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Generation of inositol phosphates, cytosolic Ca^{2+}, and secretion of noradrenaline in PC12 cells treated with glutamate

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Glutamate transiently stimulated rat pheochromocytoma PC12 cells and caused an inositol trisphosphate formation and an increase in levels of Ca^{+} in the cytosol. The rank order of potency of glutamate > *N*-methyl-D-aspartate (NMDA) > > kainate = quisqualate is characteristic of an interaction with NMDA receptors. The effect of glutamate on inositol trisphosphate formation disappeared in a low Mg^{2+} buffer and was not blocked by DL-2-amino-5-phosphonovalerate, an antagonist for NMDA receptors coupled to ion channels. Although glutamate failed to stimulate noradrenaline secretion, glutamate enhanced the effect of bradykinin, but not of Ca ionophore A23187, or KCl. These results suggest the existence of metabotropic glutamate receptors, different from previously reported receptors, in PC12 cells.

Glutamate; Phosphoinositide; Cytosolic Ca^{2+}; PC12 cell

1. INTRODUCTION

The excitatory neurotransmitter glutamate activates 3 major receptor subtypes defined by the actions of the selective agonists kainate, quisqualate, and NMDA. The stimulation of glutamate receptors has been implicated in long-term potentiation, developmental structuring, hypoxia damage and kindling (see [1,2] for review). Specific glutamate binding is detected in various central structures supposed to have glutamate neurons, such as the cerebellum, hippocampus, retina, and cerebral cortex. These receptors are directly coupled to cation-specific ion channels, and hence are known as ionotropic receptors. In addition, Sugiyama et al. [3] reported using *Xenopus* oocytes injected with rat brain messenger RNA the existence of 'metabotropic glutamate receptor' that activates GTP-binding proteins and stimulates PIs hydrolysis. Stimulation of PIs hydrolysis by excitatory amino acids has also been observed in many neuronal preparations [4–8]. It has been shown recently that [^{3}H]glutamate binding sites are located in many tissues of the central nervous system [1,2] and in some peripheral excitable tissues, including the adrenal medulla [9]. These findings led us to examine the biochemical effects of glutamate in rat pheochromocytoma PC12 cells.

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Abbreviations: NMDA, *N*-methyl-D-aspartate; PIs, phosphoinositides; NA, noradrenaline; PMA, phorbol 12-myristate 13-acetate; APV, DL-2-amino-5-phosphonovalerate; Fura-2/AM, fura-2/acetoxymethylester; DMEM, Dulbecco's modified Eagle's medium; IP_3, inositol trisphosphate; $[Ca^{2+}]_i$, the cytosolic free Ca^{2+} concentration

2. MATERIALS AND METHODS

2.1. *Materials*

PC12G cells were the gift of Dr Shyoue Furukawa, National Institute of Neuroscience. L-[7,8-^{3}H]NA (34 Ci/mmol) and *myo*-[2-^{3}H]inositol (10–20 Ci/mmol) were purchased from Amersham Corp. Glutamate, carbachol, NMDA, quisqualate, PMA, A23187 and APV were purchased from Sigma. Kainate and Fura-2/AM were purchased from Nakarai Chemicals (Japan) and Dojindo Laboratories (Japan), respectively. Other materials and chemicals were analytical or the highest available grade.

2.2. *Cell culture*

PC12G cells were cultured on collagen-coated dishes in DMEM supplemented with 5% (v/v) fetal bovine serum, 15% (v/v) horse serum, and 100 μg of streptomycin and 100 units of penicillin per ml. They were kept at 37°C in humidified 10% CO_2/90% air. The medium was changed every 48 h until the cells were grown to the subconfluent state. For experiments, subconfluent PC12 cells were further incubated for 24 h in DMEM without serum.

2.3. *PIs hydrolysis*

PC12 cell monolayers were incubated for 24 h with 3.3 μCi/ml of *myo*-[^{3}H]inositol in DMEM without serum. PC12 cells were detached from the collagen-coated dish by gently streaming DMEM over the dish by means of a Pasteur pipette or by incubation with Ca- and Mg-free phosphate-buffered saline. Detached cells were washed twice by centrifugation (200 × *g*, 2 min) at room temperature and resuspended in the Hepes buffer (137 mM NaCl, 5 mM KCl, 5.6 mM glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% bovine serum albumin, 10 mM Hepes (pH 7.4)). Cell suspensions (2×10^7 cells) were incubated at 37°C in the Hepes buffer with the various agents and 10 mM $LiCl_2$ for 1 min (IP_3 formation). The experiments were terminated by the addition of HCl (1 N, final concentration) and the preparations were stored on ice for 30 min. The preparations were centrifuged at 2000 × *g* for 5 min. The supernatant was neutralized to pH 6.2~6.8

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and applied to Dowex AG 1-X8 columns for separation of inositol phosphates as described by Berridge et al. [10].

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

Detached PC12 cells were washed and resuspended in the DMEM. The final concentration was $1\sim5 \times 10^7$ cells/ml. Loading of cells with Fura-2 was carried out by a 10-min incubation with 0.5~1.0 μM Fura-2/AM at 37°C in a water bath. The loaded PC12 cells were then diluted 1:5 with the DMEM and incubated for a further 10 min at 37°C and washed by centrifugation. An aliquot of $1\sim4 \times 10^6$ cells was used for autofluorescence measurements. The suspension was transferred to a fluorometer cuvette housed in a thermostatted holder. Fluorescence readings were taken with a Hitachi F-2000 fluorescent spectrophotometer, at excitation and emission wavelengths of 340/380 and 510 nm, respectively. At the end of each measurement, Fura-2 fluorescence changes were calibrated in terms of $[Ca^{2+}]_i$ as described by two wavelength protocols similar to that reported by Grynkiewicz et al. [11]. The K_d of the Fura-2/Ca^{2+} interaction was taken to be 224 nM.

2.5. *[^{3}H]NA secretion*

PC12 cells on dishes were incubated for 12 h with 0.9 μCi/ml of [^{3}H]NA in DMEM without serum. Detached cells were washed twice at 4°C and resuspended in the ice-cold Ca-free Hepes buffer. The labeled PC12 cells were incubated for 5 min to assay for [^{3}H]NA secretion at 37°C with 1 mM $CaCl_2$ and further additions shown in Table III. The reaction was terminated by addition of 0.5 ml of the ice-cold Ca- and Mg-free Hepes buffer containing 2 mM EGTA and 5 mM EDTA, followed by a 2-min centrifugation at 5000 × *g* at 4°C. The [^{3}H] content of the supernatant was estimated with a liquid scintillation spectrometer.

3. RESULTS

Fig. 1 shows that glutamate and NMDA evoked [^{3}H]IP_3 formation in a dose-dependent manner. During the first 1 min in glutamate-stimulated PC12 cells, IP_3 levels increased rapidly and then decreased. Inositol monophosphate and inositol diphosphate levels increased gradually over the entire 10-min test period, to levels of 140 and 160%, respectively (data not shown). Quisqualate had no effect even when using 100 μM. Kainate inhibited IP_3 formation slightly. Glutamate-stimulated IP_3 formation disappeared when assayed in the presence of 0.1 mM $MgCl_2$ (low Mg concentration), and was dependent on extracellular 1.0 mM $MgCl_2$ (Table I). In a low Mg-solution, carbachol-stimulated IP_3 was also reduced. Table II shows that glutamate-stimulated IP_3 formation was not inhibited, but slightly enhanced, by addition of APV, an antagonist for NMDA receptors coupled to ion channels. The NMDA-stimulated (data not shown) and non-stimulated IP_3 formation were not inhibited by APV.

In Fig. 2, we show the transients in $[Ca^{2+}]_i$ concentration that resulted from glutamate stimulation of PC12 cells in the presence of extracellular $CaCl_2$. Addition of glutamate to PC12 cells loaded with Fura-2/AM increased $[Ca^{2+}]_i$ without any measurable delay (Fig. 2A). In 3 independent experiments, peak levels of $[Ca^{2+}]_i$ were detected 10 ± 2 s after the addition of 100 μM glutamate. The responses to 100 μM NMDA, 500 μM carbachol, and 50 mM KCl are also shown in Fig. 2. The response to NMDA was small and slow in comparison with other stimulants. Table III shows the

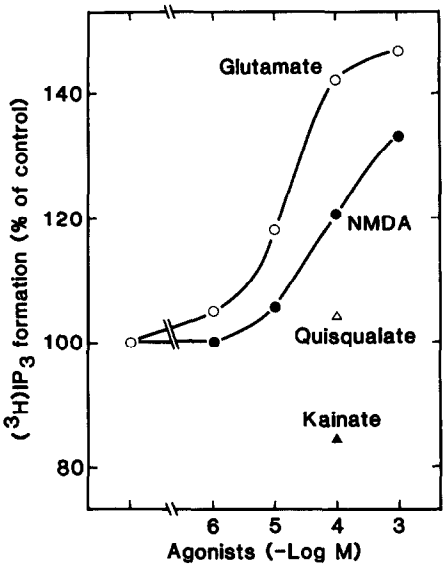

Fig. 1. Glutamate- and NMDA-stimulated IP_3 formation. The [^{3}H]inositol labeled PC12 cells were incubated with stimulants for 1 min to measure IP_3 formation. The data are the means of 3 determinations in one typical experiment. Experiments were replicated two or three times with similar results.

Table I

Effect of $MgCl_2$ on glutamate- and carbachol-stimulated IP_3 formation

Additions	[^{3}H]IP_3 formation (%)	
	0.1 mM $MgCl_2$	1.0 mM $MgCl_2$
None	100	100
100 μM glutamate	92.6 ± 4.8	134 ± 9[a]
500 μM carbachol	137 ± 6[a]	167 ± 6[a]

[a] Effects of stimulants were significant ($P < 0.01$)

The labeled PC12 cells were suspended with the Mg-free Hepes buffer and incubated with various agents in the presence of 0.1 mM or 1.0 mM $MgCl_2$. [^{3}H]IP_3 formation is plotted as percentage of the control values obtained without stimulant, which were 3215 (in 0.1 mM $MgCl_2$) and 3026 (in 1.0 mM $MgCl_2$) dpm/tube in a typical experiment, respectively. The data are the means ± SE from 3 separate experiments.

Table II

Effect of APV on glutamate-stimulated IP_3 formation

Additions	[^{3}H]IP_3 formation (%)
None	100
+ 100 μM APV	102
100 μM glutamate	136
+ 10 μM APV	137
+ 100 μM APV	145
+ 300 μM APV	138

The labeled PC12 cells were first incubated with the indicated concentrations of APV for 10 min and then incubated with or without 100 μM glutamate for 1 min to measure IP_3 formation. The assay medium was further supplemented with 1 mM $MgCl_2$. [^{3}H]IP_3 formation is plotted as percentage of the control value obtained without additions, which was 3146 dpm/tube in a typical experiment. The data are means of 3 determinations in a typical experiment. Experiments were replicated twice with similar results.

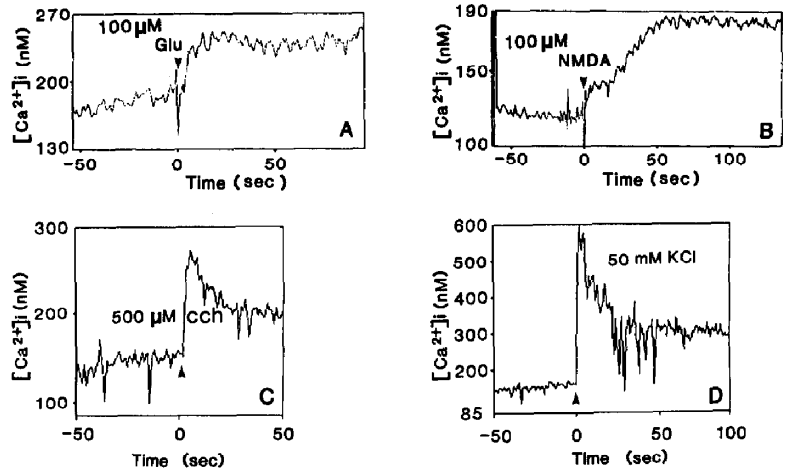

Fig. 2. Fura-2 fluorescence responses with glutamate, NMDA. Fura-2/AM loaded PC12 cells were stimulated with 100 μM glutamate (A), 100 μM NMDA (B), 500 μM carbachol (C) or 50 mM KCl (D), as indicated. The appropriate $[Ca^{2+}]_i$ calibration scale is shown at the left. Glu, glutamate; cch, carbachol.

maximal increases of $[Ca^{2+}]_i$ in response to various stimulants. The peak level of $[Ca^{2+}]_i$ after receptor stimulants such as glutamate, NMDA, carbachol, and bradykinin was lower than that after 50 mM KCl addition. One hundred μM kainate and 100 μM quisqualate had no effect on $[Ca^{2+}]_i$. We could not obtain definitive information about the role of extracellular Ca^{2+} because the magnitudes of both responses (IP_3 formation and increase of $[Ca^{2+}]_i$) to glutamate were small.

Table III

Increase of $[Ca^{2+}]_i$ by stimulants

Additions	Increase of $[Ca^{2+}]_i$ (nM)
100 μM glutamate	102 ± 31 (3)
100 μM NMDA	75.5 (2)
100 μM kainate	21 ± 18 (3)
100 μM quisqualate	24 ± 14 (3)
500 μM carbachol	109 ± 6 (5)
30 μM bradykinin	264 ± 6 (3)
50 mM KCl	313 ± 26 (5)

Data are expressed as the increment of $[Ca^{2+}]_i$. The basal (non-stimulated) value of $[Ca^{2+}]_i$ was 80 ± 25 nM. The data are the mean or the means ± SE from 2–5 separate experiments shown in parentheses.

Table IV

Glutamate- and NMDA-induced NA secretion from bradykinin-stimulated PC12 cells

Additions	$[^3H]$NA secretion (%)		
	None	100 μM glutamate	100 μM NMDA
None	100 (7.02)	100 ± 2	100 ± 1
30 μM bradykinin	100 (6.77)	126 ± 3[a]	119 ± 2[a]
10 μM A23187	100 (9.90)	106 ± 4	108 ± 5
0.1 μM PMA	100 (7.26)	112 ± 6	n.d.
30 mM KCl	100 (13.8)	102 ± 5	89 ± 7

[a] Effect of glutamate or NMDA was significant ($P < 0.01$)

The $[^3H]$NA loaded PC12 cells were incubated for 5 min with various agents. The data, expressed as percentages of the secretion in stimulated cells obtained with bradykinin, A23187, PMA, or KCl in the absence of glutamate and NMDA, are the means ± SE from 3 separate experiments. The data in parentheses are expressed as percentages of total incorporated amount in a typical experiment. n.d., not determined.

The activation of excitatory amino acid receptors often has been described to induce the release of neurotransmitter acetylcholine, γ-aminobutyric acid and NA from various preparations of brain and neurons [1,2,12]. We examined the effects of glutamate and NMDA on NA secretion from PC12 cells. PC12 cells were preincubated with $[^3H]$NA for 12 h and assayed for their ability to secrete $[^3H]$NA in response to stimulants. Under the conditions used (detached PC12 cells) 5–7% of the total $[^3H]$NA radioactivity present in the cells was secreted during the 5-min test period by unstimulated cells. A23187 and KCl stimulated NA secretion significantly (Table IV). These secretions were dependent on extracellular Ca^{2+}, in agreement with the report by Inoue and Kenimer [13]. PMA by itself stimulated NA secretion, but not significantly, and enhanced the effect of A23187 remarkably (17.4%). Bradykinin, glutamate, and NMDA had no effect by themselves. Glutamate, however, significantly increased NA release when used together with bradykinin, but not with A23187, PMA or KCl. NMDA also enhanced bradykinin-stimulated NA secretion.

4. DISCUSSION

It has been reported that excitatory amino acids influence neuronal excitation through the gating of receptor-operated ion channels [1,2]. In addition, there is a large literature on the effect of excitatory amino acids on second messenger systems. Stimulation of PIs hydrolysis and $[Ca^{2+}]_i$ mobilization by excitatory

amino acids, in particular quisqualate, has been observed in many tissues [5–8,14–16]. Neither NMDA nor kainate mobilized intracellular Ca^{2+} and both are inactive to PIs hydrolysis [4,7]. In cerebellar granule cells, omission of Mg^{2+} from the incubation buffer resulted in a 6~8-fold increase in PI hydrolysis by endogenous excitatory amino acids [17]. However, PIs hydrolysis by glutamate in PC12 cells is different from previously reported results in several points. First, glutamate and NMDA, but not quisqualate or kainate, stimulate IP_3 formation and increase $[Ca^{2+}]_i$ in PC12 cells. Second, low Mg^{2+} concentration diminished the PIs hydrolysis by glutamate and by carbachol in PC12 cells. Third, glutamate- and NMDA- (data not shown) -stimulated IP_3 formation in PC12 cells was not inhibited by addition of APV, ordinarily an antagonist for NMDA-type receptor (Table II). These data suggest that the metabotropic glutamate receptor in PC12 cells belongs to a receptor category that is apparently different from that of previously reported subtypes. The possibility that the glutamate receptor coupling to the effector system is via a GTP-binding protein, which was observed in *Xenopus* oocytes injected with rat brain messenger RNA [3], is now under study.

A cloned rat pheochromocytoma cell line, PC12, expresses many of the characteristics of adrenal chromaffin cells, including catecholamine synthetic enzymes. In our experiments, PC12 cells secrete NA in a Ca^{2+}-dependent manner in response to K^+-induced depolarization and to A23187, as previously described [18–20]. It is reported that protein kinase C appears to be involved in the regulation of NA secretion from permeabilized PC12 cells [19]. Our results that PMA potentiated the A23187-stimulated reaction and previous report [21] indicate that both Ca^{2+} and a protein kinase C regulate NA secretion from PC12 cells. It is well known that the hydrolysis of PIs accumulates two classes of second messengers, IP_3, which mediates the release of Ca^{2+} from intracellular stores, and diacylglycerol, which activates protein kinase C [22,23]. Both bradykinin (data not shown, but see [24]) and glutamate stimulated IP_3 formation derived from PIs hydrolysis and $[Ca^{2+}]_i$ increase (Table II). Appell and Barefoot [25] reported that bradykinin stimulated neurotransmitter dopamine secretion from PC12 cells attached to dishes. Bradykinin and glutamate, however, did not stimulate NA secretion from the dispersed PC12 cells when used alone (Table IV). Glutamate stimulated NA secretion when used together with bradykinin, but not with A23187 or PMA. The effect of bradykinin on increase of $[Ca^{2+}]_i$ was enhanced by addition of glutamate from 264 ± 6 nM to about 320 nM. But NA secretion by glutamate plus bradykinin was smaller than that by 30 mM KCl. Glutamate did not enhance the effect of carbachol (data not shown). The precise mechanism of glutamate to enhance bradykinin-stimulated NA secretion is unknown. Takashima and Kenimer [20] reported that receptor-stimulated NA secretion and PIs hydrolysis in PC12 cells are independent events. Recently, Wagner and Vu [26] reported the involvement of a putative Ca^{2+}-dependent kinase in NA secretion from PC12 cells, which does not appear to be protein kinase C. Glutamate may stimulate this unknown kinase. In summary, we proposed the existence of a new subtype of glutamate receptor which couples PIs hydrolysis and $[Ca^{2+}]_i$ increase and stimulates NA secretion in PC12 cells.

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