Intracellular Ca^{2+} Increase in Neuro-2A Cells and Rat Astrocytes Following Stimulation of Bradykinin B_2 Receptor

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ABSTRACT—Murine neuroblastoma cell line Neuro-2A cells and rat brain astrocytes showed a dose-dependent increase in intracellular Ca^{2+} in response to bradykinin, when assessed by a single cell image analyzing system. The Ca^{2+} increase in Neuro-2A cells by bradykinin was also examined by a suspension fluorescent assay using fura-2 loading. The Ca^{2+} increase in both cases was suppressed by a bradykinin B_2 receptor antagonist, Hoe 140, but not by a B_1 receptor antagonist, des-Arg-Hoe 140, suggesting that the effect occurred via specific B_2 receptor activation. RT-PCR for bradykinin B_2 receptor mRNA showed that both Neuro-2A cells and the astrocytes expressed B_2 receptor mRNA. Binding of [^3H]bradykinin to Neuro-2A cells was assessed, and a specific binding constant of 0.75 nM was determined. Furthermore, the increase in $[\text{Ca}^{2+}]_i$ by bradykinin could be caused by a release of Ca^{2+} from storage sites in the endoplasmic reticulum, since thapsigargin and U-73122 attenuated the effect of bradykinin in Neuro-2A as well as in astrocytes. These results indicate that both astrocytes and neuroblastoma Neuro-2A cells stimulated by bradykinin could express a bradykinin B_2 receptor-mediated intracellular Ca^{2+} increase leading to signal transduction.

Keywords: Bradykinin, Neuro-2A, Astrocyte, Intracellular Ca^{2+} , Bradykinin B_2 receptor

Bradykinin is well known as an inflammatory mediator that causes an increase in vascular permeability in various experimental animal tissues and pain receptor sensitization as well as stimulates cell growth or differentiation (1, 2). Based on studies using kininogen-deficient rat (3, 4), we have reported that bradykinin has a major role in local edema formation in carrageenin-induced rat paw edema and pleurisy, and we also showed that it causes paw edema in mice (5).

Recently, several reports have appeared suggesting that the kinin system might play a role in the central nervous system during brain injury or in the case of certain diseases (6). For example, in a cerebral ischemia model in rats (7), the kallikrein-kinin system was demonstrated to be activated to release bradykinin. Furthermore, almost all components of the system, such as prekallikrein and kininogens, have been detected in the brain by immunohistochemical techniques (8, 9). Studies have demonstrated the binding of bradykinin to cortical astrocytes and indicated that stimu-

lation with bradykinin causes mobilization of $[\text{Ca}^{2+}]_i$ (10, 11), suggesting a role for bradykinin in central nervous transmission. The responses of bradykinin in neuroblastoma cells were also described previously, but the cell types were mostly limited to rat PC12 cells.

Neuro-2A cells, which were derived from a mouse neuroblastoma (12), are widely used in neurological research. For example, one report indicates that stimulation of the $\text{P}_{2\text{Y}}$ receptors on these cells caused an increase in PI turnover and phospholipase C activation (13). However, one study has shown that bradykinin on Neuro-2A cells to cause increases in IP_3 and cGMP levels (12).

In this communication we tried to demonstrate bradykinin B_2 receptor expression not only by ligand binding assay but also by mRNA expression assay in neuroblastoma lineage cells, Neuro-2A, as well as cortical astrocytes (11) as model cells of the central nervous system. We also tried to examine B_2 -receptor functions in those cells through measuring $[\text{Ca}^{2+}]_i$ by two kinds of experimental methods, the single cell method and the cell suspension method.

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MATERIALS AND METHODS

Agents

[2,3-Prolyl-3,4-³H]bradykinin (114 Ci/mmol) and fura 2-AM were purchased from NENTM Life Science Products (Boston, MA, USA) and Dojindo (Kumamoto), respectively. Bradykinin, kallidin and Hoe 140 (Peptide Institute, Osaka) were dissolved in saline at 100 μ M. U-73122 and thapsigargin (Research Biochemicals International, Natick, MA, USA) were dissolved in dimethylsulfoxide at 2 mM and 1 mM, respectively. All drug solutions were stored at -30°C and diluted just before use in buffer solution to obtain the desired concentrations. The maximal final concentration of dimethylsulfoxide was 0.5%, which showed no effect on $[\text{Ca}^{2+}]_i$. Dibutyryl cyclic AMP (dibutyryl cAMP; Sigma, St. Louis, MO, USA) was dissolved directly in medium just before use.

Cell culture

Neuro-2A cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 in Dulbecco's modified Eagle's medium containing high glucose (25 mM), 10% fetal bovine serum (FBS) and penicillin/streptomycin. Astrocyte cultures were prepared from the cerebral cortices of embryonic day 18 or postnatal day 2 Wistar rats, as described previously (14). Astrocytes were grown in the same medium and under the same conditions as the Neuro-2A cells. After the cells became confluent, the culture medium was switched to serum-free medium, and experiments were initiated 24 h later. For *in vitro* differentiation, Neuro-2A cells were grown for 3 or 48 h in medium containing 1 mM dibutyryl cAMP. For activation of astrocytes, cells were treated with 1 μ M isoproterenol for 3 h.

Ca^{2+} measurements

Single cell image analysis system: Changes in the $[\text{Ca}^{2+}]_i$ of single cells were measured as previously described (15). Drugs were applied by a multi-barreled pipette placed close to the cells. An image analysis system (FC-200; Mitsubishi Kasei, Tokyo) was used to measure the ratio of the intensity of fluorescence excited at 340 nm (F340) to that excited at 360 nm (F360) as an index of the $[\text{Ca}^{2+}]_i$ level.

Cell suspension fluorescence assay: Subconfluent cells were washed twice and suspended by gentle scraping and pipetting in ice-cold phosphate-buffered saline containing 137 mM NaCl, 8.1 mM Na_2HPO_4 , 2.68 mM KCl and 1.47 mM KH_2PO_4 . The cells were centrifuged and resuspended in serum-free DMEM supplemented with 3 μ M fura-2-AM and further incubated at 37°C for 20 min. The cells were then washed twice with HEPES-buffered salt solution (HBSS) containing 140 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 1.2 mM MgSO_4 , 1.2 mM KH_2PO_4 , 6 mM glucose and 25 mM HEPES (pH 7.4 by NaOH) and resuspended

in HBSS at a density of 2×10^6 cells/ml. A fluorescence spectrophotometer, F-2000 (Hitachi, Tokyo), was used to measure the ratio of the intensities of fluorescence excited at 340 and 380 nm as an index of the $[\text{Ca}^{2+}]_i$ level.

Binding assays

Subconfluent Neuro-2A cells in 6-well trays were washed and then incubated at 4°C for 2 h with 0.16 to 10 nM [³H]bradykinin in 0.5 ml DMEM supplemented with 1 mM 1,10-phenanthroline and 0.1% bovine serum albumin (BSA). Nonspecific binding was determined in the presence of 5 μ M bradykinin and was subtracted from the total binding to give the specific binding. The binding reaction was terminated by aspiration of the medium, followed by three rapid washes with ice-cold PBS containing 0.1% BSA; and the cells were then solubilized with 0.5 ml of 0.1 N NaOH. A 0.45-ml fraction was transferred from each well into a scintillation vial and neutralized with 5 N HCl. Five milliliters of scintillation fluid was then added, and the radioactivity was determined by scintillation counting. The number of cells was determined by counting the cells present in the each of two wells, prepared in the same way as for the binding assay.

mRNA analysis

Total RNA was extracted from subconfluent cells with TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD, USA) and then treated with RNase-free DNase I for 15 min at room temperature. Reverse transcription was performed with 5 μ g of total RNA and 50 ng of random hexamers in a total volume of 20 μ l to produce first-strand cDNA. The quality of RNA samples was evaluated by the reverse transcriptase polymerase chain reaction (RT-PCR) using GAPDH-specific primers (5'-AGA-CAG-CCG-CAT-CTT-CTT-GT-3', 5'-CCA-CAG-TCT-TCT-GAG-TGG-CA-3'). The levels of BK B_2 receptor cDNAs were evaluated by a semiquantitative RT-PCR method (16). PCR experiments were performed with 0.5 μ l of the first-strand cDNA in a 25- μ l reaction mixture. Rat B_2 receptor cDNA was amplified with specific primers (5'-GAC-TGG-CTG-TTC-GGA-GAG-GT-3', 5'-TGG-GTG-ACA-ATA-TCC-ACG-GC-3'), based on the nucleotide sequence of the bradykinin B_2 receptor (17, 18). Primers used for RT-PCR of mouse B_2 receptor cDNA were the same ones that were used in the previous report (19). PCR cycles were titrated to establish amplification conditions for each primer, to document linearity, and to permit quantitative analyses of signal strength; the conditions for GAPDH, B_2 receptor cDNA of rat or mouse was as follows: denaturation at 94°C for 30 s; annealing at 62°C , 62°C or 56°C for 30 s; and extension at 72°C for 45 s (22, 36 or 28 cycles), respectively. PCR products were analyzed on 2% agarose gels and visualized by ethidium bromide staining.

Analysis of data

Results were expressed as means $\pm$ S.E.M. Statistical significance was evaluated by one-way analysis of variance followed by Tukey's method.

RESULTS

As for methods for measurement of $[Ca^{2+}]_i$, we used the single cell method and the cell suspension method, and found that both methods produced similar results from both Neuro-2A cells and primary cultures of astrocytes. In the cell suspension assay, Neuro-2A cells showed a dose-dependent increase in $[Ca^{2+}]_i$ in response to kinins, such as bradykinin and kallidin, as shown in Fig. 1A. This increase in $[Ca^{2+}]_i$ was inhibited dose-dependently by the kinin B₂ antagonist Hoe 140, as shown in Fig. 1B, but not by the kinin B₁ antagonist des-Arg Hoe 140 (data not shown), indicating that the effect was mediated through the kinin B₂ receptor. The $[Ca^{2+}]_i$ increase by bradykinin in these cells was also inhibited by U-73122 (Fig. 1B), an inhibitor of phospholipase C, suggesting the involvement of phospholipase C. Furthermore, thapsigargin, an inhibitor of the Ca^{2+} pump in the endoplasmic reticulum, the action of which results in the depletion of inositol 1,4,5-triphosphate (IP₃)-sensitive Ca^{2+} stores, also inhibited the $[Ca^{2+}]_i$ increase by bradykinin in Neuro-2A (Fig. 1B). The latter results suggest that the bradykinin-induced $[Ca^{2+}]_i$ increase could be mostly supplied from intracellular storage sites.

Results obtained by the single cell method are shown in

Fig. 2 and Table 1. In Fig. 2A, the ratio of responding cells / number of cells tested is shown above or below each datum point. In both cell types, the amplitude of the $[Ca^{2+}]_i$ rise and the number of responding cells increased in parallel with the bradykinin concentration. However, at the concentration of 100 nM and over, almost all of the Neuro-2A cells responded, but only approximately one-half of the astrocytes showed the increase. In Fig. 2, typical recordings for Ca^{2+} measurement in a Neuro-2A cell (B) and an astrocyte (C) are shown. Effects of Hoe 140, U-73122, and thapsigargin on the $[Ca^{2+}]_i$ increase in Neuro-2A and astrocytes assessed by the single cell method (Table 1) were mostly similar to those found by the cell suspension method shown in Fig. 1B. However, there are some differences between the data obtained by the two methods, i.e., difference in the sensitivity of the U-73122, that may most probably due to the different manipulation of cell preparation as described in the methods section.

Bradykinin binding to Neuro-2A cells was examined, as shown in Fig. 3A. The data indicate the presence of a single population of binding sites with a K_d of 0.754 nM and a B_{max} of 23,500 sites/cell. Expression of mRNA for the bradykinin B₂ receptor in both Neuro-2A cells and astrocytes was demonstrated by the RT-PCR method (Fig. 3B). Neuro-2A cells were known to be differentiated by the application of dibutyryl cAMP (20). We found some differences in the morphological change of Neuro-2A cells between 3 and 48 h of the drug application. At about 3 h, dibutyryl cAMP induced outgrowth of a number of fine

Fig. 1. $[Ca^{2+}]_i$ increase in Neuro-2A cells in response to various concentrations of bradykinin (●) or kallidin (○) (A) and inhibition of bradykinin-induced $[Ca^{2+}]_i$ increase in Neuro-2A cells by Hoe 140, U-73122 (U-7) and thapsigargin (Tg) (B). In panel A, bradykinin and kallidin at the indicated concentrations induced an increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$, nM) in Neuro-2A cells, as determined by the cell suspension method. Each value is expressed as the mean with S.E.M. from three separate experiments. In panel B, Hoe 140 (0.01, 0.1 and 1 μ M), U-7 (5 and 10 μ M), thapsigargin (Tg; 0.01, 0.03 and 0.1 μ M) were added at the indicated final concentration 1 min before the addition of bradykinin. Columns indicate percent of the value of the $[Ca^{2+}]_i$ increase elicited by bradykinin at 10 nM. Each column indicates the mean with S.E.M. from 3–5 experiments. ** shows statistically significant difference from the value of bradykinin alone at $P < 0.01$.

Fig. 2. Bradykinin-induced $[Ca^{2+}]_i$ increase measured by the single cell image analyzing system (A), and its recordings of $[Ca^{2+}]_i$ in a Neuro-2A cell (B) or an astrocyte (C). In panel A, the dose-response relationship of bradykinin causing an increase in $[Ca^{2+}]_i$ was measured in Neuro-2A cells (●) and in cortical astrocytes (○). Figures by the symbols indicate number of responding cells/number of tested cells. In panels B and C, typical recordings of $[Ca^{2+}]_i$ rise induced by repeated applications of 100 nM bradykinin (BK) and reversible inhibitory effect of Hoe 140 (HOE, 1 μ M) in a Neuro-2A cell (B) and an astrocyte (C) are shown. Vertical and horizontal bars on the right show 10 nM of $[Ca^{2+}]_i$ change (Δ) and recording time of 1 min, respectively.

Table 1. Effects of inhibitors on bradykinin-induced calcium rise in neuro-2A cells and astrocytes, when assessed by the single cell method

Cell	Inhibitor	Concentration (μ M)	Response (%)	N
Neuro-2A cells	Hoe 140	0.01	59.31 $\pm$ 5.91**	16
		0.1	63.94 $\pm$ 10.20**	22
		1.0	17.45 $\pm$ 3.36**	17
	U-73122	5.0	26.72 $\pm$ 2.62**	32
	thapsigargin	1.0	9.21 $\pm$ 1.19**	44
Astrocytes	Hoe 140	0.1	44.73 $\pm$ 5.13**	60
		1.0	15.86 $\pm$ 2.77**	4
	U-73122	5.0	20.58 $\pm$ 4.38**	5
	thapsigargin	1.0	27.07 $\pm$ 4.29**	11

The cells were pretreated with Hoe 140, U-73122 or thapsigargin for 1, 3 or 3 min, respectively, and then incubated with 100 nM bradykinin for 1 min. Responses are expressed as % of bradykinin-induced $[Ca^{2+}]_i$ change that was determined before application of the inhibitors. Data are means with S.E.M. from the responding cells (N). ** $P < 0.01$ vs bradykinin only taken as 100%.

neurites transiently in about 20% of cells and after 48 h, a few thick ones in about 35% of cells. On the other hand, almost all astrocytes were activated by isoproterenol for 3 h and showed no further morphological changes, as previously described (14). Density of the bands expressing mRNA of bradykinin B₂ receptor did not change following differentiation of Neuro-2A cells by dibutyryl cAMP or that of astrocytes by isoproterenol. We could not find any significant differences between differentiated and undifferentiated cells in the bradykinin-induced $[Ca^{2+}]_i$ increase (data not shown). The presence of bradykinin in the

medium did not affect significantly the morphology of the both cell types (data not shown).

Taken together, these data from both cell suspension and single cell methods demonstrate that bradykinin caused an increase in $[Ca^{2+}]_i$ in these cells through B₂ receptor activation to release Ca^{2+} from intracellular stores, with involvement of PLC.

DISCUSSION

We found that the data using the single cell method and

Fig. 3. Binding curve and Scatchard analysis of $[^3\text{H}]$ bradykinin ($[^3\text{H}]$ BK) binding to Neuro-2A cells (A) and expression of mRNA of bradykinin B_2 receptor in Neuro-2A cells and astrocytes (B). In panel A, specific binding ($\bullet$) was assessed by subtraction of nonspecific binding (Δ) from total binding ($\circ$) of $[^3\text{H}]$ BK. Each value is the mean of duplicates and is representative of 3 different experiments. The inset shows the Scatchard plot of the specific binding. The horizontal axis expresses $[^3\text{H}]$ BK amount as pmol/ 10^6 cells. In panel B, bradykinin B_2 receptor mRNA ($\text{B}_2\text{-R}$) was demonstrated by RT-PCR in comparison with mRNA for GAPDH. RNA was extracted from Neuro-2A cells treated with 0 or 1 mM dibutyryl cAMP (dBcAMP) for 3 or 48 h. Astrocytes were treated with 0 or 1 μM isoproterenol for 3 h, and RNA was then extracted.

the cell suspension method showed similar results, so we can use either method to evaluate $[\text{Ca}^{2+}]_i$. Especially, the cell suspension method is simple and does not require any special equipment except for a fluorometer. However, the single cell method is superior for assessment of the response in terms of which receptor population is on a cell membrane or which part of the cell population is responding. As a matter of fact, we found that some of the astrocytes did not respond to bradykinin, suggesting that part of the population expressed a lower number of B_2 receptors. However, our result is consistent with previous data demonstrating the ratio of bradykinin-responding cells among isolated astrocytes (11).

In Neuro-2A cells, we demonstrated the presence of mRNA for the B_2 receptor as well as high-affinity binding sites for kinins. The stimulation of B_2 receptor caused an increase in $[\text{Ca}^{2+}]_i$ as in astrocytes, and Ca^{2+} could be supplied from intracellular stores, in the same way as reported for other receptors such as opiate or purine receptors (13, 14).

Stimulation of the bradykinin receptor caused no morphological change in either undifferentiated Neuro-2A cells or those differentiated by dibutyryl cAMP (data not shown), and the expression level of B_2 receptor mRNA did not change following differentiation. Because it was reported that B_2 receptors in cultured cortical astrocytes were up-regulated in the reactive state induced by endothelin-1 or

dibutyryl cAMP (10), we expected that stimulation with isoproterenol might induce B_2 -receptor upregulation. However, expression of B_2 -receptor mRNA in the astrocytes also showed no change after stimulation of the cells with isoproterenol. Although it was reported that treatment of astrocytes with isoproterenol, endothelin-1 or dibutyryl cAMP induces the reactive state of the cells (10, 14), there may be some difference in induction of protein synthesis by these agents, but detailed analyses remain for future studies.

Our results demonstrate that the B_2 receptor on these cells could be stably and constitutively expressed, mediating the G-protein coupled response of signal transduction involving phospholipase C and PI turnover. These results may contribute to the consistent support for the pathophysiological role of bradykinin in the central nervous system (6).

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