

A new phospholipase-C–calcium signalling pathway mediated by cyclic AMP and a Rap GTPase

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Stimulation of phosphoinositide-hydrolysing phospholipase C (PLC) generating inositol-1,4,5-trisphosphate is a major calcium signalling pathway used by a wide variety of membrane receptors, activating distinct PLC- β or PLC- γ isoforms^{1–4}. Here we report a new PLC and calcium signalling pathway that is triggered by cyclic AMP (cAMP) and mediated by a small GTPase of the Rap family. Activation of the adenylyl cyclase-coupled β_2 -adrenoceptor expressed in HEK-293 cells or the endogenous receptor for prostaglandin E_1 in N1E-115 neuroblastoma cells induced calcium mobilization and PLC stimulation, seemingly caused by cAMP formation, but was independent of protein kinase A (PKA). We provide evidence that these receptor responses are mediated by a Rap GTPase, specifically Rap2B, activated by a guanine-nucleotide-exchange factor (Epac) regulated by cAMP^{5,6}, and involve the recently identified PLC- ϵ isoform^{7–9}.

Stimulation of PLC enzymes that hydrolyse phosphatidylinositol-4,5-bisphosphate (PtdInsP₂) can be impaired by cAMP-activated PKA, as has been demonstrated for the PLC- γ 1 and PLC- β 2 isozymes^{4,10,11}. To identify possible further mechanisms by which cAMP and cAMP-generating receptors regulate PLC enzymes, we expressed the β_2 -adrenoceptor (β_2 -AR) in HEK-293 cells, in which PLC stimulation and calcium mobilization by various membrane receptors have previously been reported^{12,13}. Surprisingly, activation of the transfected cells with 10 μ M adrenaline, the β_2 -AR agonist, rapidly and transiently increased intracellular calcium concentrations ($[Ca^{2+}]_i$) (by 285 ± 55 nM; $n = 10$, Fig. 1a) and induced a time- (data not shown) and concentration-dependent generation of inositol phosphates and inositol-1,4,5-trisphosphate (InsP₃); the immediate PLC reaction product; Fig. 1c and e). The stimulatory effects of 10 μ M adrenaline on $[Ca^{2+}]_i$ and PLC activity were fully blocked by 1 μ M propranolol, a β_2 -AR antagonist, and not observed in control, nontransfected cells (data not shown), indicating that β_2 -AR mediates this reaction. Furthermore, treatment of the cells with pertussis toxin (100 ng ml⁻¹ for 24 h), to prevent potential coupling of the β_2 -AR to G_i-type G-proteins¹⁴, did not alter the stimulatory effects of adrenaline (data not shown), suggesting that the responses to β_2 -AR activation are mediated by G_s-proteins inducing adenylyl cyclase (AC) stimulation. Consistent with this assumption, forskolin, a direct AC activator¹⁵, fully mimicked the effects of adrenaline, and was independent of β_2 -AR expression (data not shown). Increases in $[Ca^{2+}]_i$ induced by 30 μ M forskolin (by 225 ± 45 nM; $n = 8$) were slightly

delayed, but comparable in extent to those induced by adrenaline (Fig. 1b). Furthermore, forskolin increased formation of inositol phosphates and InsP₃ (Fig. 1d and e). In contrast, application of 10 μ M 2',5'-dideoxyadenosine (2',5'-dd-Ado), a P-site AC inhibitor¹⁵, reduced PLC stimulation by 50–70%, as well as the $[Ca^{2+}]_i$ increases induced by adrenaline or forskolin (data not shown). These results suggest that PLC and calcium signalling induced by the β_2 -AR is dependent on G_s-mediated stimulation of cAMP production.

Most of the cellular actions of cAMP are mediated by PKA¹⁶. Treatment of the cells with 10 μ M H-89, a specific PKA inhibitor¹⁷, however, did not significantly alter PLC stimulation by adrenaline or forskolin (Fig. 2a and b), suggesting an alternative mode of cAMP action. Epac proteins have recently been identified as direct cAMP targets; Epac proteins cause guanine-nucleotide exchange at Rap GTPases independently of PKA^{5,6}. Overexpression of Epac1 increased adrenaline- or forskolin-induced PLC stimulation by 40–50%, without altering basal PLC activity (Fig. 2c and d). The effects of Epac1 are insensitive to H-89. Furthermore, overexpression of Epac1 did not alter β_2 -AR expression and had no effects on the cellular level of the PLC substrate, PtdInsP₂, or on calcium-stimulated PLC activity *in vitro* (data not shown). Similar to PLC stimulation, overexpression of Epac1 strongly enhanced adrenaline- and forskolin-induced $[Ca^{2+}]_i$ increases, to 495 ± 85 nM ($n = 8$, $P < 0.01$) and 388 ± 65 nM ($n = 12$, $P < 0.01$), respectively (Fig. 2e and f).

The potentiating effect of Epac1 suggested that Epac-activated Rap GTPases are involved in PLC and calcium signalling by β_2 -AR. To investigate this possibility, we inactivated Rap GTPases with *Clostridium difficile* toxin B-1470 and expressed inactive mutants of several GTPases. In cells treated with toxin B-1470 (which inactivates Ras-related GTPases, including Rap GTPases, by a specific monoglucosylation of the GTPases¹⁸), PLC stimulation induced by adrenaline and forskolin was reduced by ~70% (Fig. 3a and b). Similarly, toxin B-1470 treatment strongly reduced the $[Ca^{2+}]_i$ increases induced by adrenaline and forskolin (Fig. 3c and d). In contrast, treatment with *Clostridium difficile* toxin B (100 pg ml⁻¹ for 24 h), known to inactivate Rho GTPases¹⁹, had no effect on adrenaline- and forskolin-induced PLC stimulation (data not shown). We then expressed inactive mutants of various Ras-related GTPases and toxin B-1470 substrates to identify the specific GTPase involved in β_2 -AR- and cAMP-mediated PLC stimulation. Expression of Gly26→Ala (G26A) RalA and Ser17→Asn (S17N) H-Ras, which blocked phospholipase D stimulation by receptor tyrosine kinases in these cells²⁰, did not significantly reduce PLC stimulation by adrenaline and forskolin (data not shown).

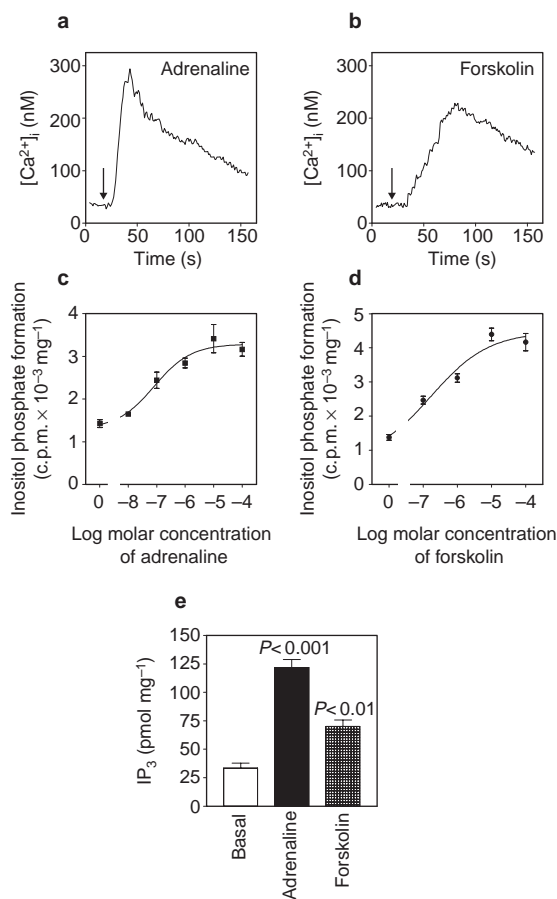


Figure 1 β_2 -AR- and forskolin-induced calcium and PLC signalling. **a, b**, Typical $[Ca^{2+}]_i$ traces in fura-2-loaded HEK-293 cells transfected with the β_2 -AR and stimulated with 10 μM adrenaline or 30 μM forskolin, as indicated by the arrows. **c, d**, Concentration-dependent stimulation of $[^3H]$ inositol phosphate formation by adrenaline and forskolin (mean \pm s.e.m. of 3 or 4 experiments). **e**, Stimulation of $InsP_3$ formation by 10 μM adrenaline (60 s) and 30 μM forskolin (120 s) (mean \pm s.e.m. of 3–5 experiments). Cells transfected with the empty pcDNA3 vector did not respond to adrenaline (data not shown).

Similarly, expression of S17N Rap1A and S17N Rap2A did not alter PLC stimulation. In contrast, expression of S17N Rap2B reduced PLC stimulation induced by adrenaline and forskolin by ~70% (Fig. 3e and f), as well as $[Ca^{2+}]_i$ increases induced by these agents (data not shown). Expression of S17N Rap2B did not alter the cellular $PtdInsP_2$ content or the Ca^{2+} -stimulated PLC activity *in vitro* (data not shown). To verify that adrenaline and forskolin induce Rap2B activation, we extracted GTP-bound Rap2B from cell lysates with immobilized RalGDS-RBD (Rap-binding domain of Ral-guanine dissociation stimulator)⁵. Fig. 3g shows that both adrenaline and forskolin increased the amount of activated Rap2B. These results indicate that the β_2 -AR and forskolin activate Rap2B, probably through the cAMP-activated guanine nucleotide-exchange factor (GEF) Epac, which leads to PLC stimulation and calcium mobilization.

The various PLC enzymes that have been identified thus far are activated by distinct mechanisms. It is generally accepted that the PLC- β isoforms are stimulated by receptors coupled to heterotrimeric G-proteins, through either activated $G\alpha_q$ or free $G\beta\gamma$ dimers, whereas the PLC- γ isoforms are activated by receptor and nonreceptor tyrosine kinases by direct tyrosine phosphorylation^{3,4}.

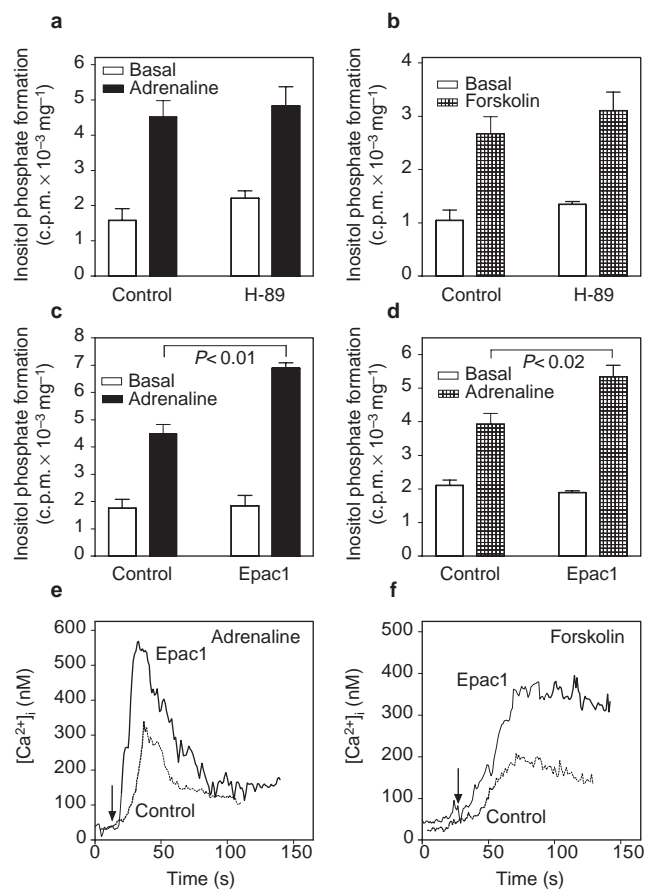


Figure 2 β_2 -AR- and forskolin-induced PLC and calcium signalling is independent of PKA and potentiated by Epac1. **a, b**, HEK-293 cells transfected with the β_2 -AR were incubated for 30 min with 10 μM H-89 or its solvent (control), followed by measurement of $[^3H]$ inositol phosphate accumulation in the absence (basal) or presence of 10 μM adrenaline or 30 μM forskolin (mean \pm s.e.m. of 3 or 4 experiments). **c, d**, $[^3H]$ inositol phosphate formation was measured in cells transfected with the β_2 -AR, either alone (control) or with Epac1, in the absence (basal) or presence of 10 μM adrenaline or 30 μM forskolin (mean \pm s.e.m. of 4 or 5 experiments). **e, f**, Typical $[Ca^{2+}]_i$ traces of cells transfected with the β_2 -AR DNA, either alone (control) or with Epac1, and stimulated with 10 μM adrenaline or 30 μM forskolin. Expression of HA-tagged Epac1 was verified by immunofluorescence and immunoblotting (data not shown).

The activation mechanisms of the PLC- δ isoforms are less clear, but could involve G_{i_n} -proteins and increases in $[Ca^{2+}]_i$ (refs 21, 22). The recently identified PLC- ϵ isoform contains, in addition to the catalytic and calcium-regulated lipid-binding domains found in all PLC isozymes, two Ras-binding domains and a Ras-GEF domain, and its PLC activity has been reported to be regulated by both heterotrimeric G-proteins ($G\alpha_{12}$) and Ras-like GTPases^{7–9}. We overexpressed different PLC isoforms to examine which isoform is involved in PLC and calcium signalling by the β_2 -AR. Expression of PLC- $\beta 1$ and PLC- $\gamma 1$ increased PLC stimulation by the transfected M_3 muscarinic receptor and the endogenously expressed epidermal growth factor receptor, respectively (data not shown). However, PLC stimulation by adrenaline and forskolin were not altered in cells overexpressing these two PLC isozymes (Fig. 4a and b). In cells overexpressing PLC- $\delta 1$, basal PLC activity was enhanced, and there was no further increase in activity induced by adrenaline or forskolin (Fig. 4c and d). In contrast, overexpression of PLC- ϵ

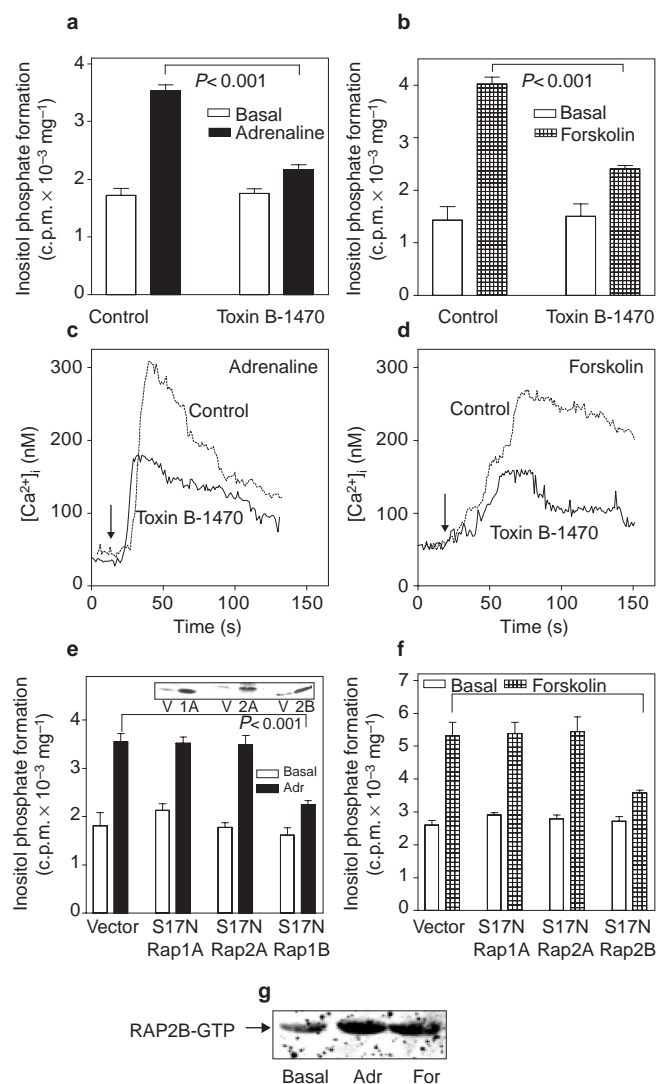


Figure 3 Inhibition of β_2 -AR- and forskolin-induced PLC and calcium signalling by toxin B-1470 and an inactive Rap2B mutant. **a–d**, HEK-293 cells transfected with the β_2 -AR were pretreated for 24 h without (control) and with 300 $\mu\text{g ml}^{-1}$ toxin B-1470, followed by measurement of [³H]inositol phosphate accumulation (mean \pm s.e.m. of 4 experiments) in the absence (basal) or presence of 10 μM adrenaline or 30 μM forskolin (**a, b**), or adrenaline- and forskolin-induced [Ca^{2+}]_i increases (**c, d**). **e, f**, [³H]inositol phosphate formation was measured in cells transfected with the β_2 -AR, either alone (vector, V) or with S17N Rap1A, S17N Rap2A or S17N Rap2B, in the absence (basal) or presence of 10 μM adrenaline or 30 μM forskolin (mean \pm s.e.m. of 4 experiments). Rap protein expression was determined by immunoblotting with specific antibodies (Santa Cruz). **g**, Activation of Rap2B by adrenaline (Adr) and forskolin (For). Shown is a typical immunoblot, repeated three times.

slightly increased basal PLC activity and nearly doubled PLC stimulation by adrenaline and forskolin (Fig. 4c and d). Consistent with increased PLC stimulation, [Ca^{2+}]_i increases induced by adrenaline and forskolin were strongly potentiated in cells overexpressing PLC- ϵ (to 680 \pm 45 nM ($n = 6$, $P < 0.001$) and 414 \pm 45 nM ($n = 6$, $p < 0.002$), respectively; Fig. 4e and f). Together, these results suggest that the β_2 -AR- and cAMP-mediated PLC stimulation and calcium mobilization are accomplished by PLC- ϵ .

The regulation of PLC- ϵ activation by Rap2B was further examined in co-transfection studies and *in vitro* PLC activity measure-

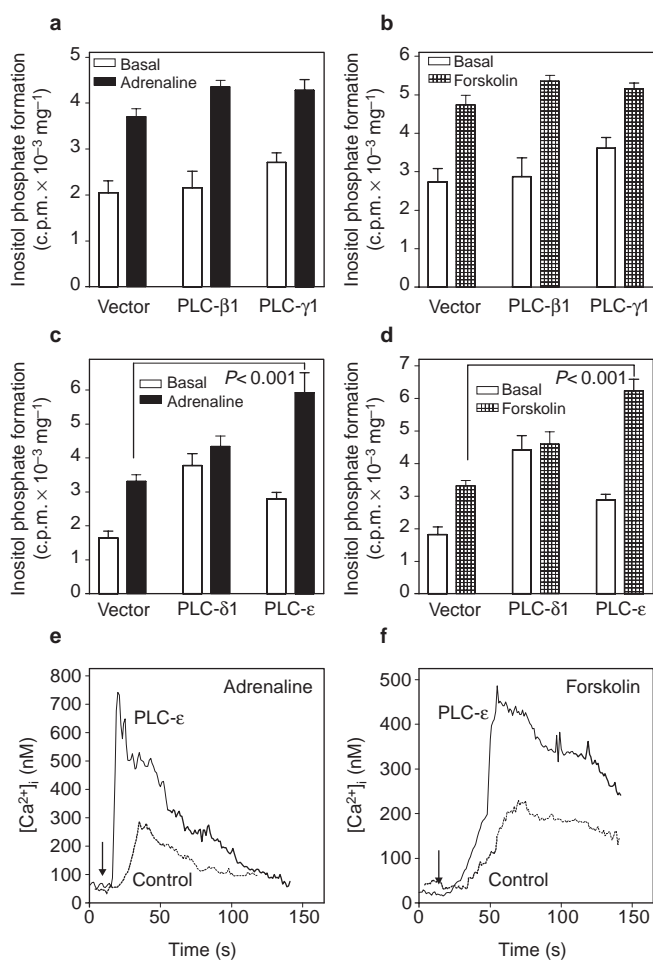


Figure 4 Potentiation of β_2 -AR- and forskolin-induced PLC and calcium signalling by PLC- ϵ . **a–d**, HEK-293 cells were transfected with the β_2 -AR, either alone (vector) or with PLC- β 1, PLC- γ 1, PLC- δ 1 or PLC- ϵ , followed by measurement of [³H]inositol phosphate formation in the absence (basal) or presence of 10 μM adrenaline or 30 μM forskolin (mean \pm s.e.m. of 3 or 4 experiments). Expression of PLC- β 1, PLC- γ 1, PLC- δ 1 and Myc-tagged PLC- ϵ was verified by immunoblotting with specific antibodies (Santa Cruz). **e, f**, Typical [Ca^{2+}]_i traces of cells transfected with the β_2 -AR, either alone (control) or with PLC- ϵ , and stimulated with 10 μM adrenaline or 30 μM forskolin.

ments. The potentiating effect of PLC- ϵ on adrenaline- and forskolin-induced PLC stimulation was fully reversed by co-expressing S17N Rap2B (Fig. 5a and b), without altering the expression level of PLC- ϵ (data not shown). In contrast, co-expression of other inactive GTPase mutants had no (Rap1A, Rap2A) or very little (H-Ras) effect (data not shown). In lysates of cells expressing S17N Rap2B or treated with toxin B-1470, GTP γ S-stimulated PLC activity (measured using exogenous PtdInsP₂ as enzyme substrate) was reduced by ~50% (Fig. 5c and d), indicating participation of Rap proteins. Furthermore, the increase in PLC activity caused by overexpression of PLC- ϵ was fully suppressed by expression of S17N Rap2B or treatment with toxin B-1470. Most importantly, addition of recombinant GppNHp-bound full-length Rap2B and carboxy-terminal-truncated Rap2B (residues 1–166; data not shown), but not Rap2A (3 μM each), fully reversed the inhibitory effect of S17N Rap2B (Fig. 5c) or toxin B-1470 (Fig. 5d). These results indicate that the regulatory effect of Rap2B on PLC- ϵ activity is specific and corroborate the findings obtained in intact cells,

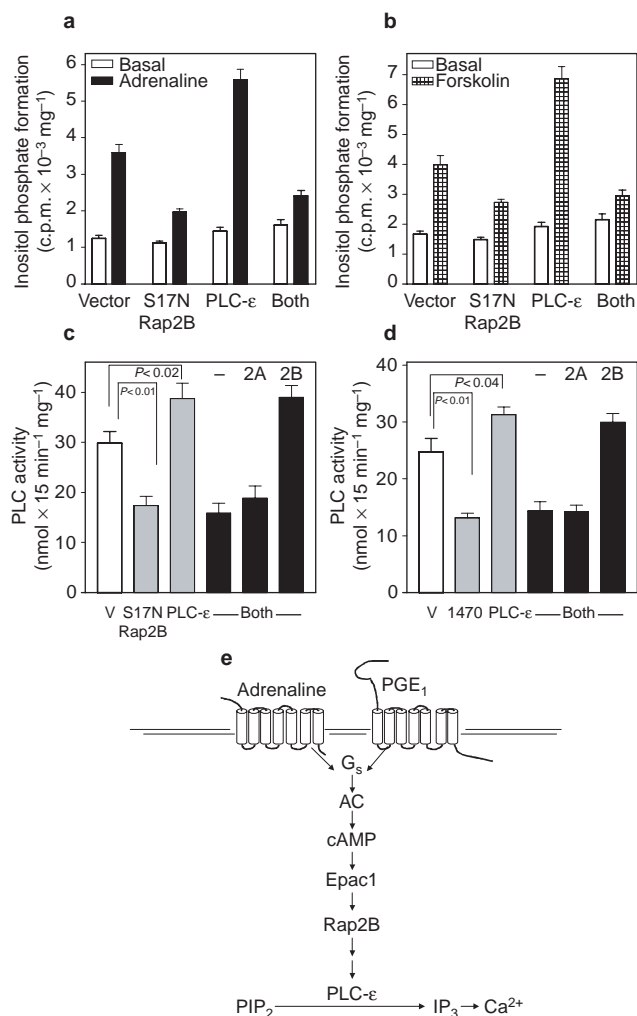


Figure 5 Influence of Rap2B on PLC-ε activity. **a, b**, HEK-293 cells were transfected with the β₂-AR, either alone (vector) or with S17N Rap2B, PLC-ε or S17N Rap2B plus PLC-ε (both), followed by measurement of [³H]inositol phosphate formation in the absence (basal) and presence of 10 μM adrenaline or 30 μM forskolin (mean ± s.e.m. of 4 experiments). **c**, HEK-293 cells were transfected without (V) and with S17N Rap2B, PLC-ε or S17N Rap2B plus PLC-ε (both). **d**, Cells transfected without and with PLC-ε were treated for 24 h without (V, PLC-ε) or with 300 pg ml⁻¹ toxin B-1470 (1470, both). Thereafter, PLC activity was determined in lysates of these cells with exogenous [³H]PtdInsP₂ in the presence of 100 μM GTPγS and without or with 3 μM recombinant GppNHp-bound Rap2A or Rap2B as indicated (mean ± s.e.m. of 3 experiments). Basal PLC activity in the absence of GTPγS (6.7 ± 0.9 nmol × 15 min⁻¹ mg⁻¹) was only slightly affected by transfection or treatment with toxin B-1470 (data not shown). **e**, Proposed model for β₂-AR- and prostanoid-receptor-mediated PLC and calcium signalling. Receptors coupling to G_s stimulate AC, resulting in elevated cAMP levels and activation of Epac1. Epac1 then catalyses GTP-loading on Rap2B, which leads to PLC-ε activation. The proposed pathway may involve additional signalling components to attain PLC stimulation. For details, see text.

in which only S17N Rap2B, but not S17N Rap2A or S17N Rap1A, although expressed at similar levels (Fig. 3e), reduced β₂-AR- and forskolin-stimulated PLC activities. Previous *in vitro* and intact cell studies on PLC-ε activation by Ras proteins suggested that stimulation of PLC activity requires the two Ras-binding domains and the Ras-GEF domain of PLC-ε and may therefore involve additional cellular components that have not yet been defined^{8,9}. Activation of

PLC-ε by Rap2B may be caused by similar complex mechanisms that need to be defined in future studies.

Prostaglandin E₁ (PGE₁) has previously been shown to increase cAMP levels as well as inositol phosphates and [Ca²⁺]_i in N1E-115 neuroblastoma cells^{23,24}. As illustrated in the Supplementary Information, PLC stimulation induced by the prostanoid receptor endogenously expressed in these cells is apparently mediated by similar mechanisms as PLC stimulation caused by the β₂-AR transfected into HEK-293 cells. Both 1 μM PGE₁ (the receptor agonist) and 30 μM forskolin (the direct AC activator) increased [Ca²⁺]_i (by 125 ± 15 nM (n = 12) and 109 ± 22 nM (n = 9), respectively) and inositol phosphate formation in N1E-115 cells to a similar extent. Treatment of the cells with the AC inhibitor 2',5'-dd-Ado (10 μM) reduced PLC stimulation by PGE₁ and forskolin by ~60%, whereas the PKA inhibitor H-89 (10 μM), did not alter the stimulatory effects of PGE₁ and forskolin. Overexpression of Epac1 increased PGE₁- and forskolin-induced PLC stimulation by 40–60%. Expression of the inactive S17N Rap2B mutant, as well as treatment of the cells with toxin B-1470 reduced PGE₁- and forskolin-induced PLC stimulation by 40–60%. Finally, overexpression of PLC-ε increased PGE₁- and forskolin-induced PLC stimulation by 50–70% (see Supplementary Information).

Stimulation of the cAMP-producing AC and the phosphoinositide-hydrolysing PLC enzymes are two common signalling pathways for membrane receptors. It is well known that products of the PLC pathway, that is, an increase in [Ca²⁺]_i and activated protein kinase C, can profoundly affect the activity of distinct AC isozymes¹⁵. In contrast, AC and its product cAMP can negatively regulate PLC stimulation, apparently by PKA-dependent phosphorylation of specific PLC isozymes^{4,10,11}. There are, however, reports in the literature that AC-coupled receptors, including the dopamine D1 receptor, the thyrotropin-releasing hormone receptor, the β-AR, prostanoid receptors and the pituitary AC-activating polypeptide type 1 receptor, can induce G_s- and cAMP-dependent PLC stimulation and/or calcium mobilization in certain cell types^{24–29}. The exact mechanisms involved in this type of PLC and calcium signalling have not been elucidated.

We propose, on the basis of our observations, a new PLC and calcium signalling pathway that is triggered by activation of G_s- and AC-coupled receptors and is dependent on cAMP production (Fig. 5e), thus establishing a positive interaction between the AC and the PLC signalling pathways. We show here that PLC and calcium signalling induced by the recombinant β₂-AR transiently expressed in HEK-293 cells or the prostanoid receptor endogenously expressed in N1E-115 neuroblastoma cells is fully mimicked by direct AC stimulation (by forskolin) and suppressed by direct AC inhibition (by 2',5'-dd-Ado), indicating that cAMP is pivotal to these receptor responses. The action of cAMP seems to be independent of PKA; instead, the cAMP-activated Rap-GEF Epac seems to serve as a cAMP effector, inducing GTP loading and, hence, activation of Rap2B, which then leads to specific activation of PLC-ε, which has been shown to interact with Rap GTPases^{7–9}. The intact cell data with the inactive GTPase mutants and the *in vitro* reconstitution results strongly suggest that Rap2B, but not the closely related GTPases Rap2A, Rap1A or H-Ras, serves as the mediator of signalling from the G_s- and AC-coupled receptors to PLC-ε. Rap GTPases can be activated by many G-protein-coupled and tyrosine kinase receptors through distinct Rap-GEFs activated by the second messengers cAMP, calcium and diacylglycerol, as well as by direct phosphorylation^{30,31}. It is an attractive hypothesis, therefore, that the Rap-dependent PLC and calcium signalling pathway reported here is not restricted to G_s- and AC-coupled receptors, such as the β₂-AR and the prostanoid receptor, but could be used by other receptors as well. In fact, preliminary data suggest that PLC stimulation by the G_q-coupled M₃ muscarinic receptor is in part mediated by Rap2B³². □

Methods

Cell culture and transfection

HEK-293 cells and N1E-115 neuroblastoma cells grown to near confluence on 145-mm culture dishes were transfected with empty vectors or 25 µg complementary DNAs each encoding the β₂-AR (in pcDNA3), Epac1 (in pMT2), PLC-β1, PLC-γ1 (both in pRK5), PLC-δ1, PLC-ε (both in pcDNA3), or 100 µg cDNAs each encoding the inactive Rap mutants (in pRK5), using the calcium phosphate method as reported previously¹³. Transfection efficiency was about 50%. Expression of the Flag-tagged β₂-AR in HEK-293 cells was ~400 fmol mg⁻¹ protein as determined by [¹²⁵I]iodocyanopindolol binding and was further verified by immunoblotting with an anti-Flag antibody (Stratagene). Expression of other proteins was monitored by immunoblotting with specific antibodies (Santa Cruz).

Measurement of [Ca²⁺]_i and PLC activity

The intracellular calcium concentration was determined in suspensions of fura-2-loaded cells as described previously¹². PLC activity in intact adherent cells was either measured for 30 min at 37 °C as [³H]inositol phosphate formation in the presence of LiCl in *myo*-[³H]inositol-prelabelled cells or for 60–120 s at 37 °C as InsP₃ mass in unlabelled cells^{13,33}. PLC activity in lysates of HEK-293 cells was determined in the presence of 40 nM free Ca²⁺ with 25 µM [³H]PtdInsP₂ as substrate for 15 min at 37 °C as described in detail previously³⁴. Experiments were generally performed 48 h after transfection in triplicate or quadruplicate culture dishes.

Measurement of Rap2B activation

To measure activation of Rap2B in HEK-293 cells, the cells were treated without and with 10 µM adrenaline (for 5 min) or 30 µM forskolin (for 10 min), followed by cell lysis in the presence of Nonidet P-40. After centrifugation, the supernatant was incubated with RalGDS-RBD bound to glutathione-Sepharose beads, and bound Rap2B was detected by immunoblotting with a specific Rap2B antibody (Santa Cruz) as described previously⁵.

Purification of recombinant Rap proteins

Recombinant Rap2A and Rap2B were prepared by transforming *Escherichia coli* with Rap2A and Rap2B cDNAs subcloned into the pGEX-4T vector, purified by adsorption of the GST-fusion proteins to glutathione-Sepharose and loaded with GppNHp as described previously³⁵.

RECEIVED 17 MAY 2001; REVISED 3 JULY 2001; ACCEPTED 23 JULY 2001;
PUBLISHED 10 OCTOBER 2001.

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Acknowledgements

We thank K. Baden, M. Hagedorn, H. Geldermann, D. Petermeyer, M. Michel and A. Rueppel for expert technical assistance, and R. Jockers, J. de Gunzburg, J. L. Bos, A. Wittinghofer and C. von Eichel-Streiber for providing various DNA constructs and proteins. This work was supported by the Deutsche Forschungsgemeinschaft, the Interne Forschungsförderung Essen (IFORES), the Fonds der Chemischen Industrie and the Council of Earth and Life Sciences and Chemical Sciences of The Netherlands Organisation for Scientific Research. Correspondence and requests for materials should be addressed to M.S. Supplementary Information is available on *Nature Cell Biology's* website (<http://cellbio.nature.com>).

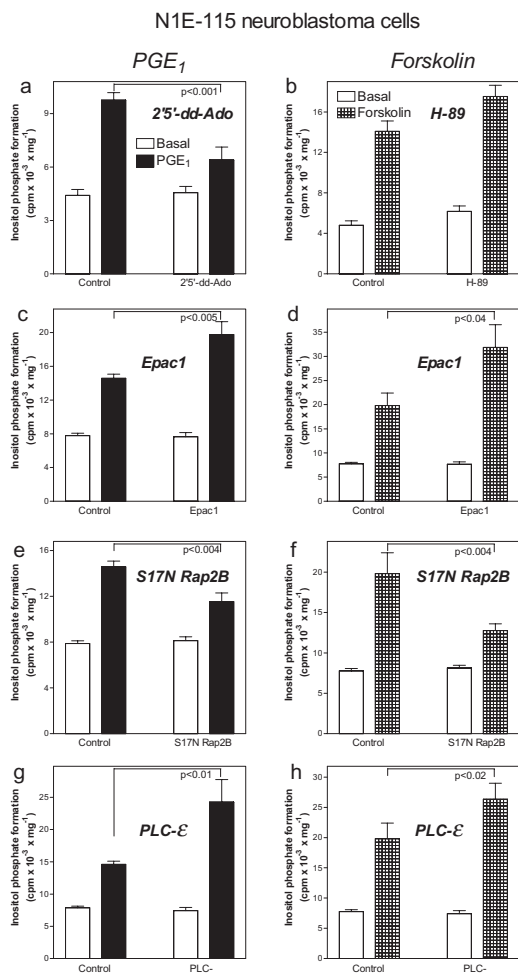


Figure S1 **PGE₁- and forskolin-induced PLC stimulation in N1E-115 neuroblastoma cells: role of cAMP, PKA, Epac1, Rap2B and PLC-ε.**

a, b, N1E-115 cells were incubated for 30 min with 10 μ M 2',5'-dd-Ado, 10 μ M H-89 or their solvents (control), followed by measurement of [³H]inositol phosphate accumulation in the absence (basal) or presence of 1 μ M PGE₁ (**a**) or 30 μ M forskolin (**b**). **c-h**, [³H]inositol phosphate formation was measured in the absence (basal) and presence of μ M PGE₁ (**c, e, g**) or 30 μ M forskolin (**d, f, h**) in N1E-115 cells transfected with empty vectors (control) or with Epac1 (**c, d**), S17N Rap2B (**e, f**) or PLC- ϵ (**g, h**). Data are mean \pm s.e.m. from 3 or 4 experiments. Expression of HA-tagged Epac1, Rap2B and PLC- ϵ was verified by immunoblotting (data not shown).