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Excitatory amino acid-induced slow biphasic responses of free intracellular calcium in human neuroblastoma cells

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Effects of an excitatory amino acid, glutamate, and of ionotropic and metabotropic glutamate receptor agonists on the levels of free intracellular calcium, and their specific receptor binding in human SH-SY5Y neuroblastoma cells were studied. The calcium response was always biphasic, except for AMPA, suggesting both stimulatory and inhibitory effects on free intracellular calcium upon glutamate receptor stimulation, both with ionotropic and metabotropic glutamate receptor agonists. Specific binding of glutamate and other glutamate receptor agonists, together with the biphasic calcium response, suggests that human SH-SY5Y neuroblastoma cells express both ionotropic and metabotropic glutamate receptors. These findings shed new light on the use of human SH-SY5Y neuroblastoma cells as a human neuronal tumor cell model.

Excitatory amino acid; Intracellular calcium; Glutamate receptor; Human neuroblastoma cell

1. INTRODUCTION

The excitatory amino acid, glutamate, causes the entry of calcium into neuronal cells through ionotropic receptor-gated calcium channels [1], and a release of calcium from intracellular nonmitochondrial calcium stores through actions on a metabotropic receptor. Glutamate metabotropic receptor stimulates phospholipase C [2], and increases the formation of two second messengers, inositol-1,4,5-trisphosphate and diacylglycerol [3,4]. Inositol-1,4,5-trisphosphate elevates free intracellular calcium which regulates a number of cellular signal transduction processes, including the activation of protein kinase C and calcium/calmodulin-dependent protein kinase. Calcium plays a critical role in the toxic cell killing and programmed cell death [5,6].

The endogenous excitatory amino acid, glutamate, activates at least three distinct ionotropic receptors in neuronal cells: the *N*-methyl-D-aspartate (NMDA), the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and the kainate receptor subtypes [1]. In addition, it also activates a metabotropic receptor that is coupled to G protein(s) and linked to inositol phospholipid metabolism [2,7–9]. A selective agonist for the metabotropic glutamate receptor is (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (1*S*,3*R*-ACPD).

The effects of glutamate, and of ionotropic and metabotropic glutamate receptor agonists, on the levels of free intracellular calcium, and their specific receptor binding, were used to assess the expression of glutamate receptors in human SH-SY5Y neuroblastoma cells.

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Abbreviations: NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; 1*S*,3*R*-ACPD, (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid; $[Ca^{2+}]_i$, free intracellular calcium.

2. MATERIALS AND METHODS

2.1. *Reagents*

L-[G-^{3}H]Glutamic acid (54 Ci/mmol) and Aqueous Counting Scintillant were obtained from Amersham (Arlington Hts., IL, USA). Fura-2/AM was obtained from Molecular Probes Inc. (Eugene, OR, USA). 1*S*,3*R*-ACPD was from Tocris Neuramin (Bristol, UK). Dulbecco's modified Eagle medium (DMEM), foetal calf serum (FCS), penicillin–streptomycin solution and Hanks' balanced salt solution (HBSS; Ca^{2+}- and Mg^{2+}-free) were obtained from Gibco (Paisley, UK). All the other chemicals were obtained from Sigma Co. (St. Louis, MO, USA).

2.2. *Cell culture*

The human neuroblastoma SH-SY5Y cell line [10] was obtained through Dr. S. Påhlman (Department of Pathology, University of Uppsala, Sweden). The cells were cultured in Dulbecco's modified Eagle medium supplemented with 7% inactivated foetal calf serum and 50 U/ml penicillin and 50 μg/ml streptomycin, and incubated at 37°C in 5% CO_2 in an air ventilated humidified incubator. The cells used in the experiments were cultured to confluency and harvested by 0.02% EDTA in phosphate-buffered saline. The cells were grown in 75 cm^2 cell culture flasks purchased from Costar (Cambridge, MA, USA).

2.3. *Measurement of free intracellular calcium ($[Ca^{2+}]_i$)*

The levels of $[Ca^{2+}]_i$ were measured using the acetoxymethyl ester of the fluorescent calcium probe, fura-2 (fura-2/AM). Aliquots (1 ml) of the cells were loaded with 3 μM fura-2/AM with a constant agitation for 45 min at 37°C. After loading the cells were washed twice with ice-cold HBSS and resuspended in HBSS (without Ca^{2+} and Mg^{2+}, including 1 mg/ml bovine serum albumin and 10 mM D-glucose) to give a final cell concentration of 3×10^6 cell/ml. One ml of the cell

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suspension and 1 ml prewarmed HBSS were placed in a quartz cuvette, in which the final cell concentration was 1.5×10^6 cells/ml. Free intracellular calcium was measured using a Hitachi F-4000 spectrofluorometer equipped with a thermostated cuvette holder and a magnetic stirrer applying the method described by Tsien et al., 1985 [11]. Free intracellular calcium was calculated using the equation.

$$[Ca^{2+}]_i = K_d (F - F_{min})/(F_{max} - F),$$

where K_d = 224 nM, F = the relative level of intracellular fluorescence, F_{min} = the relative level of fura-2 fluorescence in 7.5 mM EGTA after cell lysis with 10% sodium dodecyl sulphate, and F_{max} = the relative level of fluorescence with 7 mM calcium added after the determination of F_{min}. Before the measurements, $CaCl_2$ was added directly into the cuvette to give the final concentration of calcium of 1.2 mM in the medium. Twenty μM of $MnCl_2$ was used to quench the extracellular fura-2/AM.

2.4. *Receptor binding assay*

Specific displacement of L-[^{3}H]glutamate by glutamate or specific ionotropic or metabotropic glutamate receptor agonists was measured using intact SH-SY5Y neuroblastoma cells. All incubations were done in Tris buffer (50 mM Tris, pH 7.4 adjusted with acetic acid) to avoid Na^+ and Cl^- ions which may interfere with the binding of the agonists with the glutamate receptors [12]. The cells were simultaneously incubated with 10 nM L-[^{3}H]glutamate, and with 10 nM – 1 mM of unlabelled agonist for 15 min at 4°C. Incubation volume was 250 μl (100–200 μg protein/tube). After that, the cells were centrifuged, and the cell pellets were washed three times with ice-cold Tris-buffer. The washed pellets were agitated for 24 h in liquid scintillant, and thereafter the samples were subjected to liquid scintillation counting (LKB Wallac, Turku, Finland).

2.5. *Statistics*

The results were analyzed for statistical significance with one-way analysis of variance and with Duncan's multiple-range test. $P < 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

Functional or morphological expression of glutamate receptors in human SH-SY5Y neuroblastoma cells has not been reported earlier. We report here that both glutamate and selective ionotropic and metabotropic glutamate receptor agonists induce a slow biphasic response in free intracellular calcium ($[Ca^{2+}]_i$) in human SH-SY5Y neuroblastoma cells. A rapid initial increase is followed by a sustained decrease in cell calcium, suggesting both stimulatory and inhibitory effects on $[Ca^{2+}]_i$ upon ionotropic and metabotropic glutamate receptor stimulation. Specific binding of glutamate and other glutamate receptor agonists, together with glutamate receptor agonist-induced biphasic calcium response, suggests that human SH-SY5Y neuroblastoma cells express both ionotropic and metabotropic glutamate receptors.

High concentrations (500 μM–3 mM) of glutamate and of specific ionotropic glutamate receptor agonists induced a rapid initial increase in $[Ca^{2+}]_i$ which was followed by a sustained decrease. Low concentrations (< 500 μM) of glutamate receptor agonists caused only a sustained decrease in $[Ca^{2+}]_i$ (for individual tracings, see Fig. 1). The increase in $[Ca^{2+}]_i$ (Fig. 2) was dose-

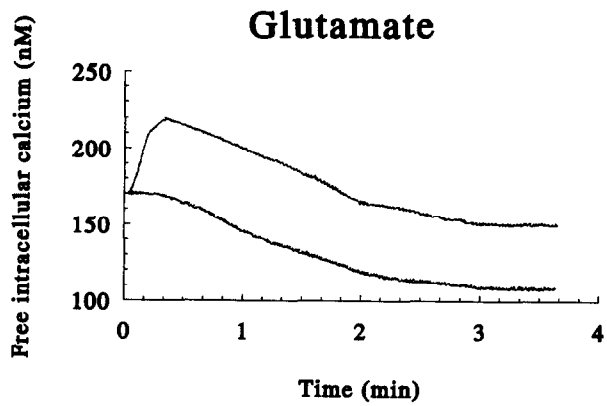

Fig. 1. Representative tracings of glutamate-induced slow oscillations of free intracellular calcium in SH-SY5Y neuroblastoma cells. Tracings of an increase (3 mM; upper tracing) and a decrease (300 μM; lower tracing) in free intracellular calcium for glutamate are shown. The tracings for other agonists closely resembled those of glutamate.

dependent after glutamate, NMDA, and kainate at a concentration range from 500 μM to 3 mM. On the contrary, AMPA did not cause a significant increase in $[Ca^{2+}]_i$. This is not, however, surprising because AMPA is known to affect mainly sodium fluxes through an action on its receptor [1]. The metabotropic glutamate receptor agonist 1*S*,3*R*-ACPD was effective only at a 3 mM concentration (Fig. 2). The time to reach the maximum of $[Ca^{2+}]_i$ decreased dose-dependently by glutamate and kainate. After glutamate, the time of the maximum in $[Ca^{2+}]_i$ decreased from 29 (500 μM) to 19 s (3 mM), whereas after kainate it decreased from 6 (1 mM) to 3 s (3 mM). On the contrary, AMPA prolonged the time for the maximum in $[Ca^{2+}]_i$ from 19 (300 μM) to 64 s (3 mM). NMDA did not have a consistent effect on the calcium peaking time which varied between 12 and 17 s.

The differences between the effects of glutamate, NMDA, kainate and AMPA on $[Ca^{2+}]_i$ are probably partially due to conformational changes that take place in receptor-gated calcium channels. The increase of $[Ca^{2+}]_i$ by 1*S*,3*R*-ACPD occurred only at a high concentration (3 mM) at 17 s. It is, however, in agreement with the time for the onset of an increase in $[Ca^{2+}]_i$ induced by other agonists coupled to calcium-mobilizing receptors [13–15]. These data suggest that ionotropic rather than metabotropic glutamate receptors predominate the temporal pattern [16] of calcium increases upon stimulation of human SH-SY5Y neuroblastoma cells by glutamate or specific ionotropic glutamate receptor agonists. In addition to the opening of receptor-gated calcium channels [17], and intracellular release of calcium [4], secondary depolarization of neuronal membrane, and subsequent opening of voltage-dependent [18,19] calcium channels may contribute to the elevation of $[Ca^{2+}]_i$. Here we report an integrated calcium response to glutamate receptor agonists, which does not exclude,

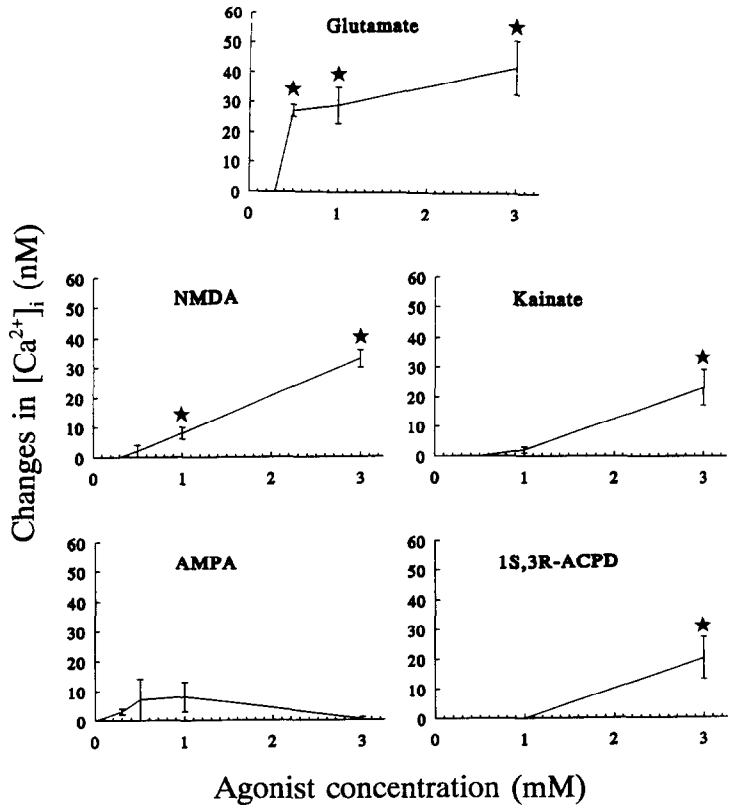

Fig. 2. Maxima of increases in free intracellular calcium induced by excitatory amino acids in SH-SY5Y neuroblastoma cells. For calcium peaking times by different glutaminergic agonists at different concentrations, see the text. Data are expressed as mean ± S.E.M. of 3–4 experiments done in duplicate. Dimethylsulfoxide (DMSO) (1%) vehicle was always used as a control. $*P < 0.05$; one-way analysis of variance and Duncan's multiple-range test.

however, rapid calcium oscillations [4] with a wide range of local alterations in $[Ca^{2+}]_i$. In fact, excitable cells are continuously susceptible to depolarizing firing via other neuronal cells, and this direct depolarization may also contribute to the elevated $[Ca^{2+}]_i$ through voltage-dependent calcium channels [18,19]. If calcium is not removed from the cytoplasm, it may reach toxic or desensitizing levels [18,20].

There are several effective mechanisms in SH-SY5Y neuroblastoma cells to stabilize $[Ca^{2+}]_i$, i.e. calcium pumps remove calcium from the cytosol to the extracellular space, or to intracellular calcium stores, such as endoplasmic reticulum, mitochondria and nucleus [18,20,21]. Moreover, calcium binding by cytosolic proteins, including calmodulin, parvalbumin, and vitamin D-dependent calcium binding protein, are effective buffering processes [20], essential for the protection of excited neuronal cells against calcium-induced cell killing [18].

An initial increase in $[Ca^{2+}]_i$ by glutamate, NMDA, kainate, AMPA, and 1*S*,3*R*-ACPD was followed by a sustained decrease in $[Ca^{2+}]_i$ which reached a maximum at 3 min; there was always a dose-dependent decrease in $[Ca^{2+}]_i$ which was, however, reversed at high doses (Fig. 3). At low agonist concentrations, however, only a decrease in $[Ca^{2+}]_i$ occurred (see Figs. 1 and 3). The levels of $[Ca^{2+}]_i$ remained decreased at least for a follow-up period of 10 min (data not shown). The basal $[Ca^{2+}]_i$ in unstimulated SH-SY5Y neuroblastoma cells was 131 ± 4 nM (mean ± S.E.M.). The maximal decrease was 30–55 nM depending on the agonist. The normal physiological range of $[Ca^{2+}]_i$ in unstimulated cells is between 50 and 200 nM [22]; maintaining of $[Ca^{2+}]_i$ in the physiological concentration range is essential for neuronal cells [23]. Inspite that the integrated decrease in $[Ca^{2+}]_i$ always remained at a physiological range, rapid oscillations in $[Ca^{2+}]_i$ are possible.

The sustained decrease of $[Ca^{2+}]_i$ below the resting level was an unexpected finding. Excessive glutamate receptor stimulation may be followed by receptor desensitization [24] which thereby may also change the balance between $[Ca^{2+}]_i$ elevations, calcium buffering, and calcium removing systems. Moreover, glutamate receptor-induced elevation of $[Ca^{2+}]_i$ may be associated with neuronal hyperpolarization [16] which would result in a relative desensitization of voltage-dependent calcium channels toward depolarizing firing. The biphasic $[Ca^{2+}]_i$ response upon glutamate receptor stimulation provides evidence that two separate processes are involved in these calcium changes.

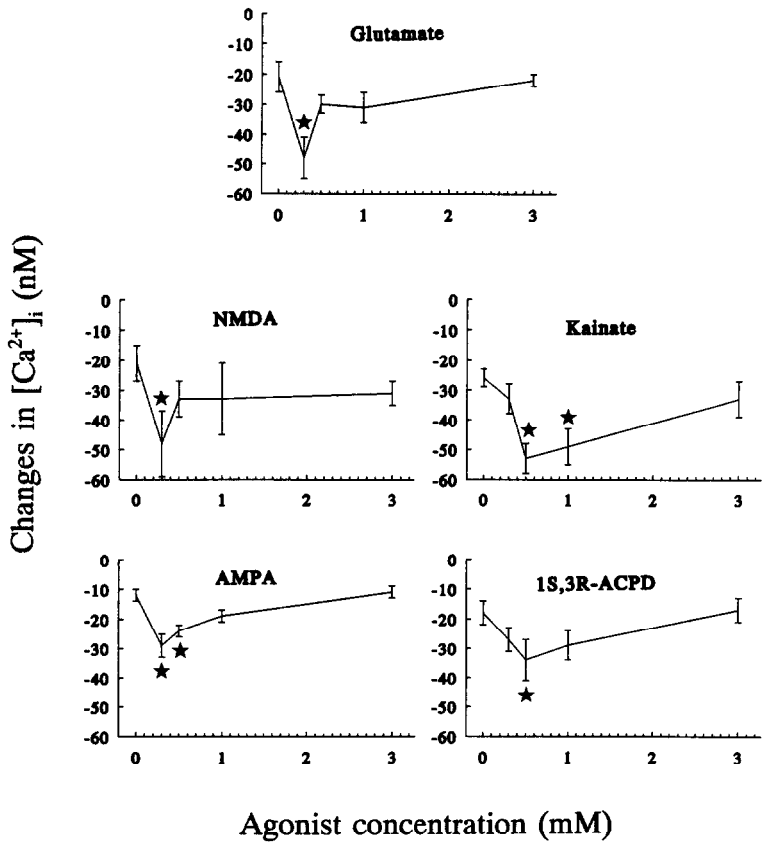

Fig. 3. Maxima of decreases in free intracellular calcium induced by excitatory amino acids in SH-SY5Y neuroblastoma cells. Calcium decrease was measured at 3 min post-stimulation of the cells by the agonists. Data are expressed as mean ± S.E.M. of 3–4 experiments done in duplicate. DMSO (1%) vehicle was always used as a control. Statistical analysis of the data is as given in the legend to Fig. 2.

The receptor binding data suggest that both ionotropic and metabotropic glutamate receptor agonists exhibit specific binding to their receptors in human SH-SY5Y neuroblastoma cells (Table I). Together with the glutamate receptor agonist-induced biphasic slow calcium responses this receptor binding data provide evidence for the expression of glutamate receptors in human SH-SY5Y neuroblastoma cells. Involvement of both receptor-gated calcium channels, and the stimulation of G protein coupled calcium-mobilizing second messenger systems, in glutamate-induced changes of $[Ca^{2+}]_i$ are likely. It increases the functional diversity and plasticity of human SH-SY5Y neuroblastoma cells upon glutamate receptor stimulation. The use of human SH-SY5Y neuroblastoma cells may be useful in exploring excitatory amino acid-induced processes in human neuronal tumor cells.

Table I

Specific displacement of L-[^{3}H]glutamate by ionotropic and metabotropic glutamate receptor agonists

Agonist	Specific displacement (fmol/mg protein)
L-Glutamate	38.0 ± 9
NMDA	23.2 ± 6
Kainate	15.8 ± 3
AMPA	23.7 ± 9
1*S*,3*R*-ACPD	23.5 ± 9

Values are expressed as mean ± S.E.M. of three experiments done in duplicate. The concentration of the L-[^{3}H]glutamate was 10 nM and that of the unlabelled agonists 1 mM. Displacement was biphasic in nature. It was the greatest both at low (10 nM) and high (1 mM) agonist concentrations, whereas only little specific displacement was found at intermediate concentrations (data not shown). Non-specific binding ranged between 9 and 26% of total binding.

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REFERENCES

[1] Monaghan, D.T., Bridges, R.J. and Cotman, C.W. (1989) Annu. Rev. Pharmacol. Toxicol. 29, 365–402.
[2] Schoepp, D., Bockaert, J. and Sladeczek, F. (1990) Trends Pharmacol. Sci. 11, 508–515.
[3] Berridge, M.J. and Irvine, R.F. (1989) Nature 341, 97–205.
[4] Berridge, M.J. (1993) Nature 361, 315–325.
[5] Orrenius, S., McConkey, D.J., Bellomo, G. and Nicotera, P. (1989) Trends Pharmacol. Sci. 10, 281–285.
[6] Orrenius, S., McCabe Jr., M.J. and Nicotera, P. (1992) Toxicol. Lett. 64/65, 357–364.

[7] Sugiyama, H., Ito, I. and Hirono, C. (1987) Nature 325, 531–533.
[8] Nicoletti, F., Wroblewski, J.T., Fadda, E. and Costa, E. (1988) Neuropharmacology 27, 551–556.
[9] Masu, M., Tanabe, Y., Tsuchida, K., Shigemoto, R. and Nakanishi, S. (1991) Nature 349, 760–765.
[10] Biedler, J.L., Helson, L. and Sprengler, B.A. (1973) Cancer Res. 33, 2643–2652.
[11] Tsien, R.Y., Rink, T.J. and Poenie, M. (1985) Cell Calcium 6, 145–157.
[12] Young, A.B. and Fagg, G.E. (1990) Trends Pharmacol. Sci. 11, 126–133.
[13] Furuya, S., Ohmori, H., Shigemoto, T. and Sugiyama, H. (1989) J. Physiol. 414, 539–548.
[14] Tuomala, M., Hirvonen, M.-R., Holopainen, M. and Savolainen, K. (1993) Toxicol. Appl. Pharmacol. 118, 224–232.
[15] Tuomala, M., Hirvonen, M.-R. and Savolainen, K. (1992) FEBS Lett. 296, 57–60.
[16] Nicoll, R.A. (1988) Science 241, 545–551.
[17] Bertolino, M. and Llinás, R.R. (1992) Annu. Rev. Pharmacol. Toxicol. 32, 399–421.
[18] Nicotera, P. and Orrenius, S. (1992) Ann. N.Y. Acad. Sci. 648, 17–27.
[19] Reuveny, E. and Narahashi, T. (1993) Brain Res. 603, 64–73.
[20] Blaustein, M.P. (1988) Trends Neurosci. 11, 438–443.
[21] Carafoli, E. (1992) J. Biol. Chem. 267, 2115–2118.
[22] Carafoli, E. (1987) Annu. Rev. Biochem. 56, 395–433.
[23] Tsien, R.W., Lipscombe, D., Madison, D.V., Bley, K.R. and Fox, A.P. (1988) Trends Neurosci. 11, 431–438.
[24] Catania, M.V., Aronica, E., Sortino, M.A., Canonico, P.L. and Nicoletti, F. (1991) J. Neurochem. 56, 1329–1335.