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Abolishment of bradykinin-induced calcium oscillations in *ras*-transformed fibroblasts by the expression of 80 kDa diacylglycerol kinase

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Our previous study showed bradykinin-induced periodic Ca^{2+} changes (Ca^{2+} oscillations) in v-Ki-*ras*-transformed NIH/3T3 (DT) cells in which protein kinase C (PKC) activity is partially down-regulated by a sustained high level of 1,2-diacylglycerol (DAG) [FEBS Lett. (1991) 281, 263–266]. In the present study, DAG kinase with 80 kDa mass (80K DGK) has been successfully transfected in DT cells, which exhibited enhanced cellular DAG kinase activities, decreased cellular DAG contents, and increased PKC activities compared to the control vector-transfected cells. Furthermore, these DGK-transfectants showed strong inhibition in bradykinin-induced Ca^{2+} oscillations. The results suggest that the sustained DAG increase down-regulates the PKC activity, thereby leading to the induction of Ca^{2+} oscillations in DT cells.

Diacylglycerol; Diacylglycerol kinase; Protein kinase C; Ca^{2+} oscillation; *ras*-Transformed NIH/3T3 cell line (DT)

1. INTRODUCTION

sn-1,2-Diacylglycerol (DAG) is known to serve as a lipid second messenger in protein kinase C (PKC)-mediated signal transduction [1]. Sustained increases in DAG have been implicated in the maintenance of the transformed phenotype in several oncogene-transfected fibroblasts [2,3]. The DAG is metabolized by the actions of either DAG lipase or DAG kinase, and the latter producing phosphatidic acid (PA), is considered to play a central role in DAG metabolism [4]. PA, on the other hand, is reported to cause mitogenesis in Swiss 3T3 cells [5].

Recently, we found that bradykinin (BK) elicits the repetitive spikes of intracellular free calcium (Ca^{2+} oscillations) in v-Ki-*ras*-transformed NIH/3T3 (DT cells), and also that activation of PKC inhibits the BK-induced oscillations, suggesting that the oscillations are negatively regulated by PKC [6]. Furthermore, PKC activity in DT cells is postulated to be partially down-regulated by sustained DAG increase [7], which is, at least in part, due to the enhanced breakdown of phosphatidylcholine [8].

In order to investigate the importance of the steady high level of cellular DAG in the signal transduction of mitogens, a DGK isozyme was transfected in DT cells to perturb cellular DAG content and PKC activity. A 14 kDa DAG kinase from *E. coli* [9,10] was transiently expressed in COS cells by Ramer and Bell [11]. In the present study, a mammalian DAG kinase isozyme with an apparent molecular mass of 80 kDa (80K DGK) [12,13] was stably transfected in DT cells, and the BK-induced $[Ca^{2+}]_i$ responses were examined in these cells.

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2. MATERIALS AND METHODS

2.1. *Cell culture*

Parent NIH/3T3 cells and DT cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS [6]. Special care was taken to maintain the cell lines at subconfluent density, and cultures were replaced at one-month intervals from frozen stocks.

2.2. *Plasmid construction and transfection*

A cDNA encoding the complete 80K DGK protein was prepared by ligation of the *Pst*I–*Acc*I fragment of pDGK21 [13] and the *Acc*I–*Eco*RI fragment of λDGK1 [13], and inserted into pSKII(+). The dG-tail part was removed by digestion with *Afl*II, followed by *Eco*RI linker ligation. The 2.5 kb *Eco*RI fragment containing 18 bp of 5′-untranslated region and 289 bp of 3′-untranslated region was inserted into a eukaryotic expression vector pSRα composed of human immunodeficiency virus promoter/enhancer and *neo* gene, and we isolated both pSRα containing sense- and antisense-oriented 80K DGK cDNA. The cDNA (5 μg) was transfected into DT cells (5×10^5 cells) as described [14]. The transfectants were selected with growth medium containing 1 mg/ml of G418. After the selection, 0.5 mg/ml G418 was used.

2.3. *Analyses of RNA and protein*

Total cellular RNA was extracted and separated on an agarose gel containing formaldehyde [15]. The UV-cross-linked membranes were

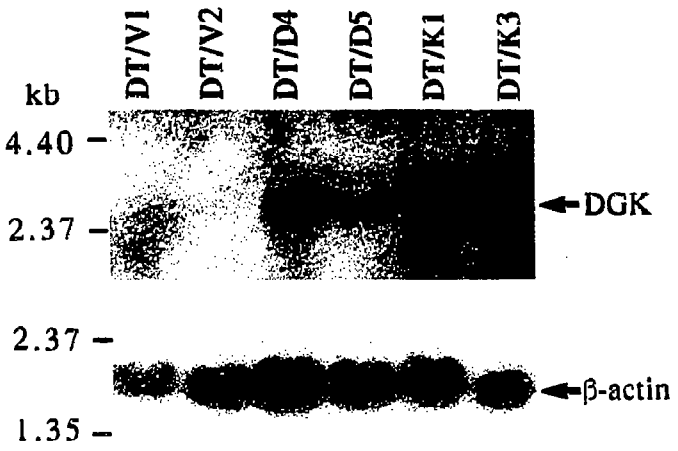

Fig. 1. Northern and immunoblot analyses of 80K DGK. (A) Total RNA (20 μg per lane) was analyzed as described in section 2. (B) Lysates of DT cells transfected with the expression vector (DT/V1 and DT/V2), the sense (DT/V1 and DT/V2) and the antisense (DT/K1 and DT/K3) cDNA of 80K DGK, were analyzed by immunoblotting with antiserum against the 80K DGK as described in section 2.

hybridized with ^{32}P-labeled DNA probes. Probes used were the 2.5 kb *Eco*RI fragment of 80K DGK cDNA and the 1.7 kb *Pst*I fragment of pA1 as a β-actin-specific probe. Cell homogenates were boiled for 3 min in Laemmli's sample buffer [16] and subjected to SDS/10%-polyacrylamide-gel electrophoresis. Proteins transferred to the membranes were immunostained using polyclonal antibody against 80K DGK as described [17] and detected by ECL detective system (Amersham).

2.4. *Assay of DAG kinase and PKC activities*

DAG kinase activities were measured by phosphorylation of 1,2-diacylglycerol with [γ-^{32}P]ATP as described [18]. PKC activities were measured as described [19].

2.5. *Measurement of cellular DAG contents and intracellular calcium concentration*

The cellular DAG mass contents were measured with *E. coli* DAG kinase using a DAG assay kit (Amersham) as described [20]. $[Ca^{2+}]_i$ was measured in fura-2-loaded single cells as described [6].

3. RESULTS AND DISCUSSION

3.1. *Establishment of stably transfected DT cell clones expressing 80K DGK*

DT cells were transfected with 80K DGK cDNA contained in a pSRα eukaryotic expression vector, followed by G418 selection. Among several independently isolated cell clones, we selected DT/D4 and DT/D5 in terms of the mRNA expression of the transfected gene. DT/K1 and DT/K3, the antisense cDNA transfectants, and DT/V1 and DT/V2, the vector transfectants, were also isolated. These selected six clones showed similar cell morphologies. Northern blot analysis with 80K DGK cDNA as a probe revealed 3.3 kb transcript in the sense- and the antisense transfectants as shown in Fig.

Table I

Effects of 80K DGK transfection on enzyme activities[a], DAG contents[a] and Ca^{2+}-oscillations[b]

Cells	DAG kinase activities pmol/mg protein/min (means ± S.E.M.) (n = 3)	DAG contents pmol/10^6 cells (means ± S.E.M.)	PKC activities pmol/mg protein/min (means ± S.E.M.) (n = 4)	Oscillating cells/total (%)	
NIH/3T3	3277 ± 52	191.0 ± 21.6 (n = 6)	883.4 ± 56.2	0/210	(0)
DT	2275 ± 176	333.1 ± 32.0 (n = 6)	388.0 ± 16.2	153/231	(66.2)
		%[c]	%	%	
Control vector					
DT/V1	2607 ± 92	305.4 ± 9.1 (n = 6)	378.3 ± 17.3	81/107	(75.7)
DT/V2	2493 ± 12	316.5 ± 21.8 (n = 4)	N.T.[d]	50/73	(68.5)
Sense		185–237	64–72	215	
DT/D4	5901 ± 530	220.7 ± 6.8 (n = 6)	814.3 ± 17.6	8/74	(10.8)
DT/D5	4825 ± 509	201.4 ± 10.5 (n = 4)	N.T.	16/66	(24.2)
Anti-sense		29–38	176–196	67	
DT/K1	952 ± 57	388.7 ± 7.7 (n = 3)	255.2 ± 14.5	77/85	(90.6)
DT/K3	760 ± 52	394.8 ± 18.6 (n = 4)	N.T.	74/75	(98.7)

[a] Cells were cultured in 10% FCS-containing DMEM for 48 h before harvesting. Activities of DAG kinase and PKC, and the mass contents of DAG, were measured as described in section 2. The data of DAG contents and PKC activities in NIH/3T3 cells and DT cells were taken from [6,8].

[b] Cells loaded with fura-2/AM were stimulated with BK (100 nM) after culturing in 10% FCS-containing DMEM for 48 h. Measurement of $[Ca^{2+}]_i$ in single cells was conducted as described in [6]. The results are obtained from at least three separate experiments.

[c] %, percent of control vector transfectants.

[d] N.T., not tested.

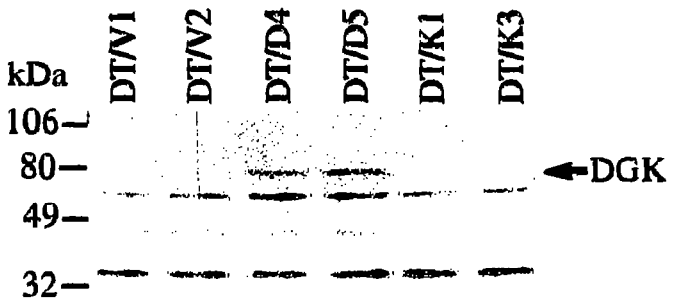

Fig. 2. $[Ca^{2+}]_i$ profile in BK-stimulated transfectants. Cells were cultured in 10% FCS-containing DMEM for 48 h prior to loading with 2.5 μM fura-2/AM for 30 min. Typical traces of single cells stimulated with 100 nM BK in NIH/3T3 cells (a), DT/V1, (b), DT/D4 (c) and DT/K1 (d) cells. Traces shown are representative examples of typical responses of single cells from at least three similar separate experiments.

1A. In contrast, no corresponding mRNA appeared in control DT/V1 and DT/V2, suggesting that mouse endogenous DGK mRNA(s) has low structural similarity to 80K DGK cDNA probe. Another possibility is that the mouse homolog is not expressed in fibroblasts, since expression of 80K DGK in pig is observed only in brain, thymus and spleen [17]. The 80 kDa protein was detected only in DT/D4 and DT/D5 cells by Western blot analysis using a polyclonal antibody against 80K DGK (Fig. 1B). Thus the 80 kDa protein could be produced from the transfected 80K DGK cDNA. Several other protein species were recognized by the antibody, but were not investigated further.

In order to know the effects of the products of transfected DNA, we measured total DAG kinase activities in vitro as shown in Table I. The DAG kinase activities in the vector-transfectants were similar to that in DT cells. In DT/D4 and DT/D5 cells, the DAG kinase activities were increased to approximately 2-fold, indicating the contribution of the transfected 80K DGK cDNA to the total activities. On the contrary, the antisense-transfectants DT/K1 and DT/K3 cells had significantly reduced DAG activities. Although there was undetectable endogenous mRNA with the cDNA probe (Fig. 1A), expression of the antisense decreased total DAG kinase activities. The results suggest that 80K DGK may share some common sequence with the endogenous DAG kinase in mouse fibroblasts. Two major species of DAG kinase of approximately 95 kDa have been reported in NIH/3T3 and the *ras*-transformed derivatives [21]. Thus we could establish the stably transfected DT cell clones expressing 80K DGK.

DT cells contain increased DAG level possibly through action of oncogenic *ras*-gene and the sustained DAG elevation is considered to be associated with the decreased PKC activity in DT cells [7,8]. Using the stable transfectants, we examined the effect of changes of DAG kinase activity on DAG level and also PKC activity. Expression of 80K DGK cDNA in DT cells was found to significantly decrease the DAG mass contents (Table I). On the other hand, the DAG mass contents in antisense-transfectants were slightly increased compared to those in control vector-transfected cells. These results supported the concept that expression of 80K DGK cDNA as well as the antisense can modify the intracellular DAG metabolism. Expression of 80K DGK cDNA in DT cells resulted in the increase of PKC activities which were comparable with those in parent NIH/3T3 cells (Table I). The observed PKC activities were inversely associated with the DAG mass contents, suggesting that intracellular DAG controls PKC activity possibly by inducing the down-regulation of PKC. These results indicate that 80K DGK is physiologically active and modulates the DAG level and PKC activity.

3.2. *Abolishment of BK-induced Ca^{2+} oscillations by 80K DGK expression*

We have recently found that mitogens, such as BK, bombesin and fetal calf serum, induce Ca^{2+} oscillations in *ras*-transformed NIH/3T3 cells including DT cells, but not in untransformed counterparts [6]. The Ca^{2+} oscillations are inhibited by short exposure of DT cells to phorbol ester (PMA) whereas the long exposure to phorbol ester and the treatment with H-7, a PKC inhibitor, accelerate the oscillations. Since 80K DGK and its antisense modulated the DAG content and PKC activity in DT cells, we investigated whether the DAG content primarily affects the BK-induced Ca^{2+} oscillations. As shown in Table I and Fig. 2, Ca^{2+} oscillations induced by BK were dramatically suppressed in DT/D4 and DT/D5 cell lines expressing 80K DGK. The fraction of oscillating cells was only 10.8–24.4%, compared with those in control cells 68.5–75.5%. In the antisense-transfectants DT/K1 and DT/K3 cells, the fraction of oscillating cells was enhanced to 90.6–98.7%. These results indicate that the increased level of DAG is important for the Ca^{2+} oscillations, and the DAG kinase-involved metabolic system could affect the intracellular Ca^{2+} homeostasis via its modulatory effect on PKC activity. Since DAG kinases have been suggested to be involved in growth signal transduction in fibroblasts [22,23] and Ca^{2+} oscillations to be related to the phospholipid metabolism, cell membrane potential changes and transformed phenotype in *ras*-transfected fibroblasts [6,24], our 80K DGK-transfectant may be a good tool for studies in these fields.

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