Down-regulation of the protein kinase A pathway by activators of protein kinase C and intracellular Ca^{2+} in fibroblast cells

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Abstract Many genes are regulated by the intracellular calcium, protein kinase C (PKC) and protein kinase A (PKA) pathways and it has been shown that these pathways synergize in some cell types, whereas they antagonize in others. Here we show that the calcium and PKC pathways suppress the effects mediated by the PKA pathway in a fibroblast cell line. The suppressing effect of elevated intracellular Ca^{2+} levels, but not of the PKC pathway, can be abrogated by the addition of cyclosporin A (CsA), indicating that the effect of Ca^{2+} is mediated by phosphatase-2B (PP-2B/calcineurin). Suppression by the PKC pathway is not mediated by the proto-oncogenes c-*fos*, c-*jun* and *junB*, as the co-transfection of these genes does not block the effects of the PKA stimulator 8-Br-cAMP. In addition, cotransfection with the catalytic subunit of PKA shows that the inhibitory effect of PKC occurs upstream of PKA activation.

Key words: Signal transduction; Gene regulation; Transcription factor; Cross-talk of signalling pathways

1. Introduction

The cell receives many signals from outside through different signalling pathways. Among the most studied pathways are the protein kinase A (PKA), protein kinase C (PKC) and Ca^{2+} pathways. The PKC and Ca^{2+} pathways nearly always synergize, as they are often triggered simultaneously by a membrane-bound receptor, whereas depending on the cell type, they sometimes antagonize or synergize with the PKA pathway [1].

The individual signalling pathways have been studied intensively; however, less work has been devoted to elucidate the mechanisms of the interactions among the different signalling pathways. To study the antagonism of the PKA, PKC and Ca^{2+} pathways in mouse fibroblasts we used defined reporter plasmids containing cAMP responsive elements (CRE) or TPA responsive elements (TRE) in front of the chloramphenicol acetyl transferase (*CAT*) reporter gene. The CREs are targets of the transcription factor CREB [2] which is activated through the PKA and Ca^{2+}/calmodulin pathways, whereas the TREs are targets of the products of the *fos* and *jun* gene families which are activated by the PKC pathway [3] and elevated levels of intracellular Ca^{2+} [4]. The results we obtained with this system show that the PKC and Ca^{2+} pathways dominate over the PKA pathway in mouse fibroblasts when they are stimulated at the same time.

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2. Material and methods

2.1. Cell culture and transient DNA transfection

L4 cells [5] which are derivatives of the mouse fibroblast cell line NIH 3T3 were grown in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% new-born calf serum (NBCS), 2.5 μg/ml amphotericin B, 5 mg/ml gentamicin, and 2 mM glutamine and were transfected by calcium phosphate co-precipitation as described in [6]

2.2. Expression vectors

The expression vector pCEV contains the catalytic subunit (c alpha) of the mouse PKA [7], whose expression is driven by the metal ion-inducible mouse metallothionein promoter. The expression of c-*fos*, c-*jun*, and *junB* was driven from the RSV (Rous sarcoma virus) enhancer of their vectors RSV-c-fos [8], RSV-c-jun [9], and RSV-junB [10]. Expression of the beta-galactosidase reference gene was driven from the CMV (cytomegalovirus) enhancer of the expression vector pCMV-beta-gal.

2.3. CAT assay

Chloramphenicol acetyl transferase (CAT) assays were performed mainly following the protocol of Gorman et al. [11] with the exception that the whole-cell extracts were heated to 65°C for 10 min to destroy deacylases and that the incubation time was extended to 16 h. As reporter genes we used 2×*SOM-CRE-CAT* [12] containing two CREs of the *somatostatin* gene [2] in front of a TATA-box and the *CAT* gene; and 5×*TRE-CAT* [13] in which five TPEs [3] have been inserted in front of a TATA-box and the *CAT* gene. For each experiment three independent transfections were carried out and each whole-cell extract obtained was tested twice for CAT activity. From the resulting six measurements the average value and standard deviation were obtained. The CAT assays were standardized by cotransfecting 0.1 μg of the expression vector pCMV-beta-gal by and measuring the beta-galactosidase activity of whole-cell extracts prepared from one-quarter of the transfected cells.

3. Results

3.1. Stimulation of gene transcription by the PKA, PKC and Ca^{2+} signalling pathways in L4 cells

We first studied the effect of 8-Br-cAMP (1 mM) on the reporter plasmid 2×*SOM-CRE-CAT* [12] in the absence and presence of thapsigargin (10 nM) a mobilizer of intracellular calcium. Fig. 1 shows that 8-Br-cAMP stimulates CAT from the 2×*SOM-CRE-CAT* plasmid approximately 5-fold. This stimulation is totally reversed when 8-Br-cAMP and thapsigargin are given simultaneously. The same effects are observed when instead of the 8-Br-cAMP treatment the catalytic subunit of PKA was co-transfected. The addition of cyclosporin A (CsA, 10 ng/ml), an inhibitor of the calcium-dependent phosphatase PP-2B (calcineurin) [14] totally relieved the suppressing effect of thapsigargin, indicating that the effect of thapsigargin is mediated by this phosphatase. The same effects are observed when instead of 8-Br-cAMP treatment the catalytic subunit of PKA is transfected into L4 fibroblasts, i.e., the approximate 6-fold stimulation by the PKA catalytic subunit is suppressed by thapsigargin and this suppression is relieved

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by CsA. These experiments indicate that the dominance of the Ca^{2+} pathway over the PKA pathway is mediated by PP-2B.

We next tested whether the PKC activator TPA had an effect on 8-Br-cAMP and PKA catalytic subunit-stimulated CAT expression from the 2×*SOM-CRE-CAT* reporter plasmid. Fig. 2 shows that the 8-Br-cAMP-stimulated *CAT* gene expression from this plasmid is totally blocked by TPA. In contrast to thapsigargin this suppression is not relieved by CsA. This result indicates that suppression of the PKA pathway by TPA is not mediated by PP-2B.

To see whether the effect of TPA is mediated by the transcription factor AP-1, we transfected expression vectors for the c-*fos* and c-*jun* gene together with the 2×*SOM-CRE-CAT* reporter plasmid into L4 fibroblast cells and treated them with mM 8-Br-cAMP. The AP-1 transcription factor is a heterodimer of the gene products of members of the *jun* and *fos* gene families and is activated by TPA. AP-1 binds to a DNA sequence which is very similar to that of CREB and it may be possible that the competition for the CREB binding sites on 2×*SOM-CRE-CAT* may lead to the reduction of the *CAT* gene expression we observed with TPA.

Fig. 2 shows that the co-transfection of expression vectors of c-*fos* and c-*jun* which form the most potent AP-1 complex has no effect on 8-Br-cAMP-stimulated gene expression. As gene expression mediated by AP-1 molecules from the CREs of 2×*SOM-CRE-CAT* may be responsible for the increased CAT expression we also transfected the *junB* gene. The JUNB protein is a quite poor transactivator [15] and often even blocks AP-1-mediated transcription [9]. Also this molecule did not block 8-Br-cAMP-mediated stimulation of gene transcription (Fig. 2), indicating that competition for DNA binding sites is not the mechanism that mediates the suppression of the PKA pathway by TPA. Control experiments with 2×*SOM-CRE-CAT* and AP-1 in the absence of 8-Br-cAMP showed that AP-1 alone could not activate *CAT* transcription from this reporter plasmid (data not shown).

We also tested the effect of TPA on the PKA catalytic subunit. Fig. 2 (lower panels) shows that in contrast to thapsigargin TPA cannot block the activity of the PKA catalytic subunit and that this molecule is not the target of TPA-induced suppression of the PKA pathway. TPA therefore must act upstream of PKA activation.

We further tested the influence of 8-Br-cAMP on TPA and thapsigargin-stimulated transcription using the plasmid

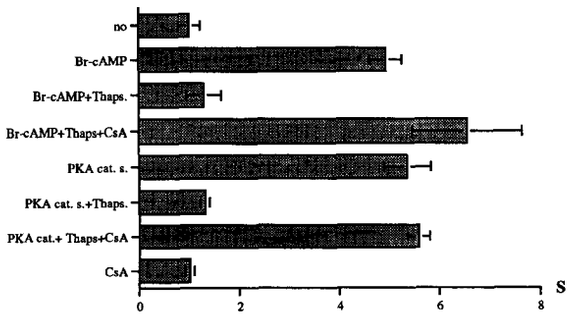

Fig. 1. Suppression of the activation (S) of gene expression by 8-Br-cAMP and the PKA catalytic subunit by the intracellular Ca^{2+} mobilizer thapsigargin in L4 fibroblast cells, using the reporter plasmid 2×*SOM-CRE-CAT*. The inducing and repressing agents are given on the left. CAT activity obtained from untreated cells (no) is set as 1.0. Standard deviation is indicated by error bars.

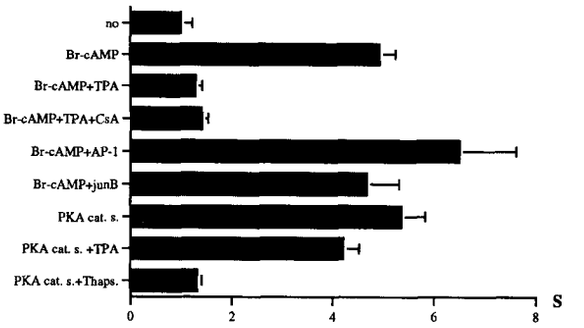

Fig. 2. The effect of TPA, AP-1, junB and CsA on 8-Br-cAMP and PKA catalytic subunit-stimulated gene transcription from the 2×*SOM-CRE-CAT* reporter plasmid. The stimulation factors (S) have been obtained as described in Fig. 1.

5×*TRE-CAT* which contains five AP-1 binding sites (TRE) in front of the *CAT* reporter gene. We found that 8-Br-cAMP cannot suppress the PKC- and Ca^{2+}-dependent signalling pathways (Fig. 3). This result implies that there is no PKA-regulated pathway which is able to block the action of the PKC and Ca^{2+} pathways in L4 cells.

4. Discussion

Our results show that there is in L4 fibroblasts an antagonism between the PKA signalling pathway on the one side and the PKC and Ca^{2+} signalling pathways on the other side. This antagonism is not reciprocal, as the latter two pathways dominate over the PKA pathway. The bias between these pathways is not due to the concentrations of the agents we used, as 1 mM 8-Br-cAMP turned out to be the optimal concentration, and concentrations higher than 1 mM 8-Br-cAMP reduced the cell growth and had a lower stimulating effect on gene transcription.

The dominance of the Ca^{2+} pathway over the PKA pathway is mediated by PP-2B as the PP-2B inhibitor CsA totally blocked the effect of the Ca^{2+} mobilizer thapsigargin. Our experiments also show that the stimulating effect of the PKA catalytic subunit can be blocked by intracellular Ca^{2+}, indicating that the catalytic subunit is one of the targets of the repression of the PKA pathway by high intracellular Ca^{2+} levels. PP-2B itself can dephosphorylate the regulatory subunit of PKA and thus favours the formation of the inactive PKA holoenzyme [16]. Eventually PP-2B does not only exert its effect by dephosphorylation of the catalytic subunit of PKA, but may also act in the same way that has been established for the glycogen metabolism [16,17] where PP-2B also de-represses the activity of the phosphatase PP-1 by inactivation of the PP-1 inhibitor 1 (I-1). PP-1 in turn dephosphorylates and inactivates substrates of PKA such as the transcription factor CREB [18]. A similar mechanism has also been found in dopaminoceptive neurons where the firing rate of the neurons is decreased by dopamine acting through cyclic AMP. This effect is overcome by glutamate acting via Ca^{2+} and this mechanism also involves derepression of PP-1. In this system DARPP 32, a homologue of I-1, is inactivated by PP2B [19,20] leading to de-repression of PP-1 which in turn inactivates substrates of PKA.

In their ability to repress PKA-dependent gene expression by Ca^{2+}, L4 fibroblast cells differ markedly from other cell types [21–23] which activate the PKA pathway by Ca^{2+}

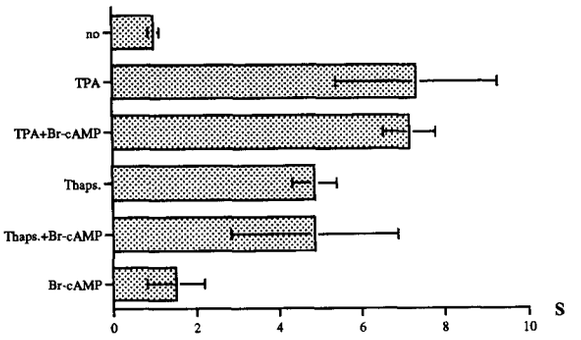

Fig. 3. The influence of 8-Br-cAMP on the TPA- and thapsigargin-induced gene transcription from the reporter plasmid $5\times$*TRE-CAT*. The stimulation factors (S) have been obtained as described in Fig. 1.

through the Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II). We suppose that the PP-2B activity in L4 cells is much higher than the one of CaM kinase II. The different activity of certain protein kinases or phosphatases may explain the phenomenon that different signalling pathways synergize in some cell types, whereas they antagonize in others [1].

PKC activation suppresses the stimulating PKA effect in another way than Ca^{2+}. The fact that CsA does not relieve the suppressing effect of the PKC pathway on the PKA activation by 8-Br-cAMP excludes that PP-2B is involved in suppression of the PKA pathway by TPA. This conclusion is corroborated by the finding that the effect of the PKA catalytic subunit is not suppressed by the PKC pathway.

The suppression of the PKA pathway by PKC activators is also not effected on the level of transcription factors, as neither AP-1 (cJUN/cFOS heterodimer) nor JUNB can suppress the stimulation of gene transcription by the PKA activator 8-Br-cAMP. Therefore it seems that these and PKA-activated transcription factors do not compete for DNA binding sites or co-factors, as it has been shown in other systems [13,24]. This result also shows that AP-1 and JUNB do not activate another activity which blocks the PKA pathway.

In summary our results show that inhibition of the PKA pathway occurs upstream of the PKA catalytic subunit and downstream of adenylate cyclase. Our findings leave only the PKA holoenzyme as a target for the PKC-mediated repression. This assumption is corroborated by the results of Gallo et al. [25] who have shown that TPA blocks the dissociation of the PKA holoenzyme and the translocation of the catalytic subunit into the nucleus.

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