Use of an activation-specific probe to show that Rap1A and Rap1B display different sensitivities to activation by forskolin in Rat1 cells

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Abstract Rap1A and Rap1B are small GTPases of the Ras superfamily whose activation can be measured using a probe that interacts specifically with the GTP-bound forms of Rap1A and Rap1B. Using this procedure we demonstrate that the cyclic AMP-elevating agent forskolin activates both Rap1A and Rap1B in Rat1 cells. Whilst the protein kinase A inhibitor H89 ablated the ability of forskolin to cause cAMP response element binding protein (CREB) phosphorylation in Rat1 cells, it did not affect the ability of forskolin to activate either Rap1A and Rap1B. Forskolin differentially activated Rap1A and Rap1B isoforms in a time- and dose-dependent manner. The cAMP-specific type 4 family phosphodiesterase inhibitor rolipram potentiated the rate of activation of both Rap1A and Rap1B by forskolin challenge of Rat1 cells. Challenge of Rat1 cells with rolipram alone was able to elicit the phosphorylation of CREB but not activation of either Rap1A or Rap1B.

Key words: Rap1; Cyclic AMP; Cyclic AMP-dependent protein kinase; Forskolin; CREB; Small GTPase; Guanine nucleotide exchange factor; Exchange protein directly activated by cAMP; cAMP-dependent GTP exchange factor; cAMP-specific phosphodiesterase of the type 4 family; cAMP phosphodiesterase; Rolipram

1. Introduction

Rap1 is a member of the Ras family of small GTPases and is involved in the control of cell growth and morphogenesis, although the downstream effectors of Rap1 still remain to be fully elucidated. [1]. There are two known forms of Rap1, called Rap1A and Rap1B, that differ in their extreme C-termini [2]. They have been shown to be activated by a wide variety of ligands, including cAMP, diacylglycerol, Ca^{2+}, bombesin as well as by changes in cell adhesion and cell density [1,2,17]. When activated, Rap1 switches from a GDP-bound, inactive state to a GTP-bound, active state. This switch is controlled by the action of both guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins [1]. Both Rap1 forms have protein kinase A (PKA) phosphorylation sites located within their C-terminal region and it was originally surmised that activation of these GTPases ensued as a result of their PKA-mediated phosphorylation. However, it has also been suggested that Rap1 proteins may be activated by cAMP through a process that does not involve PKA [3–7]. In this regard, recent attempts to delineate the pathway by which cAMP activates Rap1 have implicated the direct activation of these small GTPases by a recently discovered family of GEFs. Such cAMP-regulated GEFs have been named as either cAMP-GEFs or as EPACs (exchange protein directly activated by cAMP). These GTPase exchange proteins appear to be activated by the direct binding of cAMP and therefore represent a novel mechanism for governing signalling specificity within the cAMP cascade [4,5].

Rap1 activation has normally been followed by a GTP loading assay [6]. However, a novel activation-specific assay for Rap1 has recently been developed [8–11,19]. This assay exploits the ability of the activated, GTP-bound forms of Rap1 to bind to the Ras binding domain (RBD) of the Ral form of guanine nucleotide dissociation stimulator protein (RalGDS) [8–11]. Expression of this domain as a GST fusion protein thus allows activated GTP-bound Rap1 forms to be isolated in pull-down assays. The amount of associated Rap1 then provides a direct quantification of the degree of activation. As this precipitated material can then be blotted with an appropriate anti-Rap1 antibody, such a method allows for the selective discrimination of Rap1A activation compared to that of Rap1B.

Previous studies using this assay method analysed Rap1 activation using antisera that did not discriminate between the Rap1A and Rap1B isoforms [8–11]. Here, for the first time, we have used isoform-specific antibodies to evaluate the activation of endogenously expressed Rap1A and Rap1B isoforms in Rat1 fibroblasts. We demonstrate that the adenylyl cyclase activator forskolin causes distinct dose-dependent and time-dependent activation profiles for both Rap1 isoforms.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), newborn calf serum

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Abbreviations: CREB, cAMP response element binding protein; DMEM, Dulbecco's modified Eagle's medium; PKA, cyclic AMP-dependent protein kinase; H89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide; cAMP-GEF, cAMP-dependent GTP exchange factor; EPAC, exchange protein directly activated by cAMP; EC_{50} value, concentration at which half maximal activation ensues; rolipram, 4-{3-(cyclopentoxyl)-4-methoxyphenyl}-2-pyrrolidone; PDE, cAMP phosphodiesterase; PDE4, cAMP-specific phosphodiesterase of the type 4 family; RBD, Rap binding domain; RalGDS, the Ral form of guanine nucleotide dissociation stimulator protein

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(NCS), foetal calf serum (FCS), glutamine, penicillin/streptomycin solution, anti-goat IgG and anti-rabbit IgG horseradish peroxidase were from Sigma. *N*-[2-(*p*-Bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide (H89) was from Calbiochem. 4-{3-(Cyclopentoxyl)-4-methoxyphenyl}-2-pyrrolidone (rolipram) was a gift from Schering. Anti-mouse IgG horseradish peroxidase antibody and ECL reagents were from Amersham. Complete protease inhibitor tablets were from Boehringer Mannheim. Goat, polyclonal antibodies to Rap1A and Rap1B were from Santa Cruz. Anti-phospho cAMP response element binding protein (CREB) and anti-CREB protein antibodies were from New England Biolabs.

2.2. Construction of expression vectors

Wild-type Rap1B-HA was cloned from image clone 504434 using specific primers to the published Rap1B sequence: (sense oligo: TGCAGGAATT CACCATGCGT GAGTATAAGC TAGT and antisense oligo: CTGATCTCGA GTTAAGCGTA ATCTGGAACA TCGTATGGGT AAAGCAGCTG ACATGATG). The 3′ oligonucleotide encodes a HA epitope sequence in-frame with the terminal amino acid of the Rap1B coding region. RapV12 was constructed following PCR amplification of the full-length Rap1B-HA clone, using a mutant 5′ oligonucleotide primer (sense oligo: TGCAGGAATT CACCATGCGT GAGTATAAGC TAGTCGTTCT TGGCTCAGTA GGCGTT) resulting in a valine for glycine substitution at amino acid 12. RapN17 was constructed following PCR amplification of the full-length clone, using a mutant sense oligonucleotide primer: TGCAGGAATT CACCATGCGT GAGTATAAGC TAGTCGTTCT TGGCTCAGGA GGCGTTGGAA AGAATGCTTT GACTGTA. The resultant amplification products were subcloned into the *Eco*RI and *Xho*I sites of the mammalian expression vector pcDNA3 (Invitrogen) and then sequenced.

The Rap binding domain of RalGDS (RalGDS-RBD) was cloned by reverse transcriptase PCR. The template cDNA was synthesised from 1 μg of human embryonic kidney HEK293 cell RNA using primer ATGCCCTTGG CAATCTTGAG. Approximately 1/20th of the reverse transcribed product was used to prime a 30-cycle PCR reaction using sense, ATCTGAATTC GCGCTGCCGC TCTACAACCA, and antisense, GTACAGTCGA CTCAGGTCCG CTTCTTGAGG ACAA, primers. The amplification product was subcloned into the *Eco*RI and *Sal*I sites of pGEX6X. The fidelity of the RalGDS-RBP construct was confirmed by automated sequencing.

2.3. Cell culture

The COS1 monkey kidney cell line and Rat1 fibroblasts were maintained at 37°C in an atmosphere of 5% CO_2, 95% air in complete growth medium containing DMEM supplemented with 0.1% penicillin/streptomycin (10 000 U/ml) and 10% FCS. Details have been described previously [12,13]. For transfection, growth medium was replaced with 5 ml of DMEM containing 10% (v/v) NCS and 0.1 mM chloroquine. Plasmid DNA (10 μg) was diluted to 250 μl with TE buffer [10 mM Tris–HCl (pH 8.0)/1 mM EDTA] mixed with 200 μl of DEAE-Dextran (10 mg/ml) and incubated for 10 min at room temperature. The DNA solution was added to cells and incubated for 4 h at 37°C. The transfection medium was aspirated and the cells were shocked with 10% (v/v) dimethyl sulphoxide in phosphate-buffered saline (PBS) for 2 min at room temperature. The monolayer was then washed twice with 5 ml of PBS and then incubated for 48 h in DMEM containing 10% (v/v) FCS. Before use COS1 and Rat1 cells were incubated in serum-free medium for 16 h.

2.4. Expression and purification of RalGDS as a GST fusion protein

An overnight culture of JM109 bacteria bearing the vector pGEX6X-RalGDS-RBP was diluted 1:10 (v/v) in LB (10 g/l (w/v) tryptone, 10 g/l (w/v) NaCl, 5 g/l (w/v) yeast extract) containing 50 μg/ml ampicillin and grown for a further hour at 37°C. Protein synthesis was induced for 4 h with the addition of 1 mM IPTG. Cells were then pelleted (4000×*g*, 10 min, 4°C) and frozen at −80°C overnight to break bacterial cell walls. The following day cells were resuspended in 10 ml of 50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol containing protease inhibitor cocktail. Cell suspensions were sonicated on ice to shear DNA (4×30 s bursts), after which 0.02% Triton X-100 was added and then cell debris was pelleted by centrifugation (13 000×*g*, 10 min, 4°C). The supernatant was transferred to a 10 ml centrifuge tube and incubated for 30 min on a rotary shaker at 4°C with 1 ml of pre-equilibrated glutathione Sepharose beads (Pharmacia). Beads were recovered by centrifugation at 2000×*g* for 1 min, washed in resuspension buffer containing 0.02% (v/v) Triton X-100 and fusion protein eluted in 2.4 ml of 5 mM glutathione, 50 mM Tris, pH 8.0. To remove glutathione eluates were dialysed, with three changes against 50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 5% (v/v) glycerol and stored at −80°C until use.

2.5. Rap1 activation assay and immunoblotting

GST-RalGDS-RBD was used to determine the relative amount of active GTP-bound Rap1 as previously described [11]. Detergent extracts of whole cells were prepared by solubilisation in 55 mM Tris–HCl, pH 7.4, 132 mM NaCl, 22 mM NaF, 11 mM sodium pyrophosphate, 10 mM magnesium chloride, 1% Nonidet P40 containing complete protease inhibitor cocktail (Boehringer). Cell extracts were incubated with 10 μg of immobilised GST-RalGDS-RBD for 60 min at 4°C. The beads were then washed four times in lysis buffer, followed by SDS–PAGE and immunoblotting with anti-HA antiserum, anti-Rap1A or anti-Rap1B antibodies all used at a concentration of 1:500 (v/v). CREB activation in cell lysates was detected with phospho-CREB antibodies as described [14].

3. Results and discussion

3.1. Evaluating the RalGDS binding assay for assessing Rap1 activation

Recently it has been suggested that RalGDS-RBD, when expressed as a GST fusion protein in *Escherichia coli*, can be used to isolate specifically GTP-bound Rap1 forms and thus serve as a specific probe to determine Rap1 activation [11]. We set out to evaluate independently the fidelity of this novel system. To do this we first expressed RalGDS-RBD as a GST fusion protein in *E. coli* and purified it on glutathione agarose (Fig. 1a). We then transiently transfected COS1 cells with a wild-type form of Rap1B. To ensure that we were able to monitor its activation independently of any endogenous Rap1 proteins expressed in COS cells we generated a HA epitope-tagged form of Rap1 and detected it with an antibody that was specific for this tag. We then treated these cells with the diterpene forskolin (1 μM), which is known to activate adenylyl cyclase in these cells and to elevate cAMP levels [15,16]. The transfected COS cells were then lysed and the GTP-bound form of Rap1B was isolated with the GST fusion protein of RalGDS-RBP with detection done by immunoblotting with a HA-specific monoclonal antibody in order to detect the bound, epitope-tagged Rap1B.

This clearly showed that forskolin treatment of transfected COS cells led to a robust activation of transiently transfected HA epitope-tagged Rap1B (Fig. 1a,b). In order to provide a control to evaluate the authenticity of this assay we generated the N17 mutant form of Rap1B in order to provide a dominant negative form that should not interact with RalGDS-RBP [1], again this was HA epitope-tagged so that we could detect it specifically. This demonstrated that the N17-Rap1B form failed to bind in forskolin-challenged cells (Fig. 1b,c). In order to gauge the maximal level of interaction in this assay and to provide and independent positive control, we generated the constitutively activated V12-Rap1B mutant form, again as a HA epitope-tagged form. The V12 mutation inactivates the Rap1 GTPase and thus allows Rap1 to accrue in a fully GTP-bound form in cells [1,2]. Consistent with this, we were able to show (Fig. 1b,c) that this form of Rap1B was able to associate with RalGDS-RBP in cells that had not been challenged with forskolin. Indeed, the degree of association was similar to that seen for wild-type Rap1B subsequent to forskolin challenge

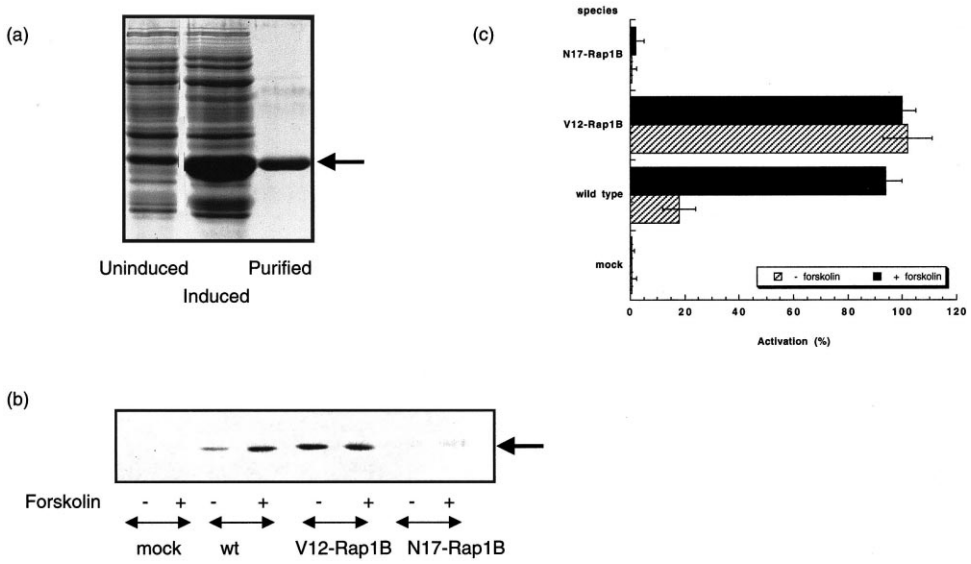

Fig. 1. Activation of Rap1B in transiently transfected COS1 cells. a: Coomassie blue protein stain of an SDS gel analysing a lysate from *E. coli* cells that had either been treated (induced) or not (uninduced) with IPTG to induce expression of RalGDS-GST fusion protein (major staining band in induced track). Also shown is a track for the specific eluate from a glutathione affinity column, showing the purification of RalGDS-GST. b: Confluent COS1 cells (Mock) and COS1 cells transfected with cDNAs encoding HA epitope-tagged versions of wild-type (Rap1B-HA), constitutively active (V12-Rap1B-HA) and dominant negative (N17-Rap1B-HA) forms of Rap1B were challenged with 1 μM forskolin for 10 min. Cells were then lysed and active, GTP-bound forms of Rap1 were isolated with RalGDS (see Section 2). The bound, and hence active, forms of Rap1 were specifically detected by immunoblotting with anti-HA antibody. c: The data from three experiments of the form described in b were analysed densitometrically. Maximal activation (100%) was taken for that described by the V12-Rap1B form (means ± S.D.; $n = 3$).

(Fig. 1c). This implies that such forskolin treatment of COS cells sufficed to maximally activate the transfected Rap1B. Consistent with this, we noted that forskolin failed to increase the amount of constitutively activated V12-Rap1B associated with RalGDS-RBP in transfected COS cells (Fig. 1).

These results demonstrate that the RalGDS-RBP probe can be used to identify active Rap1 generated by forskolin treatment of wild-type Rap1.

3.2. Forskolin and 8-bromo-cAMP activation of endogenous Rap1 isoforms in Rat1 fibroblasts

We set out to examine whether forskolin might lead to the activation of endogenously expressed Rap1A and Rap1B isoforms. To do this we employed antisera that were capable of discriminating between Rap1A and Rap1B isoforms (Fig. 2a). We chose to evaluate Rat1 fibroblasts, as we were able to show, immunologically, that these cells expressed the 21 kDa forms of both Rap1A and Rap1B (Fig. 2b).

In order to determine whether increased levels of cAMP activated these forms, we treated Rat1 cells with forskolin (1 μM) and isolated the GTP-bound forms of activated Rap1 in pull-down assays with RalGDS-RBD. The active forms of both Rap1A and Rap1B were then separately detected with isotype-specific antisera able to recognise the unique C-termini of Rap1A and Rap1B. Challenge with forskolin increased the level of Rap1A and Rap1B immunoreactivity associated with RalGDS-RBD, indicative of Rap1 activation in these treated cells (Fig. 2b).

Forskolin-induced activation of Rap1A and Rap1B was completely unaffected by the PKA inhibitor H89 (Fig. 2b), indicating that such activation was independent of PKA activation in these cells [22]. However, we wished to ascertain whether H89 did, indeed, serve as an effective inhibitor of PKA action in these cells under the conditions utilised here. To do this we showed that challenge of Rat1 cells with the adenylyl cyclase activator forskolin, increased the phosphorylation of the transcription factor CREB (Fig. 2c). PKA activation, as a consequence of increased cAMP levels, has been shown to phosphorylate this protein at a single site, Ser-133 [23]. Consistent with this, H89 served to totally ablate this action of forskolin on CREB phosphorylation (Fig. 2c). It would thus appear that the Rap1 forms in Rat1 cells are activated by forskolin in a PKA-independent fashion.

To evaluate whether increased cAMP levels did indeed activate these Rap1 isoforms we also challenged the Rat1 cells with the cell-permeable cAMP analogue, 8-bromo-cAMP (10 μM). This too was capable of activating both Rap1A and Rap1B isoforms and, as with forskolin, this process was unaffected by the PKA inhibitor H89 (Fig. 2d).

We then set out to determine the dose dependence of activation of these Rap1 forms by forskolin. This, intriguingly, demonstrated that forskolin appeared to activate Rap1B at lower concentrations than caused Rap1A activation (Fig. 3a). Thus the EC_{50} value for forskolin activation of Rap1A was 0.2 ± 0.1 μM and for Rap1B was strikingly different at 0.01 ± 0.05 μM (mean ± S.D.; $n = 3$ separate experiments). A similar differential effect was observed when we employed 8-bromo-cAMP, with the EC_{50} values for activation of Rap1A being 0.12 ± 0.01 μM and for Rap1B at 0.011 ± 0.003 μM (mean ± S.D.; $n = 3$ separate experiments). There are many possible explanations for such differences. For example, it could be that either different EPACs/cAMP-GEFs control the activation of these two Rap1 forms and that these have different affinities for activation by cAMP or that different levels of occupancy of the EPAC/cAMP-GEF by cAMP are needed to elicit Rap1A and Rap1B activation. However, insight into this apparent difference can be gleaned from observations concerning the rate of activation of these two Rap1

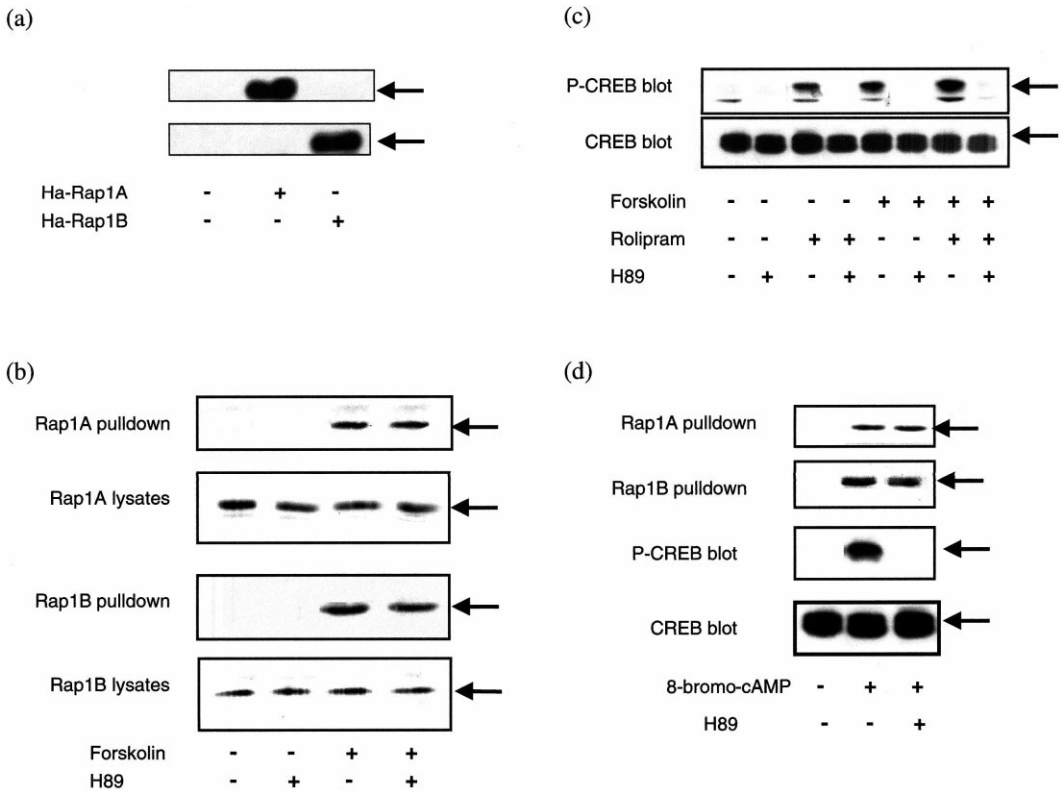

Fig. 2. Activation of Rap1 isoforms in Rat1 cells. a: COS1 cells were transfected to express HA epitope-tagged forms of either Rap1A or Rap1B. Then lysates from either untransfected cells (track 1), Rap1A-transfected (track 2) or Rap1B-transfected cells (track 3) were immunopurified with the HA-specific monoclonal antibody and Western blotted with either the Rap1A-specific antiserum (upper panel) or the Rap1B-specific antiserum (lower panel). The Rap1A-specific antiserum identified a single 21 kDa immunoreactive species only in the Rap1A-transfected cells and vice versa for the Rap1B-specific antiserum. b: Rat1 cells were grown to confluence, and stimulated with, as indicated, 10 μM forskolin, 10 μM H89 or a combination of the two agents for 20 min. Cells were lysed and GTP-bound forms of Rap1 were isolated using the Ral-GDS pull-down assay and detected with specific antisera to Rap1A and Rap1B as described in Section 2 (first and third panels). In addition, equal amounts of cell extract (10 μg) were subjected to SDS–PAGE and immunoblotted with antisera to Rap1A and Rap1B (second and fourth panels). c: Rat1 cells were treated as above except, where indicated, the PDE4-selective inhibitor rolipram was added (10 μM). Lysates were then treated with antibodies that recognise the phosphorylated form of CREB (upper band indicated with arrow). In addition, lysates were blotted using an antibody that detects both phosphorylated and unphosphorylated forms of CREB (lower panel) in order to gauge that equal loading was done. Results are representative of an experiment carried out on three separate occasions. d: Rat1 cells were treated with 8-bromo-cAMP (20 μM) ± H89 (10 μM), as above, with Ral-GDS pull-down and blotting analyses done, as above, for Rap1A and Rap1B activation. In addition, blotting was done, as above, to gauge CREB phosphorylation and total CREB levels. These blots are typical of experiments done at least three times.

isoforms in forskolin-challenged Rat1 cells. Thus, in the presence of maximally effective dose of forskolin (10 μM), activation of Rap1B preceded that of Rap1A (Fig. 3b,c). Thus the apparent diminished sensitivity of Rap1A to activation by forskolin may reflect the fact that in using a single time point to determine activation we may have underestimated Rap1A activation, relative to Rap1B activation, especially at the lower doses of forskolin.

It is then very intriguing that the activation of the Rap1A isoform is slow compared to activation of Rap1B. We then set out to evaluate if inhibiting cAMP degradation could augment the rate of Rap1 activation by forskolin. It is well established that inhibition of cAMP phosphodiesterase (PDE) activity can potentiate the ability of adenylyl cyclase activators, such as forskolin, to elevate intracellular cAMP levels [18]. At least six different gene families encode PDE enzymes able to hydrolyse cyclic AMP [15]. However, the activity of members of the type 4 family of PDE (PDE4) provides the major PDE activity in Rat1 cells (M.D. Houslay, E. Huston, C. Shepherd, unpublished). These enzymes can be selectively inhibited with the anti-inflammatory and anti-depressant compound rolipram [18]. The addition of rolipram to Rat1 cells, at a concentration (10 μM) known to cause maximal inhibition of this family of phosphodiesterases [18], potentiated the rate of activation of both Rap1A and Rap1B by forskolin but did not increase the maximal level of activation (Fig. 3b,c). Thus the presence of rolipram served to emphasise the different rates of activation of these Rap1 isoforms, with forskolin and rolipram together activating Rap1B within 2 min, whilst Rap1A activation only began to be evident at around 10 min. This effect of rolipram was unaffected by the PKA inhibitor H89 (data not shown), indicating that rolipram exerted its action of these Rap1 forms in a fashion that was independent of PKA activation.

The addition of rolipram (10 μM) alone to Rat1 cells for 20 min led to CREB phosphorylation (Fig. 2c) up to levels comparable to that achieved using forskolin (10 μM; data not shown). In contrast to this, treatment of Rat1 cells with rolipram (10 μM; 20–40 min) did not lead to the activation of either Rap1A or Rap1B (data not shown). This indicates a functional compartmentalisation of cAMP signalling in these cells.

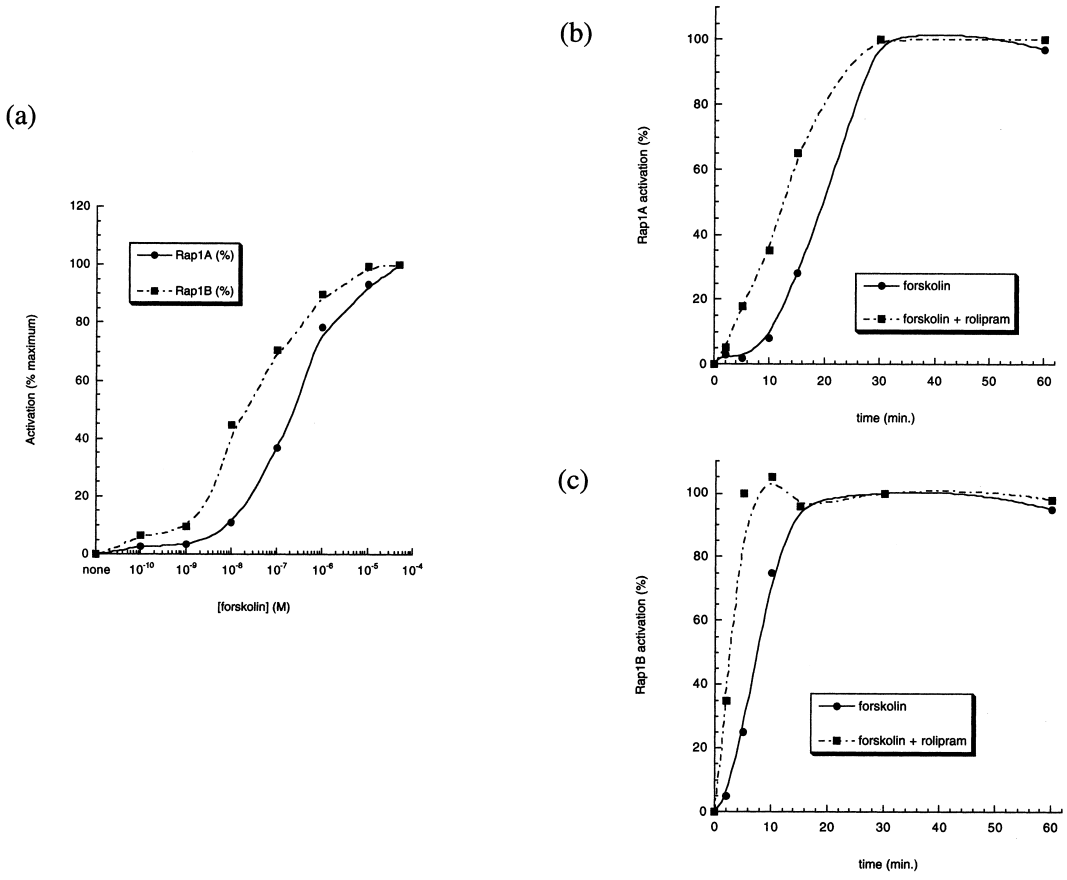

Fig. 3. Dose-dependent activation of Rap1A and Rap1B isoforms by forskolin in RAT1 cells. Serum-starved Rat1 cells were treated, as above, in order to gauge Rap1 activation by association of the GTP-bound forms of Rap1A and Rap1B with RalGDS-RBP-GST in pull-down assays with visualisation using isoform-specific antibodies. a: Experiments done with a range of forskolin concentrations for Rap1A and Rap1B. b: Experiments done over the indicated times with 10 μM forskolin ± 10 μM rolipram for Rap1A. c: Experiments done over the indicated times with 10 μM forskolin ± 10 μM rolipram for Rap1B. Quantification was done by densitometric analysis under conditions where linear responses were determined. Results are means ± S.D. of $n = 3$ experiments.

4. Conclusions

Using an independent assessment system, we show here that binding to RalGDS-RBP provides an effective means of determining Rap1 activation in intact cells. We then have extended this to show that it can be used to make independent assessments of the separate activation of Rap1A and Rap1B isoforms in Rat1 cells. This could be triggered in a PKA-independent fashion using either forskolin to activate adenylyl cyclase or using the cell-permeable cAMP analogue, 8-bromo-cAMP. This is consistent with the notion that Rap1 proteins can be converted to their GTP-bound forms and thus activated through the action of EPACs/cAMP-GEFs as recently suggested [4,5].

Our ability to discriminate between the activation of Rap1A and Rap1B, however, indicated that forskolin caused the activation of Rap1B at a faster rate than it caused the activation of Rap1A. In each case, the rate of activation was augmented by the presence of the PDE4 cAMP phosphodiesterase inhibitor rolipram. This can be expected to potentiate the level of cAMP accumulation sustained by forskolin activation of adenylyl cyclase and hence the degree of occupancy of EPACs/cAMP-GEFs. The differences in rates of activation of these two Rap1 isoforms could reflect their coupling to EPACs/cAMP-GEFs with different affinities for cAMP or differences in affinity of the Rap1 isoforms for cAMP-bound EPACs/cAMP-GEFs. This functional compartmentalisation of cAMP signalling in Rat1 cells also appears to extend to the effect of PDE4 enzyme inhibition of the Rap1 enzymes compared to CREB activation. For inhibition of PDE4 enzymes by rolipram appears to suffice in itself to elicit CREB phosphorylation whilst adenylyl cyclase activation is needed to elicit Rap1 activation whereupon PDE4 inhibition can then serve to augment it.

Whatever provides the underlying molecular mechanism, our study emphasises the need not only to evaluate separately Rap1A and Rap1B activation but also to take particular note of temporal differences in activation rates that may occur. Failure to do this may lead to erroneous conclusions concerning the regulation of these two species. In any event, it would seem that we have made the novel observation that these two Rap1 isoforms can be differentially regulated by changes in cAMP levels. In that way the Rap1 system is analogous to the PKA system, where the binding of RII-PKA isoforms to anchor proteins allows for the selective regulation of PKA sub-populations through their responses to intracellular gradients of cAMP compartmentalised [15,20,21].

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