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Hypothesis

Differential feedback regulation of the MAPK cascade underlies the quantitative differences in EGF and NGF signalling in PC12 cells

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Abstract Although epidermal growth factor (EGF) induces transient activation of Ras and the mitogen-activated protein kinase (MAPK) cascade in PC12 cells, whereas nerve growth factor (NGF) stimulates sustained activation, the basis for these contrasting responses is not known. We have developed a computer simulation of EGF-induced MAPK cascade activation, which provides quantitative evidence that feedback inhibition of the MAPK cascade is the most important factor in determining the duration of cascade activation. Hence, we propose that the observed quantitative differences in EGF and NGF signalling can be accounted for by differential feedback regulation of the MAPK cascade.

Key words: Epidermal growth factor; Nerve growth factor; PC12 cell; Ras; Mitogen-activated protein kinase; Son of sevenless

1. Introduction

The PC12 rat phaeochromocytoma cell line has been used extensively for studying nerve growth factor (NGF) signal transduction, but is also responsive to other growth factors, including epidermal growth factor (EGF); the latter stimulates the proliferation of PC12 cells, whereas NGF stimulates differentiation [1]. Both EGF and NGF activate a cytosolic signal transduction pathway comprising the small guanine nucleotide binding protein, Ras, and a mitogen-activated protein kinase (MAPK) cascade formed by Raf, MAPK or ERK kinase (MEK) and extracellular signal-regulated kinase (ERK) protein kinases. In PC12 cells however, EGF causes transient activation of Ras, MEK and ERK, whilst NGF induces sustained activation of these signalling molecules [1]. The basis for these quantitative differences in MAPK cascade activation by EGF and NGF has not yet been established, although differences in down-regulation of the respective cell-surface receptors have been implicated as the cause [2]. This explanation assumes that growth factor signalling is attenuated by receptor internalisation, but it has recently been shown that internalised EGF receptor (EGFR) is as effective as the cell-surface receptor in activating Ras [3], and hence a mechanism acting downstream of the EGFR is more likely to be responsible for down-regulation of the signal generated by EGF. One possible explanation for the contrasting patterns of MAPK cascade activation generated by EGF and NGF may therefore be that the intracellular signals induced by these growth factors are subject to differential feedback regulation. Other possibilities that have been suggested include differences in binding affinities of the specific receptors for substrate molecules, or in receptor tyrosine kinase (RTK) activities [4], or differential modulation of some factor that negatively regulates ERK, such as Ras GTPase activating protein (GAP) or an ERK phosphatase [5]. In order to investigate the factors influencing the kinetics of MAPK cascade activation, we have developed a computer simulation of the EGF signal transduction pathway in PC12 cells. Our analysis of this pathway indicates that negative feedback inhibition of the MAPK cascade is the most important factor in determining whether cascade activation is transient or sustained, and that differences in feedback regulation are likely to underlie the characteristic patterns of EGF- and NGF-induced Ras, MEK and ERK activation in PC12 cells.

2. Model description

We have developed a computer simulation of the EGF signal transduction pathway [6] that has been implemented using the biochemical kinetics simulation software package, Gepasi 3.2 [7]. The model is defined in terms of a number of signalling molecules and reaction steps, for which rate equations and kinetic constants are specified. The software utilises these data to calculate the change in the concentrations of signalling intermediates over time.

The initial steps of the simulated pathway (Fig. 1) constitute a self-contained simulation of EGF-induced EGFR activation and internalisation. This is based upon a mathematical model of the EGFR endocytic pathway [8] that has been modified to include ligand-induced receptor dimerisation [9,10]. The first step is the binding of EGF (L in the reaction scheme) to the monomeric cell-surface EGFR (Rs), to form a receptor–ligand complex (RL) [10]. After ligand binding, RL complexes associate to yield the R2L2 dimer [9,10]. Activation of the intrinsic protein tyrosine kinase occurs simultaneously with dimerisation [11]. Only the active R2L2 species is intern-

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Abbreviations: EGF, epidermal growth factor; NGF, nerve growth factor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK or ERK kinase; EGFR, EGF receptor; GAP, Ras GTPase activating protein; RTK, receptor tyrosine kinase; Shc, Src homology and collagen domain protein; Grb2, growth factor receptor binding protein 2; SOS, Son of sevenless homologue protein; TrkA, high affinity NGF tyrosine kinase receptor; PTPase, protein tyrosine phosphatase; PP2A, protein phosphatase 2A

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alised via a ligand-induced pathway [12], through binding to cell-surface coated pit adaptor proteins (CPP). Internalisation of R2L2 yields an intracellular dimeric species (R2i), which rapidly dissociates to form the monomeric species (Ri). Inactive Rs and RL complexes are internalised through a constitutive mechanism [12], as are R2L2 complexes not associated with coated pits. Once internalised, the ligand dissociates from the receptor (Li) and is degraded. Internalised Ri is then recycled to the cell-surface.

Activated receptors (R2L2, R2i and R2-CPP) catalyse the tyrosine phosphorylation of the adaptor protein, Src homology and collagen domain protein (Shc) [13], yielding ShcP, which then associates with a constitutive complex formed by the adaptor protein, growth factor receptor binding protein 2 (Grb2), and the guanylnucleotide exchange factor, Son of sevenless homologue protein (SOS) (GS) [14], to form the ternary complex, ShcGS. It is assumed that only uncomplexed ShcP may be dephosphorylated by cellular protein tyrosine phosphatases (PTPases), as it is not known whether complexed ShcP is a target for phosphatase activity. Formation of ShcGS recruits SOS to the plasma membrane where Ras is localised [14]. Interaction of RasGDP with ShcGS forms a Ras–ShcGS complex, and enables SOS to stimulate the conversion of inactive RasGDP to active RasGTP [14].

RasGTP may bind to GAP, yielding a Ras–GAP complex and stimulating the intrinsic GTPase activity of Ras, so that RasGTP is converted to RasGDP [15]. Alternatively, RasGTP may bind to Raf to generate the Ras–Raf complex. This recruits Raf to the plasma membrane and facilitates Raf kinase activation [16]; the latter is associated with growth factor-induced serine phosphorylation of Raf [17]. Activated Raf* catalyses the phosphorylation of two MEK serine residues; both MEKP and MEKPP are catalytically active [18]. MEKP and MEKPP activate ERK by catalysing the phosphorylation of a tyrosine and a threonine residue, but only ERKPP is active [19]. Dephosphorylation of Raf*, MEKP, MEKPP and ERKPP is catalysed by the same serine/threonine phosphatase, protein phosphatase 2A (PP2A), however, ERKP/ERKPP is also dephosphorylated by an, as yet, unidentified PTPase [20]. Feedback regulation of the pathway is mediated by the inhibitory serine/threonine phosphorylation of SOS, catalysed by ERKPP, resulting in dissociation of the ShcGS complex to yield ShcP and GSP [21]. GS is regenerated by dephosphorylation, although the specific phosphatases involved in this process are not known.

All enzyme-catalysed reactions follow Michaelis–Menten kinetics, whereas all other reactions are represented by mass–action kinetics. Parameter values are available at http://bms-mudshark.brookes.ac.uk.

To assess the sensitivity of the behaviour of the pathway to variations in parameter values, we changed the value of a selected parameter away from its original value (whilst maintaining all other parameters at their original values) and observed the effect on the activation state of MAPK cascade components. The initial values of the selected parameters are given in Table 1. These values were varied over ranges consistent with either measured or estimated values specified in the literature [22–26].

3. Results and discussion

Numerous reports have demonstrated that EGF stimulation of PC12 cells results in a rapid, transient activation of Ras, MEK and ERK. The level of RasGTP rapidly reaches a maximum of up to 20% of total Ras within 2 min exposure to EGF, but declines to less than 10% within 10 min and gradually returns to the basal level within 60 min [27]. Similarly, MEK and ERK activation are maximal within 2–5 min, but MEK activation rapidly declines to less than 15% of the peak value within 30 min [22,28], whilst ERK activation declines to less than 50% of the maximum level by 30 min, and to less than 25% within 60 min [22,28–30]. In order to compare the time-dependent behaviour of the computer simulation of EGF signal transduction with these experimental observations, the time courses of Ras, MEK and ERK activation were computed over 60 min continuous exposure to 100 nM EGF. Fig. 2 demonstrates that the simulated time course patterns of Ras, MEK and ERK activation are compatible with those typical

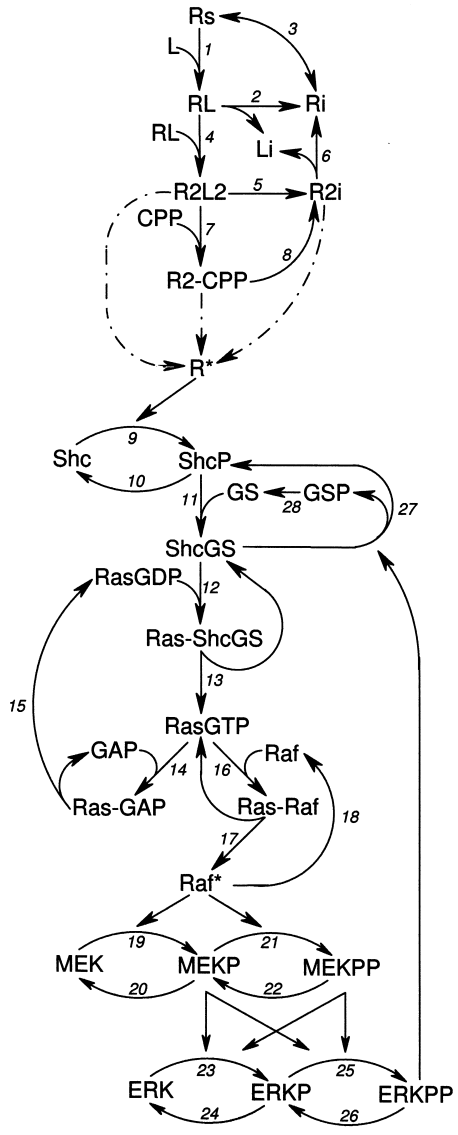

Fig. 1. Schematic representation of the computer simulation of EGF signal transduction. The three active species of receptor (R2L2, R2i and R2-CPP) are modelled as separate entities in the simulation, but for convenience these are denoted in the reaction scheme by the generic term, R*. The numbering of reactions is arbitrary.

Table 1
Parameter values of selected steps in the EGF signal transduction pathway, and the effect of variations in these parameters on the duration of MAPK cascade activation over 60 min simulated continuous exposure to EGF

Reaction number	Parameter	Value	Reference	Change in value	Sustained activation		
					Ras	MEK	ERK
1	total receptors, [Rs+Ri]	1.5×10^4 molecules/cell	[22]	↑	×	×	×
2, 3, 5, 6, 8	ke, receptor internalisation	0.7/min	[8]	↓	×	×	×
9	km, Shc phosphorylation	816 molecules/cell	[39]	↓	×	×	×
9	*k*, Shc phosphorylation	12/min	[39]	↑	×	×	×
27	*k*, SOS phosphorylation	1.2/min	[26][a]	↓	√	√	√
27	*V*, SOS dephosphorylation	75 molecules/cell/min	[23,25,26][a]	↑	√	√	√
15	*k*, GAP	720/min	[40,41][a]	↓	√	√	√
24, 26	*V*, ERK PTPase	2.5×10^5 molecules/cell/min	[23,25,26][a]	↓	×	×	√
18	*V*, PP2A	9.7×10^4 molecules/cell/min	[23,25,26][a]	↓	×	√	√
20, 22	*V*, PP2A	9.7×10^5 molecules/cell/min	[23,25,26][a]	↓	×	√	√
24, 26	*V*, PP2A	2.5×10^5 molecules/cell/min	[23,25,26][a]	↓	×	√	√
1	[EGF]	100 nM	[29]	–	–	–	–

[a]The value assigned to the parameter is consistent with a range of measured or estimated values given in the literature.

of EGF-stimulated PC12 cells, indicating that the computer simulation is an adequate representation of EGF-induced MAPK cascade activation in this cell type.

The pattern of NGF-stimulated MAPK cascade activation in PC12 cells differs from that induced by EGF; NGF has been shown to induce a rapid activation of Ras, MEK and ERK that may be largely sustained for up to several hours [22,27–30]. In order to investigate the basis for this difference in EGF and NGF signalling through the MAPK kinase cascade, we utilised the computer simulation of EGF signal transduction in a quantitative analysis of the factors affecting the time course of cascade activation. By individually varying the values of kinetic parameters governing a number of signalling events, we have been able to determine the specific effect of each of these events on the pattern of Ras, MEK and ERK activation. A summary of the results of this analysis is given in Table 1.

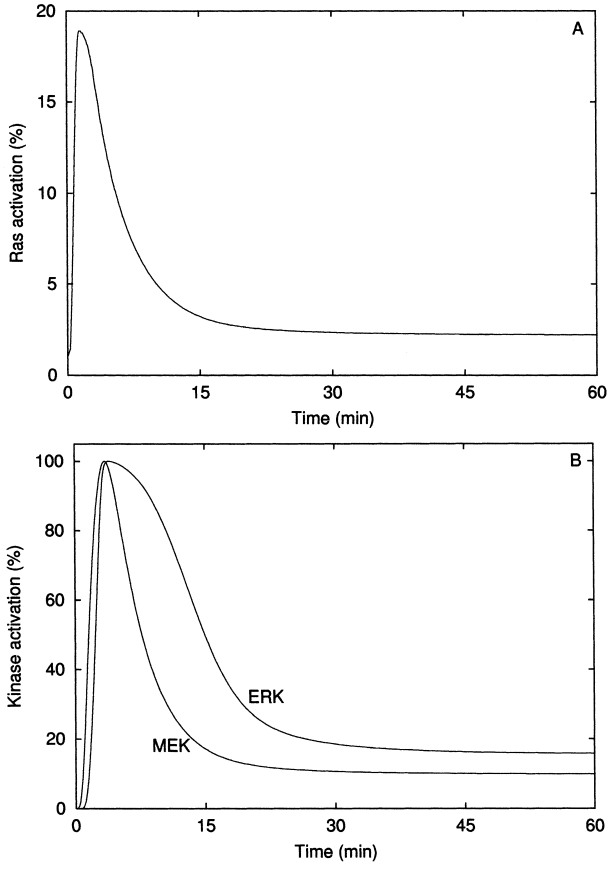

Fig. 2. Simulated time courses of MAPK cascade activation in PC12 cells over 60 min continuous exposure to EGF. A: Percentage of total Ras in the GTP-bound form. B: Percentage of maximal MEK and ERK activation. Simulation parameters as given in Table 1.

It has been proposed that the transient activation of Ras and ERK by EGF in PC12 cells may be due to attenuation of the signal at or near the level of the EGFR [22,28]. Thus, sustained Ras and ERK activation may possibly be due to prolonged activation of high affinity NGF tyrosine kinase receptor (TrkA) compared to EGFR [31]. Studies involving manipulation of the expression or down-regulation of growth factor receptors appear to confirm the view that the number of active cell-surface receptors [2], and hence the strength of the signal generated [32], determines whether Ras and ERK activation are sustained. Consequently, we investigated the influence of factors affecting the number of activated cell-surface receptors on the duration of Ras, MEK and ERK activation, these being the total number of receptors and the rate of receptor internalisation. Increasing the number of receptors up to 50-fold had no effect on the time course of MAPK cascade activation (Table 1), despite prolonging Shc phosphorylation (data not shown). Decreasing the rate of receptor internalisation was also without effect. These results are in agreement with a recent study indicating that EGFR internalisation is not likely to be the predominant mechanism for the rapid attenuation of EGF-induced Ras activation [3].

It has also been suggested that differences in the kinetics of receptor interaction with intracellular signalling intermediates might determine the duration of MAPK cascade activation [4]. Thus, we also examined the influence of the affinity of the receptor for the adaptor protein, Shc, and the rate of RTK-catalysed phosphorylation of this protein, but found that these factors also had no effect on the time course of Ras, MEK or ERK activation (Table 1), even though Shc phosphorylation was enhanced (data not shown). Hence, our results suggest that specific differences in the number of activated receptors at the cell-surface, or in the intensity of the signals generated by the receptors, do not underlie the quantitative differences in EGF and NGF signalling in PC12 cells,

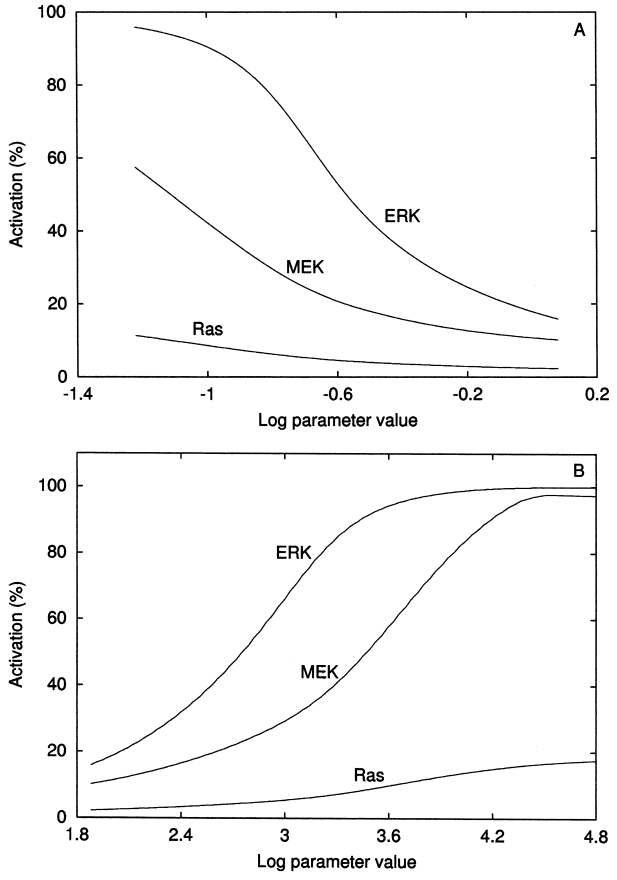

Fig. 3. Sensitivity of the duration of MAPK cascade activation to variations in the values of kinetic parameters governing the feedback phosphorylation of SOS. The effect of (A) decreasing k, SOS phosphorylation, and (B) increasing V, SOS dephosphorylation on the activation state of the MAPK cascade after 60 min simulated continuous exposure to EGF. The curves indicate the percentage of Ras in the GTP-bound form, and the percentage of maximal MEK and ERK activation.

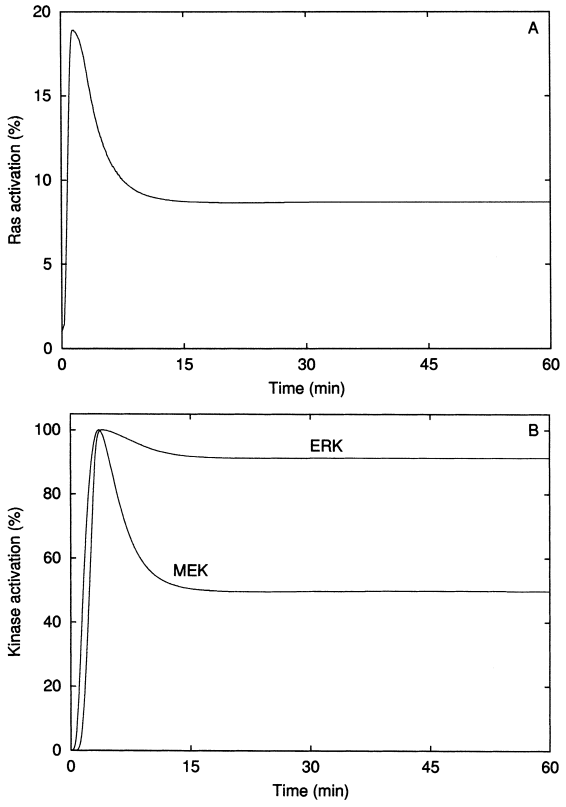

Fig. 4. Sustained activation of the MAPK cascade resulting from a 40-fold increase in the rate of SOS dephosphorylation, over 60 min simulated continuous exposure to EGF. Time courses of (A) percentage of total Ras in the GTP-bound form, and (B) percentage of maximal MEK and ERK activation. V, SOS dephosphorylation = 3×10^3 molecules/cell/min. Other parameter values as given in Table 1.

as manipulating the kinetic parameters of the computer simulation to increase the magnitude of ligand-induced signalling did not generate sustained MAPK cascade activation.

We next examined the influence of factors directly affecting the persistence of the Shc–Grb2–SOS complex on the duration of Ras, MEK and ERK activation, since it is likely that EGF signalling is in fact down-regulated through feedback desensitisation [3], mediated by MEK-dependent serine/threonine phosphorylation of SOS and resulting in dissociation of the signalling complex formed by phosphorylated Shc, Grb2 and SOS [21]. Fig. 3 shows that decreasing the rate of feedback SOS phosphorylation, or increasing the rate of subsequent SOS dephosphorylation, and thereby enhancing signalling via the Shc–Grb2–SOS complex, resulted in sustained activation of Ras, MEK and ERK (Table 1). Furthermore, Fig. 4 illustrates that a 40-fold increase in rate of SOS dephosphorylation generated a time course of MAPK cascade activation that is strikingly similar to that observed when PC12 cells are stimulated with NGF, rather than EGF, with the level of RasGTP being sustained at around 8% of total Ras [27], MEK activation maintained at around 50% of the peak value [22] and ERK activation remaining at almost the maximal level for at least 60 min [22,28–30]; none of the other parameters investigated had a comparable effect on the time course of MAPK cascade activation. These results are consistent with experimental data showing that inhibition of the feedback phosphorylation of SOS prolongs Ras activation [33], and imply that some difference in feedback regulation of the signals initiated by EGF and NGF could account for the quan-

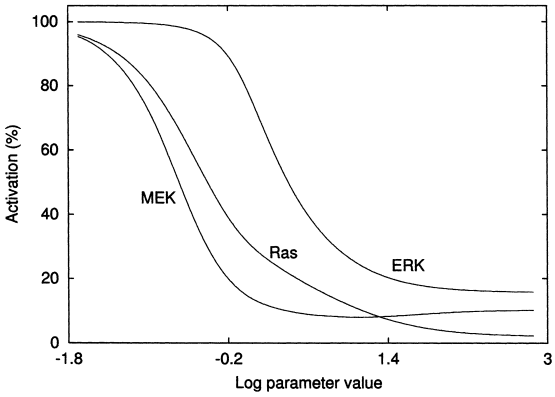

Fig. 5. Sensitivity of the duration of MAPK cascade activation to decreasing GAP activity. The effect of decreasing k, GAP on the activation state of the MAPK cascade after 60 min simulated continuous exposure to EGF. The curves indicate the percentage of Ras in the GTP-bound form, and the percentage of maximal MEK and ERK activation.

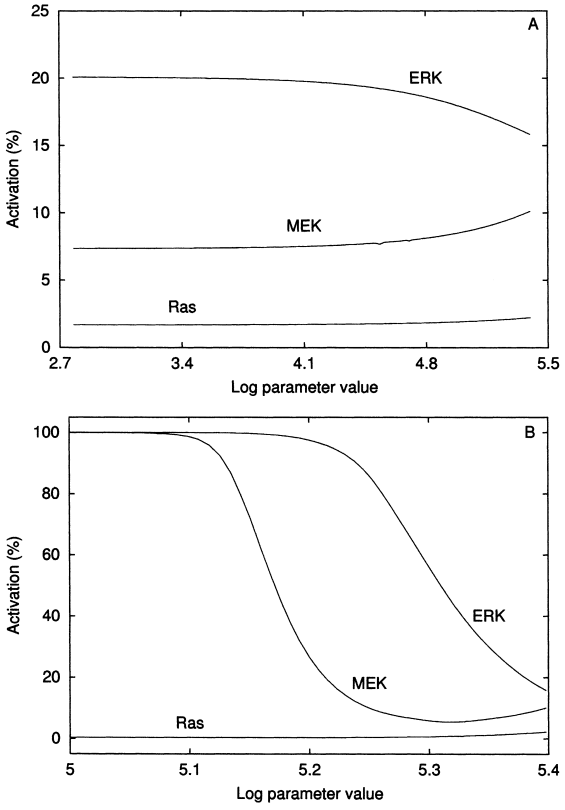

Fig. 6. Sensitivity of the duration of MAPK cascade activation to variations in the values of kinetic parameters governing ERK PTPase and PP2A activity. The effect of (A) decreasing V, ERK PTPase, and (B) decreasing V, PP2A on the activation state of the MAPK cascade after 60 min simulated continuous exposure to EGF. The curves indicate the percentage of Ras in the GTP-bound form, and the percentage of maximal MEK and ERK activation.

titative differences in EGF and NGF signalling in PC12 cells. Furthermore, our results support the hypothesis that NGF, but not EGF, enhances phosphatase activity towards phosphorylated SOS, resulting in sustained signalling through the Shc–Grb2–SOS complex. Although ERK is generally implicated in the feedback phosphorylation of SOS, there is evidence that additional kinases may be involved, such as the downstream target of ERK, p90 Rsk-2 [34]. Moreover, the kinases responsible for catalysing SOS phosphorylation appear to vary with the cell-surface receptor, with the effect on the stability of the signalling complex depending upon the site of phosphorylation [35]. Hence, it is possible that EGF and NGF may induce the phosphorylation of SOS by different kinases, and thereby at distinct phosphorylation sites, resulting in differing effects on complex regulation [34]; in the case of NGF, this might generate a substrate that is more readily dephosphorylated by cellular phosphatases, thus increasing the rate at which functional Shc–Grb2–SOS is reconstituted. Differential regulation of the Grb2–SOS complex by EGF and NGF in PC12 cells has in fact been observed, with EGF stimulating a small transient increase in Grb2–SOS association, and NGF inducing an initial decline in detectable Grb2–SOS complexes, followed by a phase of sustained Grb2–SOS association [36].

Another potential mechanism for regulating the duration and magnitude of MAPK cascade activation would be modulation of the activity of negative regulators of the cascade [5], but this was not supported by our analysis of the influence of the activity of GAP and protein phosphatases on the duration of cascade activation. Reducing the activity of GAP increased the duration of Ras, MEK and ERK activation (Table 1), although in order to sustain MEK and ERK activation at a substantial level it was necessary to virtually eliminate GAP activity, resulting in Ras activation being maintained at a level far in excess of that observed experimentally (Fig. 5). As no differential effect of EGF and NGF on GAP activity in vivo has been described, it therefore seems improbable that modulation of GAP activity represents a physiological mechanism for regulating the duration of MAPK cascade activation. Reducing ERK PTPase activity marginally enhanced ERK activation, although this consequently amplified the feedback inhibition of Ras, and thereby diminished the activation of both Ras and MEK (Table 1; Fig. 6A). Consistent with PP2A being the predominant phosphatase activity acting at all points in the MAPK cascade [20], reducing PP2A activity had a more pronounced positive effect on the duration of both MEK and ERK activation, but again resulted in a reduction in Ras activation (Table 1; Fig. 6B). Thus, in conjunction with conflicting experimental data [30], our analysis indicates that negative modulation of either ERK PTPase or PP2A activity by NGF is also unlikely to play a major role in sustaining MAPK cascade activation.

4. Conclusions

Through a quantitative analysis of a computer simulation of the EGF signal transduction pathway in PC12 cells, we have presented evidence that the characteristic differences in the pattern of EGF- and NGF-induced MAPK cascade activation observed in PC12 cells are unlikely to simply be due to differences in the persistence or intensity of cell-surface receptor signalling, or in the modulation of GAP or MAPK cascade phosphatase activity. Instead, we conclude that some difference in the negative feedback regulation of the cascade is predominantly responsible for determining whether activation is transient or sustained. Although other regulatory mechanisms may be involved, these are likely to play a secondary role. Our results clearly demonstrate that unless feedback inhibition of the signal is counteracted, intensifying the signal at the cell-surface level is likely to be without effect, and enhancing ERK activation alone paradoxically reduces Ras and MEK activation, generating a pattern of activation that is incompatible with experimental observation. Our analysis therefore suggests a model that accounts for the sustained activation of the MAPK cascade in PC12 cells by NGF, but not EGF. Both EGF and NGF initially activate the Ras/Raf/MEK/ERK cascade via formation of the Shc–Grb2–SOS signalling complex. In the case of the EGF, this signal is rapidly terminated through the negative feedback phosphorylation of SOS, resulting in dissociation of the Shc–Grb2–SOS complex, mediated by a kinase downstream of MEK. Similarly, in the case of NGF there is initial down-regulation of the signal through Shc–Grb2–SOS, resulting in a transient decline in the level of MEK and ERK activation by 30 min exposure to growth factor, which has been observed in certain studies [22,29]. However, the rapid reconstitution of functional Grb2–SOS, through enhanced SOS dephosphorylation, facilitates a second sustained phase of MEK and ERK activation, which has again been observed [22,29]. Shc-independent pathways

for Ras activation (not represented in the computer simulation), such as via the association of activated TrkA with the lipid-anchored docking protein, *suc*1-associated neurotrophic factor target/fibroblast growth factor receptor substrate 2 [5], in combination with the adaptors Grb2 and Crk, and guanyl-nucleotide exchange factors, SOS and C3G [37], may also contribute to sustained MAPK cascade activation, since these are presumably not subject to the same negative feedback regulation as signalling through the Shc–Grb2–SOS complex. Finally, although it has been acknowledged that initial activation of the MAPK cascade is mediated by Ras, a parallel pathway involving activation of the Ras-like GTPase, Rap1, via Crk and C3G, has been linked with the sustained phase of ERK activation [38]. Although this Ras-independent pathway may be sufficient to enhance MEK and ERK activation, the evidence presented here implies that this alone would be associated with a decline in the Ras activation level, which is in contrast to experimental observation. Hence, suppression of the feedback inhibition of Ras activation, possibly mediated through enhanced SOS dephosphorylation, is also necessary to account for all the experimentally observed effects of NGF stimulation of PC12 cells on Ras/Raf/MEK/ERK cascade activation.

Although many growth factor signal transduction mechanisms have been characterised qualitatively, there is still a lack of quantitative data. Whilst our conclusions are based largely on a theoretical analysis of a current issue in growth factor signalling, this work has demonstrated that by taking a more quantitative approach to studying the regulation of cellular signalling, it is possible to gain valuable insights into areas that are still not fully understood, and to determine new directions for future experimental work.

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