Volume 258, number 1, 150–152 FEB 07858 November 1989

Guanine nucleotides modify calcium entry induced by insulin-like growth factor-I

Itaru Kojima, Masafumi Kitaoka and Etsuro Ogata

Cell Biology Research Unit, Fourth Department of Internal Medicine, University of Tokyo School of Medicine, Tokyo 112, Japan

Received 26 September 1989

When calcium influx rate was measured in the cell suspension system instead of confluent monolayer culture, IGF-I (1 nM) augmented calcium entry independent of the cell cycle. In a cell suspension system, NaF increased calcium entry in a dose-dependent manner, which was sensitive to tetramethrin. When GTP-γS was introduced into the cells by employing ATP^{4-}, calcium influx rate was elevated whereas ATP-γS was without effect. In cells loaded with GDP-βS, IGF-I did not stimulate calcium entry. These results suggest that a G-protein is involved in the signal transduction of IGF-I action.

Insulin-like growth factor-I; Calcium; G-protein

1. INTRODUCTION

Insulin-like growth factor-I (IGF-I) is a polypeptide growth factor which promotes cell cycle progression [1–3]. We have recently shown that IGF-I stimulates calcium entry by activating a calcium-permeable cation channel in Balb/c 3T3 cells in IGF-sensitive state [4]. Since blockade of the cation channel results in an inhibition of mitogenic action of IGF-I, we have postulated that calcium influx is an intracellular message of the mitogenic action of IGF-I. Of interest is a fact that pretreatment of the cells with pertussis toxin completely abolishes IGF-I-mediated calcium influx and DNA synthesis [5]. Moreover, pertussis toxin-induced inhibition of the IGF-I action correlates well with the toxin-induced ADP-ribosylation of a 40 kDa protein [5,6], an observation suggesting that a pertussis toxin-sensitive G-protein plays a critical role in signal transduction of the IGF-I action. The present study was conducted to further examine an involvement of G-protein in IGF-I-mediated calcium gating.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Balb/c 3T3 cells kindly provided by Dr C.D. Stiles (Dana-Farbor Cancer Institute; Boston, MA) were cultured in Dulbecco's modified Eagle's medium (DME medium) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Quiescent cells and IGF-sensitive primed competent cells were obtained as described previously [4].

Correspondence address: I. Kojima, Fourth Department of Internal Medicine, University of Tokyo School of Medicine, 3-28-6 Mejirodai, Bunkyo-ku, Tokyo 112, Japan

2.2. *Measurement of calcium influx*

Unidirectional calcium influx rate was determined by measuring an initial uptake of [^{45}Ca] using cell suspension as described elsewhere [7]. Quiescent and primed competent cells were detached by using trypsin and were suspended in DME medium [8]. Protein concentration was measured by the method of Bradford [9]. Statistical significance was analyzed by Student's *t*-test.

2.2. *Loading of guanine nucleotides into cells*

Guanine nucleotides were loaded into the cells by making plasma membrane reversibly permeable using ATP^{4-}. Procedures were essentially similar to the method for the loading of the calcium-sensitive photoprotein aequorin [8]. In short, cells were incubated in the following solutions (in mM) sequentially for 20 min at 4°C: KCl, 120; EGTA, 10; Na_2ATP, 5; $MgCl_2$, 2; Hepes (pH 7.1), 20 (solution I); KCl, 120; EGTA, 0.1; Na_2ATP, 5; $MgCl_2$, 2; Hepes (pH 7.1) 20; and various concentrations of nucleotide as specified in the legends of tables 3 and 4 (solution II). Cells were then incubated for 40 min at 4°C in a solution containing (in mM) KCl, 120; EGTA, 0.1; Na_2ATP, 5; $MgCl_2$, 10; Hepes (pH 7.1), 20. Cells were centrifuged and were then incubated for 60 min at room temperature in a solution containing (in mM) NaCl, 120; KCl, 3.5; $NaHCO_3$, 5; NaH_2PO_4, 1.4; $MgCl_2$, 10; Hepes (pH 7.4), 10; and glucose, 5.5. The loading procedure did not affect the viability of cells as described previously [8]. Metabolism of [^{35}S]GTP-γS was studied as described by [10].

2.3. *Materials*

Partially purified platelet-derived growth factor was obtained by the method of Hasegawa-Sasaki. Epidermal growth factor was obtained from Collaborative Research (Lexington, MA). Synthetic IGF-I was kindly provided by Fujisawa Pharmaceutical. ATP-γS, GTP-γS and GDP-βS were purchased from Sigma (St. Louis, MO). [^{45}Ca] and [^{35}S]GTP-γS were obtained from DuPont New England Nuclear (Boston, MA).

3. RESULTS AND DISCUSSION

We previously reported that IGF-I stimulates calcium influx in primed competent cells but not in quiescent cells [4]. Those studies were carried out using

Published by Elsevier Science Publishers B.V. (Biomedical Division)

Table 1

Effect of IGF-I on calcium influx rate measured in cell suspension

Treatment	Calcium influx rate (nmol/min/mg protein)
Quiescent cells	
None	0.68 ± 0.20
IGF-I	1.32 ± 0.24*
Primed competent cells	
None	0.51 ± 0.28
IGF-I	1.16 ± 0.24*

Quiescent and primed competent cells were detached and were incubated for 10 min in DME medium in the presence and absence of 1 nM IGF-I. Calcium influx rate was measured as described in section 2. Values are the mean ± SE for 4 determinations. * $P < 0.05$ vs none

Table 3

Effect of nucleotides on calcium influx

Treatment	Calcium influx rate (nmol/min/mg protein)
None	0.55 ± 0.18
ATP-γS (200 μM)	0.62 ± 0.26
GTP-γS (20 μM)	0.68 ± 0.20
GTP-γS (200 μM)	1.18 ± 0.14
GTP-γS (200 μM) + tetramethrin (50 nM)	0.70 ± 0.32

Quiescent cells were detached and adenine and guanine nucleotides were loaded into the cells as described in section 2. Nucleotides were included in solution II at the concentrations shown in parentheses. Results are the mean ± SE of 3–4 determinations

cells in confluent monolayer culture. However, when cells were detached, IGF-I-mediated calcium entry was observed independent of cell cycle. As shown in table 1, IGF-I increased calcium influx rate approximately 2-fold in cell suspension obtained from quiescent cells in monolayer culture. Our unpublished observations also indicate that IGF-I augments calcium entry independent of the cell cycle when cells were cultured sparsely. Hence, cell-to-cell contact seems to be necessary for cell cycle-dependent action of IGF-I. The following experiments were done using cell suspension obtained from quiescent cells. To study a possibility of an involvement of G-protein in the transduction system of IGF-I, we first examined the effect of NaF on calcium influx. If a G-protein acts as a regulatory of the IGF-sensitive cation channel, it would be expected that NaF activates the calcium gating system. As expected, NaF increased calcium influx rate in a dose-dependent manner and, at 10 mM, NaF caused an approximately 2-fold stimulation. As in the case for IGF-I, NaF-mediated calcium influx was almost completely blocked by tetramethrin, a compound which inhibits IGF-sensitive cation channels [11] (table 2).

In the following sets of experiments, we attempted to load non-hydrolyzable nucleotides into the cells. To this end, we employed ATP^{4-} to make plasma membrane reversibly permeable. Using this method, we could introduce calcium-sensitive photoprotein aequorin without significant changes in cell functions [8]. More recently, Barrit and colleages loaded guanine nucleotides into hepatocytes by using ATP^{4-} [10]. When ATP-γS was introduced into the cells, calcium influx rate was similar to that in unloaded cells. This observation indicates that calcium permeability of plasma membrane is maintained after the permeabilization by using ATP^{4-}. In contrast, intracellularly applied GTP-γS augmented calcium entry (table 3). Thus, calcium influx rate was 220% in cells loaded with GTP-γS. GTP-γS-mediated calcium influx was inhibited by tetramethrin, suggesting that IGF-sensitive channel is activated. Of note is an effect of GTP-γS in pertussis toxin-treated cells. We have reported that IGF-I does not stimulate calcium influx in the toxin-treated cells [5]. However, GTP-γS increased calcium influx rate approximately 2-fold in the toxin-treated cells. When [^{35}S]GTP-γS was introduced into the cells, approximately 70% of intracellular [^{35}S]GTP-γS remained intact after 60 min incubation at 37°C (data not shown). We also examined the effect of GDP-βS, which inhibits receptor-mediated activation of G protein. As depicted in table 4, GDP-βS did not affect calcium entry in the absence of IGF-I. However, GDP-βS completely abolished IGF-I-mediated stimulation of calcium entry.

Table 2

Effect of NaF on calcium influx rate

Treatment	Calcium influx rate (nmol/min/mg protein)
None	0.68 ± 0.32
NaF (1 mM)	0.56 ± 0.24
NaF (5 mM)	0.84 ± 0.20
NaF (10 mM)	1.29 ± 0.38
NaF (10 mM) + tetramethrin (50 nM)	0.74 ± 0.41

Quiescent cells were detached and were incubated for 10 min in DME medium in the presence and absence of various concentrations of NaF. Calcium influx rate was then measured as described in section 2. Results are the mean ± SE for 3 determinations

Table 4

Effect of GDP-βS on IGF-I-induced calcium influx

Treatment	Calcium influx rate (nmol/min/mg protein)
None	0.49 ± 0.14
IGF-I (1 nM)	0.56 ± 0.22
BAYK8644 (10 μM)	1.42 ± 0.34

Quiescent cells were detached and GDP-βS was loaded into the cells as described in section 2. 200 μM GDP-βS was included in solution II. Loaded cells were then incubated for 10 min with or without either IGF-I or BAYK8644 and calcium influx rate was measured. Results are the mean ± SE for 3 determinations

Likewise, GDP-βS inhibited NaF-induced calcium entry (data not shown). It should be mentioned that BAYK8644 could stimulate calcium entry in GDP-β-S-loaded cells. These results provide evidence for an involvement of a G-protein and activation of the calcium gating system by IGF-I.

Recent studies have shown that a pertussis toxin-sensitive G-protein participates in the signal transduction system of some but not all of the actions of insulin. For example, some of the actions of insulin are blocked by pertussis toxin [12]; insulin alters sensitivity of pertussis toxin-catalyzed ADP-ribosylation of α-subunit of G-protein [13], an observation suggesting that the insulin causes dissociation of α-subunit from $\beta\gamma$-subunits; and insulin phosphorylates G-proteins of different classes [14,15]. Given that the insulin receptor resembles the IGF-I receptor in its structure, it is possible that a certain G-protein also acts as a transducer and participates in the signal transduction system of IGF-I. It remains to be elucidated how binding of a ligand to the IGF-I receptor leads to an activation of the putative G-protein.

Acknowledgements: This study was supported by grants from the Ministry of Education, Science and Culture of Japan, the Ministry of Health and Welfare of Japan and the Workshop on Calcium Signal in the Cardiovascular System. Authors thank Ms Akiko Watanabe for her secretarial assistance.

REFERENCES

[1] Pledger, W.J., Stiles, C.D., Antoniades, H.N. and Scher, C.D. (1977) Proc. Natl. Acad. Sci. USA 74, 4481–7785.
[2] Stiles, C.D., Capone, G.T., Scher, C.D., Antoniades, H.N., Van Wyk, J.J. and Pledger, W.J. (1979) Proc. Natl. Acad. Sci. USA 76, 1279–1283.
[3] Loef, E.B., Wharton, W., Van Wyk, J.J. and Pledger, W.J. (1982) Exp. Cell. Res. 141, 107–115.
[4] Kojima, I., Matsunaga, H., Kurokawa, K., Ogata, E. and Nishimoto, I. (1988) J. Biol. Chem. 263, 16561–16567.
[5] Nishimoto, I., Ogata, E. and Kojima, I. (1987) Biochem. Biophys. Res. Commun. 148, 407–412.
[6] Nishimoto, I., Hata, Y., Ogata, E. and Kojima, I. (1987) J. Biol. Chem. 262, 12120–12126.
[7] Kojima, I., Kojima, K. and Rasmussen, R. (1985) J. Biol. Chem. 260, 9171–9176.
[8] Nishimoto, I., Ohkuni, Y., Ogata, E. and Kojima, I. (1987) Biochem. Biophys. Res. Commun. 142, 275–286.
[9] Bradford, M.M. (1976) Anal. Biochem. 72, 248–253.
[10] Hughes, B.P. and Barritt, G.T. (1989) Biochem. J. 257, 591–598.
[11] Matsunaga, H., Nishimoto, I., Kojima, I., Yamashita, N., Kurokawa, K. and Ogata, E. (1988) Am. J. Physiol. 255, C442–C446.
[12] Luttrell, L.M., Hewlett, E.L., Romeo, G. and Rogol, A.D. (1988) J. Biol. Chem. 263, 6134–6141.
[13] Rothenberg, P.L. and Kahn, C.R. (1988) J. Biol. Chem. 263, 15546–15552.
[14] O'Brien, R.M., Houslay, M.D., Milligan, G. and Siddle, K. (1987) FEBS Lett. 212, 281–288.
[15] Krupinski, J., Rajaram, R., Lakonishok, M., Benovic, J.L. and Cerione, R.A. (1988) J. Biol. Chem. 263, 12333–12341.