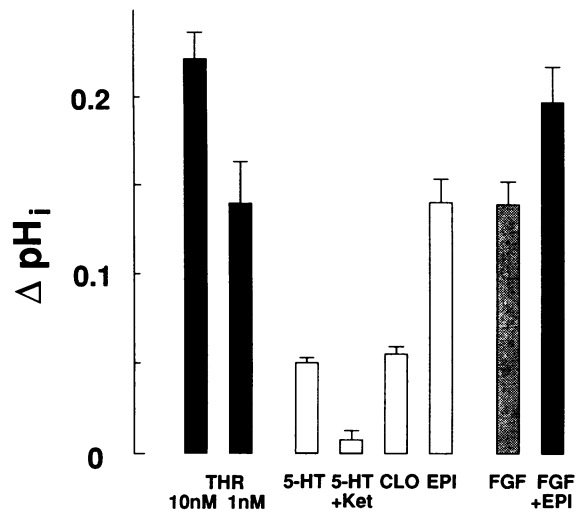


Figure 5. Activation of Na^+/H^+ exchange. Experiments were performed as described in the methods section. $39\alpha_2$ -21 cells were stimulated with α -thrombin (THR, concentrations indicated), epinephrine (EPI, $1 \mu\text{M}$), clonidine (CLO, $1 \mu\text{M}$), serotonin (5-HT, $1 \mu\text{M}$) alone or in the presence of ketanserin (Ket, $1 \mu\text{M}$) and FGF (50 ng/ml). The ordinate shows the intracellular pH change relative to unstimulated control cells. Error bars indicate SEM for triplicate determinations.

from receptor stimulation, we cannot rule out the hypothesis that PI breakdown also contributes to the mitogenic effect elicited by α_2 -adrenergic agonists. For the following reasons, however, we believe that this is not the case: first, although epinephrine is a much stronger PLC agonist than clonidine (Figures 3 and 4), it does not lead to a substantially stronger DNA synthesis reinitiation response (Figure 2). Second, the proliferative responses evoked by clonidine or epinephrine are not additive to the one evoked by serotonin, which acts through 5-HT_{1b} -receptors inhibiting AC (Seuwen *et al.*, 1988a). These data suggest that the mitogenic response to the α_2 -agonists is not due to their moderate stimulatory effect on PI turnover but correlates well, as in the case of serotonin, with the inhibition of AC. In fact, experiments carried out recently in our laboratory on CCL39 cells transfected with muscarinic M1 - and 5-HT_{1c} -receptors indicate that even a strong activation of the PLC signaling pathway, comparable with thrombin, is not sufficient to induce a mitogenic response by itself, although early events like activation of Na^+/H^+ exchange and early gene expression are maximally induced. When tested in the presence of FGF, carbachol or serotonin (in the presence of 5-HT_{1b} antagonists) increases the proliferative response only weakly (Seuwen and Pouysségur, 1990; Seuwen *et al.*,

in preparation). Working on the mitogenic effects of lysophosphatidic acid in Rat-1 cells, van Corven *et al.* (1989) recently arrived at similar conclusions.

As we have discussed for serotonin (Seuwen *et al.*, 1988a; Seuwen and Pouysségur, 1990), an open question remains whether the mitogenic effects of epinephrine and clonidine reported here are in fact due to the inhibition of cAMP formation or to the activation or inhibition of another G protein-modulated pathway. Indeed, agents stimulating AC like PGE_1 have been shown to inhibit cell proliferation in CCL39 cells, but this effect is independent of the growth factor employed (Magnaldo *et al.*, 1989). In platelets (Haslam *et al.*, 1978) as well as in pancreatic islet cells (Ullrich and Wollheim, 1988), the biological activities of α_2 -agonists have been dissociated from their effects on intracellular cAMP levels. Effectors different from AC and directly or indirectly regulated by G_i proteins include PLA_2 and various ionic channels. Many others probably exist but have not yet been identified. Discovering these messenger systems and determining their relevance for growth control will be an important issue for future work.

In conclusion, our results add support to the hypothesis outlined in the introduction, namely that receptors coupled to G_i proteins contribute to growth signaling in fibroblasts. Interestingly, a mutated form of the G_{i2} α -subunit has been found recently in adrenal cortical tumors (Lyons *et al.*, 1990), and, by analogy with the previously identified G_s mutants (Landis *et al.*, 1989), it probably represents an α -subunit with reduced GTPase activity. From our data we predict that this putative oncogene acts by increasing cellular responses to growth factors of the tyrosine kinase class.

Materials and methods

Highly purified human α -thrombin and recombinant basic FGF were generous gifts of Dr. J. W. Fenton II (New York State Department of Health, Albany, NY) and Dr. D. Gospodarowicz (University of California Medical Center, San Francisco, CA), respectively. PTX was from List Biological Laboratories (Campbell, CA). Myo-[2- ^3H]-inositol, [methyl- ^3H]-thymidine, and [^3H]-clonidine were from Amersham (Les Ulis, France) and [^{14}C]-benzoic acid from DuPont-NEN (Paris, France). All other substances used were purchased from Sigma (La Verpillière, France).

Cells and culture conditions

CCL39 cells are an established line of Chinese hamster lung fibroblasts (American Type Culture Collection). Cells expressing human α_2 -adrenergic receptors were obtained by transfection with the use of the pMAM-neoexpression vector

(Clontech, Palo Alto, CA) containing the α_2 -C10 gene (Cotecchia *et al.*, 1990). Transfection was carried out by the use of the calcium phosphate coprecipitation technique (Graham and van der Eb, 1973). Cell clones resistant to the antibiotic G418 (500 μ g/ml) were isolated as described earlier (Seuwen *et al.*, 1988b) and screened for expression of functional receptors by testing the capacity of clonidine to inhibit AC. The cell clone chosen for this study (39 α_2 -21) was found to express ~500 000 receptors in the absence of dexamethasone (DEX), as estimated by Scatchard analysis of [3 H]-clonidine binding data. Receptor expression could be markedly increased (~3-fold) by addition of 0.1 μ M DEX; however, the glucocorticoid strongly inhibited cell proliferation at this concentration. All experiments shown were therefore conducted at a low but defined concentration of 1 nM DEX. Under these conditions, receptor number per cell increased by 20%.

All cells were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Cergy Pontoise, France)—supplemented with 10% FCS, antibiotics (50 units/ml penicillin and 50 μ g/ml streptomycin), and 25 mM sodium bicarbonate—at 37°C in a 5% CO₂, 95% air atmosphere. To obtain quiescent cells arrested in the G0/G1 phase of the cell cycle, cultures were incubated for 24 h in serum-free medium containing 1 nM DEX. When cells were to be pretreated with PTX, the toxin (50 ng/ml) was added 4 h before the beginning of the experiments described below.

Measurement of AC activity

Quiescent cells in 12-well plates prelabeled with [3 H]-adenine (2 μ Ci/ml, 24 h) were incubated in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered DMEM (pH 7.4). AC was stimulated with PGE₁ (1 μ M), and, after 20 min, agonists (epinephrine or clonidine) were added. After another 5 min, isobutyl-methylxanthine (IBMX) was added at 1 mM to inhibit the phosphodiesterase, and, 10 min later, the cells were extracted with ice-cold trichloroacetic acid (TCA, 5%). [3 H]-ATP and [3 H]-cAMP were separated by sequential chromatography on Dowex and alumina columns (Bio Rad, Paris, France) (Magnaldo *et al.*, 1988).

Measurement of DNA synthesis reinitiation

Quiescent cells in 24-well plates were stimulated in a serum-free DMEM/Ham's F12 medium (1:1) containing [3 H]-thymidine (3 μ M, 0.5 mCi/ml) with the hormones and purified growth factors indicated. After 24 h of incubation, the cells were fixed and washed four times with ice cold TCA (5%). The TCA-precipitated material was recovered with 0.1 N NaOH and the radioactivity incorporated counted.

PI breakdown assay

Quiescent cells in 12-well plates prelabeled with [3 H]-inositol (2 μ Ci/ml, 24 h) were incubated in HEPES-buffered DMEM (pH 7.4) containing 20 mM Li⁺. After 10 min, agonists were added. After indicated times, cells were extracted with 10 mM formic acid and the total level of IPs measured by anion-exchange chromatography and liquid scintillation counting, as described by Seuwen *et al.* (1988b).

Determination of intracellular pH changes

Quiescent cells in 24-well plates were washed once and equilibrated for 1 h in HEPES-buffered DMEM (pH 7) at 37°C. Medium was changed once more to eliminate traces of bicarbonate, and the indicated growth factors and [14 C]-benzoic acid (3 mCi/ml) were added. After 10 min of incubation, external medium was aspirated, and the wells were rapidly

washed four times with ice-cold phosphate-buffered saline. Radioactivity was recovered from cells lysed in 0.1 N NaOH. The difference in intracellular pH (Δ pH) between stimulated and unstimulated cells was calculated from the equilibrium distribution of the weak acid, as previously described (L'Allemain *et al.*, 1984).

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References

- Ashkenazi, A., Winslow, J.W., Peralta, E.G., Peterson, G.L., Schimerlik, M.I., Capon, D.J., and Ramachandran, J. (1987). An M2 muscarinic receptor subtype coupled to both adenylyl cyclase and phosphoinositide turnover. *Science* 238, 672–675.
- Ashkenazi, A., Peralta, E.G., Winslow, J.W., Ramachandran, J., and Capon, J. (1989). Functionally distinct G proteins selectively couple different receptors to PI hydrolysis in the same cells. *Cell* 56, 487–493.
- Chambard, J.C., Paris, S., L'Allemain, G., and Pouyssegur, J. (1987). Two growth factor signalling pathways in fibroblasts distinguished by pertussis toxin. *Nature* 326, 800–803.
- Cotecchia, S., Kobilka, B.K., Daniel, K.W., Nolan, R.D., Lapetina, E.Y., Caron, M.G., Lefkowitz, R.J., and Regan, J. (1990). Multiple second messenger pathways of α -adrenergic receptor subtypes expressed in eukaryotic cells. *J. Biol. Chem.* 265, 63–69.
- Fargin, A., Raymond, J.R., Regan, J.W., Cotecchia, S., Lefkowitz, R.J., and Caron, M.G. (1989). Effector coupling mechanisms of cloned 5-HT_{1A} receptor. *J. Biol. Chem.* 264, 14 848–14 852.
- Graham, F.L., and van der Eb, A.J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52, 456–467.
- Haslam, R.J., Davidson, M.M.L., and Desjardins, S.V. (1978). Inhibition of adenylyl cyclase by adenosine analogues in preparations of broken and intact human platelets. Evidence for the unidirectional control of platelet function by cyclic AMP. *Biochem. J.* 176, 83–95.
- Hoyer, D., and Middlemiss, D.N. (1989). Species differences in the pharmacology of terminal 5-HT autoreceptors in mammalian brain. *Trends Pharmacol. Sci.* 10, 130–132.
- Isom, L.L., Cragoe, E.J., and Limbird, L.L. (1987). Multiple receptors linked to inhibition of adenylyl cyclase accelerate Na⁺/H⁺ exchange in neuroblastoma glioma cells via a mechanism other than decreased cAMP accumulation. *J. Biol. Chem.* 36, 17 504–17 509.

- Jakobs, K.H. (1979). Activation and inhibition of adenylyl cyclase by hormones and neurotransmitters. *Mol. Cell. Endocrinol.* **16**, 147–156.
- Katada, T., and Ui, M. (1981). Islet activating protein. A modifier of receptor-mediated regulation of rat islet adenylyl cyclase. *J. Biol. Chem.* **256**, 8310–8317.
- Kavanaugh, W.M., Williams, L.T., Ives, H.E., and Coughlin, S.R. (1988). Serotonin-induced DNA synthesis in vascular smooth muscle cells involves a novel pertussis toxin-sensitive pathway. *Mol. Endocrinol.* **2**, 599–605.
- Kobilka, B.K., Matsui, H., Kobilka, T.S., Yang-Feng, T.L., Francke, U., Caron, M.G., Lefkowitz, R.J., and Regan, J.W. (1987). Cloning, sequencing and expression of the gene coding for the human platelet α_2 -adrenergic receptor. *Science* **238**, 650–656.
- L'Allemain, G., Paris, S., and Pouyssegur, J. (1984). Growth factor action and intracellular pH regulation in fibroblasts. Evidence for a major role of the Na^+/H^+ antiporter. *J. Biol. Chem.* **259**, 5809–5815.
- Landis, C.A., Masters, S.B., Spada, A., Pace, A.M., Bourne, H.R., and Vallar, L. (1989). GTPase inhibiting mutations activate the α chain of G_s and stimulate adenylyl cyclase in human pituitary tumours. *Nature* **340**, 692–697.
- Letterio, J.J., Coughlin, S.R., and Williams, L.T. (1986). Pertussis toxin-sensitive pathway in the stimulation of c-myc expression and DNA synthesis by bombesin. *Science* **234**, 1117–1119.
- Lyons, J., Landis, C.A., Harsh, G., Vallar, L., Grunewald, K., Feichtinger, H., Duh, Q.-Y., Clark, O.H., Kawasaki, E., Bourne, H.R., and McCormick, F. (1990). Point mutations in $G_{\alpha s}$ and $G_{\alpha i}$ genes in human tumors. *Science* (in press).
- Magnaldo, I., Pouyssegur, J., and Paris, S. (1988). Thrombin exerts a dual effect on stimulated adenylyl cyclase in hamster fibroblasts: an inhibition via a GTP-binding protein and a potentiation via activation of protein kinase C. *Biochem. J.* **253**, 711–719.
- Magnaldo, I., Pouyssegur, J., and Paris, S. (1989). Cyclic AMP inhibits mitogen-induced DNA synthesis in hamster fibroblasts regardless of the signalling pathway involved. *FEBS Lett.* **245**, 65–69.
- Masters, S.B., Martin, M.W., Harden, K., and Brown, J.H. (1985). Pertussis toxin does not inhibit muscarinic-receptor-mediated phosphoinositide hydrolysis of calcium mobilization. *Biochem. J.* **227**, 933–937.
- Michel, M.C., Brass, L.F., Williams, A., Bokoch, G.M., LaMorte, V.J., and Motulsky, H.J. (1989). α_2 -Adrenergic receptor stimulation mobilizes intracellular Ca^{2+} in human erythroleukemia cells. *J. Biol. Chem.* **264**, 4986–4991.
- Paris, S., and Pouyssegur, J. (1986). Pertussis toxin inhibits thrombin-induced activation of phosphoinositide hydrolysis and Na^+/H^+ exchange in hamster fibroblasts. *EMBO J.* **5**, 55–60.
- Pouyssegur, J., Chambard, J.C., L'Allemain, G., Magnaldo, I., and Seuwen, K. (1988). Transmembrane signalling pathways initiating cell growth in fibroblasts. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **320**, 427–436.
- Pouyssegur, J. (1990). G proteins in growth factor action. In: Iyengar, R., Birnbaumer, L., eds., *G proteins*, New York: Academic Press, 555–571.
- Regan, J.W., Kobilka, T.S., Yang-Feng, T.L., Caron, M.G., Lefkowitz, R.J., and Kobilka, B.K. (1988). Cloning and expression of a human kidney cDNA for an α_2 -adrenergic receptor subtype. *Proc. Natl. Acad. Sci. USA* **85**, 6301–6305.
- Sardet, C., Counillon, L., Franchi, A., and Pouyssegur, J. (1990). Growth factors induce phosphorylation of the Na^+/H^+ antiporter, a glycoprotein of 110-kD. *Science* **247**, 723–726.
- Seuwen, K., Magnaldo, I., and Pouyssegur, J. (1988a). Serotonin stimulates DNA synthesis in fibroblasts via 5-HT_{1B} receptors coupled to a G_i -protein. *Nature* **335**, 254–257.
- Seuwen, K., Lagarde, A., and Pouyssegur, J. (1988b). Deregulation of hamster fibroblast proliferation of mutated *ras* oncogenes is not mediated by constitutive activation of phosphoinositide-specific phospholipase C. *EMBO J.* **7**, 161–168.
- Seuwen, K., and Pouyssegur, J. (1990). Serotonin as a growth factor. *Biochem. Pharmacol.* **39**, 985–990.
- Ullrich, S., and Wollheim, C.B. (1988). GTP-dependent inhibition of insulin secretion by epinephrine in permeabilized RINm5F cells. *J. Biol. Chem.* **263**, 8615–8620.
- van Corven, E.J., Groenink, A., Jalink, K., Eichholtz, T., Moolenaar, W.H. (1989). Lysophosphatidate-induced cell proliferation: identification and dissection of signaling pathways mediated by G proteins. *Cells* **59**, 45–54.
- Zachary, I., Millar, J., Nanberg, E., Higgins, T., and Rozengurt, E. (1987). Inhibition of bombesin-induced mitogenesis by pertussis toxin: dissociation from phospholipase C pathway. *Biochem. Biophys. Res. Commun.* **146**, 456–463.