Evidence for a Ca^{2+}-independent hydrolysis of phosphatidylinositol 4,5-bisphosphate in neuron-like cell line NG108-15 cells

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The addition of bradykinin to $^{32}P_i$-labeled neuroblastoma × glioma hybrid NG108-15 cells caused a substantial loss of radioactivity from phosphatidylinositol 4,5-bisphosphate (PI-4,5-P_2). The bradykinin-induced hydrolysis of PI-4,5-P_2 was almost equally observed even when extracellular Ca^{2+} was depleted with EGTA (100 μM). On the other hand, high K^+ depolarization of the cells, which allows Ca^{2+} influx through voltage-dependent Ca^{2+} channels, failed to induce any significant decrease in the radioactivity of PI-4,5-P_2. These data indicate that the bradykinin-stimulated PI-4,5-P_2 hydrolysis in NG108-15 cells is independent of extracellular Ca^{2+} and also that PI-4,5-P_2 hydrolysis is not stimulated by an elevation of intracellular Ca^{2+} concentration.

Neuroblastoma × glioma *Bradykinin* *Potassium depolarization* *Phosphoinositide* *Calcium*

1. INTRODUCTION

Neuroblastoma × glioma hybrid NG108-15 cells are unique in that they express neural properties including synaptogenesis in in vitro culture [1], and this cell line has therefore been employed for studying the effects of a variety of agents on the neuronal functions [2–5]. A previous report from our laboratory [6] revealed that bradykinin, which has recently emerged as a potential neurotransmitter in the central nervous system [7], provokes a transient hyperpolarization followed by a prolonged depolarization in the hybrid cells, ultimately resulting in the release of acetylcholine.

In the course of search for biochemical mediator(s) of the physiological responses in the bradykinin-activated NG108-15 cells, we found that bradykinin causes a profound phosphodiesteratic hydrolysis of phosphatidylinositol 4,5-bisphosphate (PI-4,5-P_2) [6,8]. Agonist-induced PI-4,5-P_2 breakdown has been assigned the role of a mobilizer of cellular Ca^{2+} [9,10]. In particular, inositol trisphosphate, one of the two products of phosphodiesteratic hydrolysis of PI-4,5-P_2, has been shown to release non-mitochondrial Ca^{2+} in an increasing number of cell types [11]. This Ca^{2+}-mobilizing action of PI-4,5-P_2 hydrolysis would be pertinent in cell systems where the PI-4,5-P_2 hydrolysis per se is not dependent on Ca^{2+}, and so far the majority of cell systems examined exhibit the Ca^{2+}-independent hydrolysis of PI-4,5-P_2 [10]. However, there is evidence to indicate that agonist-activated phosphoinositide breakdown is Ca^{2+}-dependent in some cell types such as neutrophils [12], pancreatic islets [13] and adrenocortical fasciculata cells [14].

The aim of this study is, therefore, to examine whether the bradykinin stimulation of PI-4,5-P_2 breakdown in NG108-15 cells is dependent on Ca^{2+}. Our results indicate that PI-4,5-P_2 hydrolysis in response to bradykinin occurs in-

Abbreviations: PI-4,5-P_2, phosphatidylinositol 4,5-bisphosphate; TBS, Tris-buffered saline

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dependently of extracellular Ca^{2+}. It is also shown that an increment of intracellular [Ca^{2+}] by virtue of high K^+ depolarization in the hybrid cells does not induce any significant hydrolysis of PI-4,5-P_2.

2. MATERIALS AND METHODS

2.1. *$^{32}P_i$ labeling of NG108-15 cells*

The culture method for NG108-15 cells was essentially the same as in [2,15]. Before starting the culture, the culture dishes (60 mm) were either coated or not coated with polyornithine [16] (see below). The hybrid cells were grown for 4–6 days in Dulbecco's modified Eagle's medium (Gibco) with 5% fetal calf serum (Gibco), in either the presence or absence of 10 μM prostaglandin E_1 (Ono Pharmaceutical, Osaka) and 1 mM theophylline (Sigma) (see below). During this period, cell density reached 1.0–1.5 $\times$ 10^6 cells/dish. The culture medium was then replaced with 2 ml Tris-buffered saline (TBS), which consisted of 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$ and 25 mM glucose, supplemented with 20 μCi of $^{32}P_i$ (HCl and carrier-free, New England Nuclear). The cells were labeled with $^{32}P_i$ for 90 min at 37°C, and then washed 3 times with 2-ml volumes of TBS.

2.2. *Effect of bradykinin on PI-4,5-P_2 hydrolysis in the Ca^{2+}-depleted medium*

Cells were grown for 6 days in culture dishes coated with polyornithine in the absence of prostaglandin E_1 and theophylline. The $^{32}P_i$-labeled cells were bathed in 2 ml of either TBS or Ca^{2+}-depleted TBS which contains 100 μM EGTA without any added Ca^{2+}. Treatment of the culture dishes with polyornithine was to prevent cell detachment from the dishes which is caused by deprivation of extracellular Ca^{2+} [6]. After preincubating the labeled cells at 37°C for 5 min, cell stimulation was initiated by the addition of 20 μl of 1 mM bradykinin (Protein Research Foundation, Osaka) solution. After various periods of time, the reaction was terminated by adding 2 ml chilled methanol.

2.3. *Effect of high K^+ depolarization on PI-4,5-P_2* radioactivity

Cells were cultured for 4–6 days in dishes untreated with polyornithine in the presence of 10 μM prostaglandin E_1 and 1 mM theophylline to convert the cells into a differentiated state [1,2,17], which is represented by the appearance of voltage-dependent Ca^{2+} channel activity [1]. The $^{32}P_i$-labeled cells were preincubated in 1.8 ml TBS at 37°C for 5 min. High K^+ depolarization was induced by the addition of 0.4 ml of an isotonic solution consisting of 20 mM Tris-HCl (pH 7.4), 156 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$ and 25 mM glucose. This procedure increased the extracellular [K^+] from 5.4 to 32.8 mM whereas the extracellular [Na^+] was reduced from 150 to 122.8 mM. After incubation for various periods, 2 ml chilled methanol was added to stop the reaction.

2.4. *Lipid analysis*

Lipid extraction, separation of individual phospholipids and counting of radioactivity in individual phospholipids were all carried out as described [6,18].

3. RESULTS AND DISCUSSION

NG108-15 cells acquire the activity of voltage-sensitive Ca^{2+}-channels when grown in the presence of agents which raise the intracellular cyclic AMP level, thus leading the cells to a differentiated state [1]. In [6] we demonstrated that not only differentiated but also undifferentiated NG108-15 cells respond to bradykinin with a stimulated influx of extracellular Ca^{2+}, and suggested that this cell line may possess an alternate mechanism of Ca^{2+} inflow which appears to be coupled with bradykinin-receptor interaction. It was also shown that the temporal profile of PI-4,5-P_2 degradation induced by bradykinin stimulation is similar to that of the receptor-coupled Ca^{2+} influx. Therefore, this study was designed to examine whether the bradykinin-induced PI-4,5-P_2 degradation is dependent on extracellular Ca^{2+}, and thus on the bradykinin stimulation of the receptor-coupled Ca^{2+} influx. For this purpose, effect of bradykinin on the radioactivity in PI-4,5-P_2 of the $^{32}P_i$-labeled undifferentiated cells was investigated in the Ca^{2+}-containing (1.8 mM) and -depleted media. To prevent cell detachment from culture dishes in the Ca^{2+}-depleted medium, the dishes were coated

with polyornithine before starting cell culture (see section 2). As shown in fig.1, bradykinin provokes a loss of radioactivity from PI-4,5-P_2 by 26 ± 9 and 37 ± 6% at 10 and 30 s, respectively, in the Ca^{2+}-depleted medium. There was no significant difference between the magnitude of the bradykinin-stimulated PI-4,5-P_2 degradation with Ca^{2+} present (table 1) and under Ca^{2+}-depleted conditions. It should be noted, however, that the basal incorporation of $^{32}P_i$ into PI-4,5-P_2 during the preincubation period and the magnitude of loss of radioactivity from PI-4,5-P_2 upon addition of bradykinin were different between cells which had been grown in polyornithine-coated and non-coated dishes, as summarized in table 1. This difference might reflect an alteration in the metabolic state of the cells grown in polyornithine-coated dishes. Nevertheless, the data shown in fig.1 and table 1 indicate that the bradykinin-induced PI-4,5-P_2 degradation in NG108-15 cells is not affected by very low extracellular [Ca^{2+}] which prevents the bradykinin stimulation of receptor-coupled Ca^{2+} influx.

High K^+ depolarization of the differentiated NG108-15 cells is known to induce the inward transport of Ca^{2+} through voltage-dependent Ca^{2+} channels [1]. Thus, we have examined the effect of high K^+-induced depolarization on the level of PI-4,5-P_2 in $^{32}P_i$-labeled NG108-15 cells which had been grown in the presence of 10 μM prostaglandin E_1 and 1 mM theophylline, to assess further the causal relationship between PI-4,5-P_2 degradation and enhanced transmembrane influx of extracellular Ca^{2+}. Under the present experimental conditions, extracellular [K^+] was increased from 5.4 to 32.8 mM upon stimulation. This change of extracellular [K^+] caused membrane depolariza-

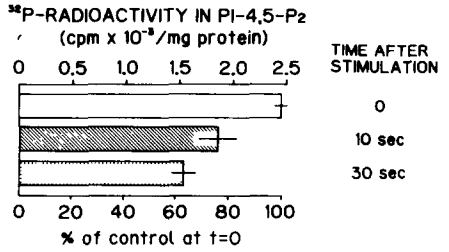

Fig.1. Effect of bradykinin on PI-4,5-P_2 hydrolysis in Ca^{2+}-depleted medium. NG108-15 cells which had been grown for 6 days in polyornithine-coated dishes were prelabeled with 20 μCi $^{32}P_i$ for 90 min at 37°C. After preincubation for 5 min at 37°C, $^{32}P_i$-labeled cells were stimulated with bradykinin at a final concentration of 10 μM for 10 and 30 s in Ca^{2+}-depleted medium containing 100 μM EGTA. Lipid extraction and separation of PI-4,5-P_2 were carried out as described in section 2. Each value represents the mean ± SE of triplicate cultures of a representative from two experiments giving similar results.

Table 1

Difference in $^{32}P_i$-labeling of PI-4,5-P_2 between cells grown in polyornithine-coated and non-coated dishes

Cells grown in	Basal incorporation of $^{32}P_i$ into PI-4,5-P_2 during 90 min preincubation (cpm/mg protein)	Loss of PI-4,5-P_2 radioactivity upon bradykinin addition (% of control)	
		10 s	30 s
Polyornithine-coated dishes[a]	2450 ± 110	31 ± 8	40 ± 5
Non-coated dishes[b]	5510 ± 430	48 ± 5	55 ± 6

[a] Mean ± SE of triplicate cultures of a representative from two experiments
[b] Mean ± SE of two experiments performed in triplicate cultures

NG108-15 cells which had been grown for 6 days in either polyornithine-coated or non-coated dishes were prelabeled with 20 μCi $^{32}P_i$ for 90 min at 37°C. For stimulation with bradykinin, the $^{32}P_i$-labeled cells were incubated with bradykinin at a final concentration of 10 μM for the designated period in Ca^{2+}-containing (1.8 mM) medium. Lipids were extracted and PI-4,5-P_2 separated as described in section 2

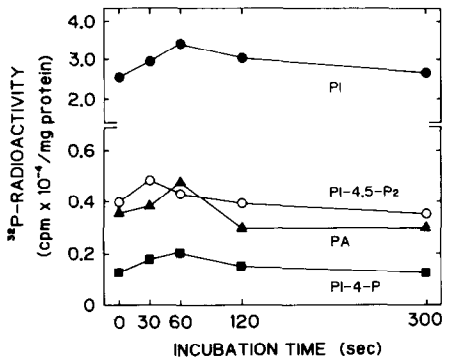

Fig.2. Time course of effect of high K^+ depolarization on radioactivity of $^{32}P_i$-labeled phospholipids from NG108-15 cells. NG108-15 cells which had been grown for 4–6 days in the presence of 10 μM prostaglandin E_1 and 1 mM theophylline were prelabeled with 20 μCi $^{32}P_i$ for 90 min at 37°C. After preincubation of $^{32}P_i$-labeled cells for 5 min at 37°C in 1.8 ml TBS, 0.4 ml of an isotonic solution was added to change the extracellular $[K^+]$ from 5.4 to 32.8 mM. The reaction was then continued for the indicated time. Lipid extraction and separation of phospholipids were performed as described in section 2. Each value is the mean of duplicate cultures of a representative from two experiments giving similar results. PI, phosphatidylinositol; PA, phosphatidic acid; PI-4-P, phosphatidylinositol 4-phosphate.

tion in differentiated NG108-15 cells by approx. 15 mV (not shown). Fig.2 demonstrates that in such a depolarized state, which is sufficient to enhance the Ca^{2+} influx through voltage-dependent Ca^{2+} channels [1,6], no decrease but rather a small increase is seen in the radioactivity of inositol phospholipids. This result is consistent with our finding [6] that the ionophore A23187 failed to provoke PI-4,5-P_2 hydrolysis in NG108-15 cells, indicating that the activation of PI-4,5-P_2 hydrolysis is not due to increased intracellular $[Ca^{2+}]$.

Our data provide evidence that the bradykinin-activated hydrolysis of PI-4,5-P_2 in NG108-15 cells occurs independently of extracellular Ca^{2+} and that the elevation of intracellular $[Ca^{2+}]$ due to high K^+ depolarization does not activate PI-4,5-P_2 hydrolysis. We have shown that the bradykinin-induced PI-4,5-P_2 degradation in NG108-15 cells produces two putative second messengers, inositol trisphosphate and diacylglycerol [8]. In many cell systems, it seems likely that these two messenger substances synergistically activate cells by the mobilization of intracellular Ca^{2+} and the activation of protein kinase C, which are mediated by inositol trisphosphate and diacylglycerol, respectively [10,19]. Furthermore, inositol trisphosphate has been reported to provoke electrophysiological responses in ventral photoreceptors of *Limulus* [20,21] and in *Xenopus* oocytes [22] and to enhance protein phosphorylation in a variety of cells [23,24]. It thus appears that the Ca^{2+}-independent phosphodiesteratic hydrolysis of PI-4,5-P_2 is the initial event which links bradykinin-receptor interaction to the neuronal activities in NG108-15 cells.

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