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Calcium oscillation associated with reduced protein kinase C activities in *ras*-transformed NIH3T3 cells

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We show here novel intracellular Ca^{2+} oscillation in v-K-*ras*-transformed NIH3T3 cells induced by mitogenic peptide hormones, bradykinin and bombesin, as well as fetal calf serum. Induction of the Ca^{2+} oscillation is strongly correlated with the malignant properties and inversely with PKC activities in vitro and in vivo. These results suggest that the mitogen-induced Ca^{2+} oscillation is negatively regulated by PKC, which modulates Ca^{2+} influx in v-K-*ras*-transformed NIH3T3 cells.

Ca^{2+} oscillation; *ras*-Transformed fibroblast; Protein kinase C

1. INTRODUCTION

Earlier studies on the function of v-*ras* oncogene products (v-Ras) in the malignant transformation of fibroblasts suggest that the expression of v-*ras* stimulates phosphatidylinositol and phosphatidylcholine metabolism to elicit sustained increases of 1,2-diacylglycerol [1–5], accompanied by reduced activities of protein kinase C (PKC) [2,3,5]. Although intracellular Ca^{2+} is thought to play an important role in cell proliferation [7–9], involvement of v-Ras in Ca^{2+} responses is poorly understood except that bombesin-induced transient increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]$) is Ras-dependent [10].

The Ca^{2+} oscillation is observed in various cell types [11–14], though its precise molecular mechanism has not yet been disclosed. Oscillation can arise from fluctuations either in the entry of external Ca^{2+} or in the release from internal stores. In addition, the necessity of extracellular Ca^{2+} for the induction and maintenance of Ca^{2+} oscillation and involvement of PKC have been reported [11]. Woods et al. [15] have first indicated the possible involvement of PKC in repetitive $[Ca^{2+}]_i$ transients by showing the decreased frequency, but the effects of PKC appear to depend on the cell type [11].

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Abbreviations: BK, bradykinin; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; EGTA, [ethylenebis(oxyethylene nitrilo)]tetraacetic acid; fura-2/AM, fura-2 acetoxymetyl ester; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; IP_3(1,4,5), inositol 1,4,5-trisphosphate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate

We here demonstrate the agonist-induced Ca^{2+} oscillation in v-K-*ras*-transformed NIH3T3 cells, which was apparently regulated by PKC. The relationship between the Ca^{2+} oscillation, Ras levels and colony formation in soft agar were investigated in a series of NIH3T3 cell clones transfected with v-K-*ras*.

2. MATERIALS AND METHODS

2.1. *Materials*

BK and bombesin were purchased from Sigma (St. Louis, MO). Fura-2/AM and fura-2 from Dojin Laboratories (Kumamoto, Japan). $[\gamma-^{32}P]$ATP and ^{32}P were obtained from Amersham. All other chemicals were obtained from commercial sources and were of the highest quality available.

2.2. *Cell Culture*

Parent NIH3T3 cells and v-K-*ras*-transformed derivatives of NIH3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS). Special care was taken to maintain the cell lines at subconfluent densities at all times.

2.3. *Measurement of* $[Ca^{2+}]_i$

Cells were plated at a density of 2×10^4 cells/chamber on a glass coverslip attached to a Flexiperm-Disc (Heraeus Biotechnology, Hanau, Germany) and precoated with gelatin. After 48 h culture at 37°C, the cells were kept in the serum-free medium for 2 h unless otherwise indicated. The cells were labeled with fura-2/AM (2.5 μM) and fluorescence images were obtained, and conversion from the ratio (340/360 nm) into the absolute value of $[Ca^{2+}]_i$ was made as described [16].

2.4. *PKC activity assay*

The in vitro PKC activities of total homogenate were measured as previously reported [17]. $^{32}P_i$-labeled cells were solubilized for two-dimensional gel electrophoresis as described [2]. Relative $^{32}P_i$-incorporation into the 80 kDa protein was compared with the unstimulated 57 kDa protein, and determined with a BAS3000 bio-image analyzer (Fuji Film, Japan). Western blot analyses of PKC using monoclonal antibodies against PKC subtypes were performed as previously described [18].

3. RESULTS AND DISCUSSION

$[Ca^{2+}]_i$ in normal and v-K-*ras*-transformed NIH3T3 cells labeled with a fluorescence indicator fura-2/AM was traced with a Ca^{2+}-imaging analyzer. As shown in Fig. 1b, bradykinin (BK) did induce Ca^{2+} oscillation following the initial rapid $[Ca^{2+}]_i$ increase in significant proportion (65%) of v-K-*ras*-transformed NIH3T3 cells (DT cells), whereas parent NIH3T3 cells showed a transient $[Ca^{2+}]_i$ increase (Fig. 1a). Another mitogen, bombesin (Fig. 1c), as well as FCS (Fig. 1d) caused fluctuating Ca^{2+} responses to the same proportion of cells as BK stimulation, with some variety in frequency and amplitude. On the contrary, NIH3T3 cells transiently responded to bombesin and FCS, and Ca^{2+} oscillation in the cells was not observed under the same conditions.

Since there was only a transient formation of IP_3 (1,4,5) peaking at 30 s by BK in DT cells (data not shown), a model that oscillation is driven by fluctuating IP_3 (1,4,5) formation [19,20] seems not to be applicable to the current case. In addition, the pretreatment with 10 μM ryanodine for 30 min, which inhibits the Ca^{2+}-induced Ca^{2+} release [13], did not affect the oscillation in DT cells (data not shown). Thus the Ca^{2+}-induced Ca^{2+} release model [11] does not fit the case either.

Furthermore, we examined whether Ca^{2+} influx process was involved in the oscillation of DT cells. The pretreatment of DT cells with 1 mM EGTA for 3 min completely abolished the BK-induced oscillation, and this abolishment was recovered by the addition of excess Ca^{2+} (data not shown). However, the possibility cannot be ruled out that EGTA inhibits some Ca^{2+}-dependent process which is important for the oscillation. Thus a Mn^{2+} quench method was used, by which Mn^{2+} inflow through a divalent cation channel was monitored [22]. BK, in the presence of Mn^{2+}, caused a decrease in the fluorescence intensity of fura- 2-loaded DT cells, whereas no changes were observed in NIH3T3

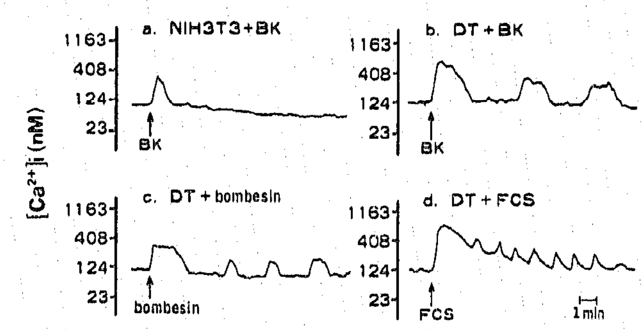

Fig. 1. Changes in $[Ca^{2+}]_i$ in NIH3T3 and DT cells following the addition of mitogens. (a) BK (100 nM) was added to NIH3T3 cells; (b) BK (100 nM) added to DT cells; (c) Bombesin (2.5 nM) added to DT cells; (d) FCS (10%) added to DT cells. Each trace represents the results from 8 separate experiments and more than 200 cells were examined. Approximately 60-65% of the DT cells exhibited Ca^{2+} oscillation in response to these mitogens (b–d).

Table I

Relationship between PKC activity, BK-induced $[Ca^{2+}]_i$ oscillation, Ras level and colony formation in soft agar.

Cell line	PKC activity* (pmol/min/mg protein) (mean ± SD)	BK-induced $[Ca^{2+}]_i$ oscillation	Ras level**	Colony-forming efficiency in soft agar(%) (mean ± SD)
NIH3T3	883.4 ± 112.3	−	+	< 0.1
DT	388.0 ± 33.2	+	+ + + +	75.8 ± 12.6
A1	369.7 ± 38.4	+	+ + + +	47.6 ± 3.6
B1	518.9 ± 22.5	−	+ + + +	5.2 ± 2.2
b6	856.5 ± 57.8	−	+	< 0.1
C11	387.3 ± 76.6	+	+ + + +	< 0.1

* Subconfluent cultures were washed with cold PBS and scraped with a rubber policeman in PBS. The PKC activities were measured in vitro as described in section 2.

** Ras levels and colony-forming efficiencies in soft agar have been shown by Sugimoto et al. [25].

cells (data not shown). These results suggest the role of Ca^{2+} influx in the oscillation in DT cells.

For regulating Ca^{2+} influx, there could be three major mechanisms including voltage-dependent calcium channels, second messenger-mediated channels and the receptor-mediated channels [12]. Although the voltage-gated Ca^{2+} channel (L-type) has been reported to be involved in Ca^{2+} oscillation [23,24], the pretreatment with 5 μM nifidipine for 5 min had no effect on the mitogen-induced oscillation in DT cells (data not shown). Since continuous presence of BK is required for the maintenance of oscillation, the Ca^{2+} influx is likely through mitogen receptor-mediated opening of Ca^{2+} channels rather than through the second messenger-mediated channels.

To test whether the observed Ca^{2+} oscillation is associated with the *ras*-transformed phenotypes, we used NIH3T3 cell clones freshly infected with helper-free Kirsten murine sarcoma virus [25] and a *ras*-revertant C11 clone [26]. The malignant properties of A1, B1, b6 and C11 cells and their Ras protein levels are given in Table I. Fig. 2a shows the BK-induced oscillation in highly malignant A1 cells, while B1 (Fig. 2b) and b6 (Fig. 2c) cells exhibiting lower malignant properties did not show the oscillation. However, the oscillation was detected in the C11 revertant (Fig. 2d). Kamata et al. [2] reported that PKC activities in DT and C11 cells were significantly lower than their parent NIH3T3 cells. Although the effects of PKC are cell type-dependent [11,27], the mitogen-induced Ca^{2+} oscillation in fibroblasts is likely to be inhibited by PKC activation. To examine the involvement of PKC in the oscillation, we pretreated DT cells with phorbol 12-myristate 13-acetate (PMA) and a PKC inhibitor H-7 [28]. The pretreatment with PMA for 5 min shortened the duration of the first $[Ca^{2+}]_i$ peak and completely abolished the subsequent oscillation (Fig. 2e). This treatment also inhibited the Mn^{2+} quenching, further suggesting the

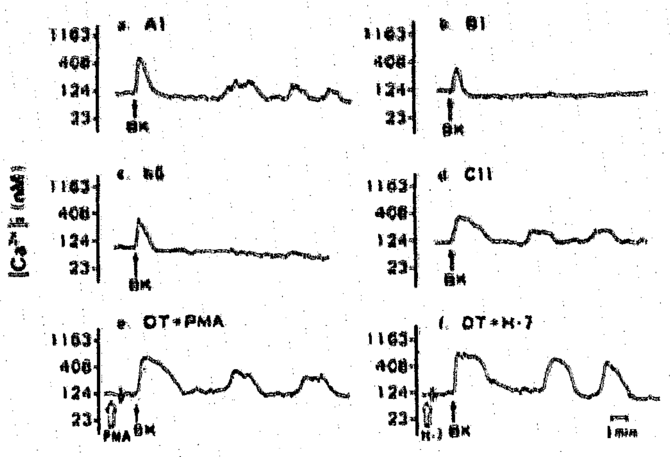

Fig. 2. Effect of BK on $[Ca^{2+}]_i$ response in *ras*-infected NIH3T3 cell lines. BK (100 nM) was added to A1 (a), B1 (b), b6 (c), C11 (d) and DT (e and f). (e) DT cells were pretreated with PMA (100 ng/ml) for 5 min (broken line); DT cells without pretreatment (solid line). (f) DT cells pretreated with H-7 (50 μM) for 30 min. The results shown are typical of at least 6 separate experiments. Approximately 40% and 65% of A1 and C11 cells exhibited Ca^{2+} oscillation, respectively and the pretreatment with H-7 increased the proportion of DT cells exhibiting BK-induced Ca^{2+} oscillation from 65% to 90%.

involvement of Ca^{2+} influx in the oscillation. On the other hand, H-7 markedly prolonged the falling phase of the initial $[Ca^{2+}]_i$ peak induced by BK and most of the responding cells showed an oscillation pattern (Fig. 2f). This prolonged falling phase was also observed in bombesin-stimulated PKC down-regulated Swiss 3T3 cells [29]. The pretreatment with PMA for 24 h induced similar results to those with H-7 (data not shown). These results strongly suggest that inhibition of PKC enhances the Ca^{2+} oscillation. However, the pretreatment of NIH3T3 cells either with H-7 for 30 min or with PMA for 24 h did not cause the BK-triggered oscillation (data not shown).

The in vitro PKC activity in DT cells was significantly lower than that in NIH3T3 cells (Table I). However, relative proportions in membrane and cytosol of PKC activity were almost equal in both DT and NIH3T3 cells. Western blot analysis of PKC with monoclonal antibodies (MC-1a, MC-2a, MC-3a, which selectively interact with type I, II and III of PKC) revealed that the major subtype PKC III was lower in DT cells than the parent cells (data not shown). As shown in Table I, low activities of PKC are in good agreement with induction of the BK-induced Ca^{2+} oscillation. High expression of v-K-*ras* was necessary but not sufficient for induction of the oscillation. Furthermore, the in vivo PKC activity was estimated by monitoring phosphorylation of the 80 kDa protein [30,31] (Fig. 3). Relative incorporation of ^{32}P into the 80 kDa protein in unstimulated DT cells was much lower than that in unstimulated NIH3T3 cells. Although the treatment with PMA for 5 min enhanced the phosphorylation, the level in DT cells was still lower than that in unstimulated NIH3T3 cells.

Lloyd et al. [10] have reported that the expression of

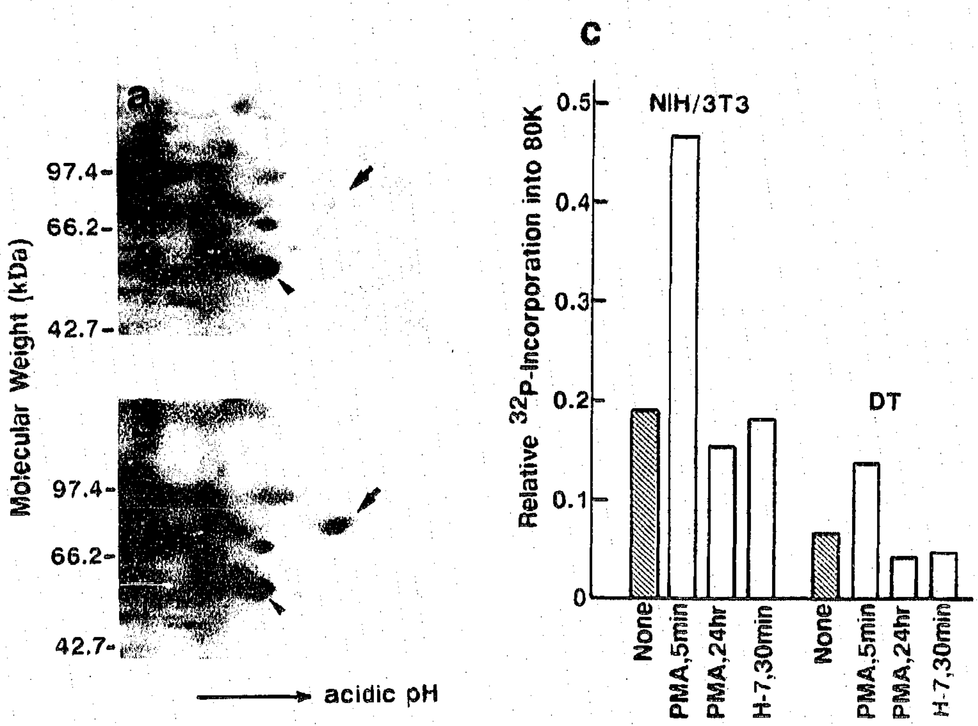

Fig. 3. In vivo assay for PKC activity as determined by ^{32}P-incorporation into the 80 kDa protein. Relative ^{32}P-incorporation into the 80 kDa protein (➡) was compared with the unstimulated 57 kDa protein (▶). (a) ^{32}P-autoradiogram of unstimulated NIH3T3 cells; (b) ^{32}P-autoradiogram of NIH3T3 cells treated with PMA (100 ng/ml) for 5 min. (c) NIH3T3 and DT cells were treated with either PMA (100 ng/ml) for 5 min or 24 h, H-7 (50 μM) for 30 min.

N-*ras* is associated with transient IP_3 (1,4,5) formation induced by bombesin leading to an elevated release of intracellularly stored Ca^{2+}. However, Ca^{2+} oscillation was not observed in their system. A recent report by Polverino et al. [32] demonstrated that the bombesin-induced $[Ca^{2+}]_i$ increase was inhibited in the *ras*-transformed NIH3T3 cells by a mechanism involving PKC activation. Such discrepancies in our observations may indicate the presence of an unknown consequence induced by *ras* expression. For example, our previous experiments [25] suggest that Thy-1 inhibits the colony formation in soft agar of NIH3T3 cells expressing high amounts of v-Ras. Interestingly Thy-1 is not detectable in either DT, A1 or C11 cells exhibiting the mitogen-induced Ca^{2+} oscillation. In NIH3T3, B1 and b6 cells producing Thy-1, on the other hand, the oscillation was not observed. These observations suggest a link between the colony-forming potency in soft agar, induction of the Ca^{2+} oscillation and Thy-1 levels, except for *ras*-revertant C11 cells which retain the ability to grow on media containing low concentrations of serum (1%) as DT cells [26]. It is possible that the Ca^{2+} oscillation is one of the malignant phenotypes of *ras*-transformed NIH3T3 cells.

Since the Ca^{2+} signal is essential for cell proliferation [7–9], repetitive Ca^{2+} oscillation may be advantageous for low concentrations of mitogens to sustain efficient growth signals. This may partly explain the finding that DT cells are able to grow in the presence of low serum. Finally we propose that the reduced PKC activity in *ras*-transformed cell lines is important for malignant growth, and the consequence of the reduced activity could elicit other events which positively affect proliferation.

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REFERENCES

[1] Fleischman, L.E., Chahwara, S.B. and Cantly, L. (1987) Science 213, 407–410.
[2] Kamata, T., Sullivan, N.F. and Wooten, M.W. (1987) Oncogene 1, 37–46.
[3] Wolfman, A. and Macara, I.G. (1987) Nature 325, 359–361.
[4] Lacal, J.C., Moscat, J. and Aaronson, S.A. (1987) Nature 330, 269–272.
[5] Huang, M., Chida, K., Kamata, N., Nose, K., Kato, M., Homma, Y., Takenawa, T. and Kuroki, T. (1988) J. Biol. Chem. 263, 17975–17980.
[6] Price, B.D., Morris, J.D., Marshall, C.J. and Hall, A. (1989) J. Biol. Chem. 264, 16638–16643.
[7] Moolenaar, W.H., Tertoolen, L.G.J. and de Laat, S.W. (1984) J. Biol. Chem. 259, 8066–8069.
[8] Hesketh, T.R., Moore, J.P., Morris, J.D.H., Taylor, M.V., Rogers, J., Smith, G.A. and Metcalfe, J.C. (1985) Nature 313, 481–484.
[9] Morgan, J.I. and Curran, T. (1986) Nature 322, 552–555.
[10] Lloyd, A.C., Davies, S.A., Crossley, I., Whitaker, M., Houslay, M.D., Hall, A., Marshall, C.J. and Wakelam, M.J.O. (1989) Biochem. J. 260, 813–819.
[11] Berridge, M.J. (1990) J. Biol. Chem. 265, 9583–9586.
[12] Rink, T.J. and Hallam, T.J. (1989) Cell Calcium 10, 385–395.
[13] Malgaro, A., Fesce, R. and Meldoles, J. (1990) J. Biol. Chem. 265, 3005–3008.
[14] Richter, J., Olsson, I. and Anderson, T. (1990) J. Biol. Chem. 265, 14358–14363.
[15] Woods, N.M., Cuthbertson, K.S.R. and Cobbold, P.H. (1987) Biochem. J. 246, 619–623.
[16] Fu, T., Okano, Y., Wei, Z., Ozeki, T., Mitsui, Y. and Nozawa, Y. (1990) Biochem. J. 272, 71–77.
[17] Nagao, S., Nagata, K., Kohmura, Y., Ishizuka, T. and Nozawa, Y. (1987) Biochem. Biophys. Res. Commun. 142, 645–653.
[18] Fu, T., Okano, Y., Hagiwara, M., Hidaka, H. and Nozawa, Y. (1989) Biochem. Biophys. Res. Commun. 162, 1279–1286.
[19] Meyer, T. and Stryer, L. (1990) Proc. Natl. Acad. Sci. USA 85, 5051–5055.
[20] Berridge, M.J. (1990) in: The Biology and Medicine of Signal Transduction (Nishizuka, Y. ed.) pp. 108–113, Raven, New York.
[21] Jacob, R., Merritt, J.E., Hallam, T.J. and Rink, T.J. (1988) Nature 335, 40–45.
[22] Hallam, T.J., Jacob, R. and Merritt, J.E. (1988) Biochem. J. 255, 179–184.
[23] Ueda, S., Oiki, S. and Okada, Y. (1986) J. Membr. Biol. 91, 65–72.
[24] Harootunian, A.T., Kao, J.P.Y. and Tsien, R.Y. (1988) Cold Spring Harbor Symp. Quant. Biol. 53, 935–943.
[25] Sugimoto, Y., Ikawa, Y. and Nakauchi, H. (1991) Cancer Res., in press.
[26] Noda, M., Selinger, Z., Scolnick, E.M. and Bassin, R.H. (1983) Proc. Natl. Acad. Sci. USA 80, 5602–5606.
[27] Kikkawa, U., Kishimoto, A. and Nishizuka, Y. (1989) Annu. Rev. Biochem. 58, 31–44.
[28] Hidaka, H., Inagaki, M., Kawamoto, S. and Sasaki, Y. (1984) Biochemistry 23, 5036–5041.
[29] Corps, A.N., Cheek, T.R., Moreton, R.B., Berridge, M.J. and Brown, K.D. (1989) Cell Regul. 1, 75–86.
[30] Rozengurt, E., Rodriguez-Pena, M. and Smith, K.A. (1983) Proc. Natl. Acad. Sci. USA 80, 7244–7248.
[31] Blackshear, P.J., Witters, L.A., Girard, P.R., Kuo, J.F. and Quama, S.N. (1985) J. Biol. Chem. 260, 13304–13315.
[32] Polverino, A.J., Hughes, B.P. and Barritt, G.J. (1990) Biochem. J. 271, 309–315.