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Effects of Bifemelane on the Calcium Level and ATP Release of the Human Origin Astrocyte Clonal Cell

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Abstract. The effect of bifemelane hydrochloride (bifemelane) was examined on human origin astrocyte clonal cells (Kings-1). Bifemelane (125 – 1000 μM) induced a dose-dependent increase in the intracellular calcium concentration ($[Ca^{2+}]_i$). In the highest concentration (1000 μM), the drug caused the second large increase in $[Ca^{2+}]_i$ during the washing. The increase that occurred during the administration partially remained in the Ca^{2+}-free medium and was blocked by 2-aminoethoxydiphenyl borate (2-APB), an IP_3-receptor blocker, indicating that the source of Ca^{2+} for the increase could be ascribed to the intracellular store. The increase in $[Ca^{2+}]_i$ was not observed during washing with Ca^{2+}-free medium, but was observed when the washing was performed with Ca^{2+}-containing medium. Bifemelane caused a dose-dependent ATP release, but histamine and carbachol, which induced a large increase in $[Ca^{2+}]_i$, had no effects on the ATP release. The effects on the $[Ca^{2+}]_i$ were blocked by pretreatment with pyridoxal phosphate-6-azophenyl-2',4' disulfonic acid, a P2-receptor antagonist. Although the mechanisms of ATP release induced by the drug have not been elucidated yet, the present results demonstrate that the increase in $[Ca^{2+}]_i$ induced by bifemelane is not due to its direct effect on the cells, but is dependent upon the ATP released from the cells.

Keywords: human astrocyte, bifemelane, ATP release, store operated Ca^{2+} entry, intracellular Ca^{2+} store

Introduction

Recent studies on astrocytes have revealed that they express neurotransmitter receptors and release neurotransmitter (1). Tremendous numbers of synapses in the brain have been shown to be covered with the lamella developed from a single astrocyte (2), which not only supports synaptic structure in the brain, but also regulates the efficiency of synaptic transmission by inter-communication among neurons and astrocytes (3). The structure consisting of astrocytes, pre-synaptic and post-synaptic neurons has been emphasized by coining of the term "tripartite synapse", which participates in information processing in the brain (4). However, the interaction between astrocytes and neurons may build much larger scale machineries than the tripartite synapse and may participate in the higher order information processing in the brain.

In our previous paper on the cultured rat cerebral astrocytes, we found that bifemelane hydrochloride (bifemelane), which had been developed as a nootropic drug, induced a characteristic increase in the intracellular calcium concentration ($[Ca^{2+}]_i$), from intracellular storage sites (5). At higher concentrations, the drug facilitated Ca^{2+} influx from the extracellular space through the store operated Ca^{2+} channels (SOCC) during washing. Interestingly, the drug induced rapid onset and highly reversible blocking effects on the SOCC activated in the cells treated with thapsigargin and bathed in normal medium (5). Although the dose required to cause the effect seemed to be much higher than its therapeutic dose, the effects of bifemelane on the $[Ca^{2+}]_i$ of astrocytes found in our previous study might have some

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relationship to the pharmacological and therapeutic efficiency of the drug (6, 7).

In the present study, we investigated the mechanisms of actions of bifemelane on Kings-1 cells, human astrocyte clonal cells, which are stable and reliable human astrocyte models for studying the effects of drugs and their mechanisms of actions. Our results suggested that bifemelane might cause the increase in $[Ca^{2+}]_i$ of the cells by the indirect action of ATP released from the cells.

Materials and Methods

Cell culture

Kings-1, a human origin astrocyte clonal cell, was purchased from Health Sciences Research Resource Bank (Tokyo). The cells were cultured, harvested and separated into tubes and stocked in a deep freezer (−70°C). The cells were plated on the glass cover slip (Matsunami #1; Matsunami Glass, Ind., Ltd., Kishiwada) at a density of 1.0×10^4 cells per well. The culture was maintained for 4 days in an incubator with a medium composed of Dulbecco's modified Eagles's (cat. No. 430-2100, containing no L-glutamate and L-aspartate; GIBCO BRL, Life Technologies, Inc., Rockville, MD, USA), 5% v/v precolostrum new born calf serum (Mitsubishi Chemical Co., Tokyo), and 5% v/v heat-inactivated horse serum (GIBCO BRL, Life Technologies, Inc.).

Intracellular Ca^{2+} measurement

Intracellular Ca^{2+} measurement on cultured cells was performed according to previous papers (8, 9). The cultured cells were washed twice with fresh balanced salt solution (BSS: 130 mM NaCl, 5.4 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.5 mM glucose, and 20 mM HEPES-NaOH; pH 7.3); and then they were treated for 45 min at 32°C with 7.5 μM fura-2 acetoxymethyl-ester form (fura-2/AM) (Dojindo Co., Kumamoto), which was prepared from a 1 mM stock solution in DMSO by diluting in BSS supplemented with 0.01% Cremophor EL (Sigma-Aldrich Japan KK, Tokyo) to facilitate the solubility. After washing out the dye-containing medium, the cells were incubated for 60 min and used in the following image analysis.

The cultured cells loaded with the Ca^{2+} indicator were placed on the stage of an inverted fluorescence microscope (IMT-2; Olympus Co., Tokyo) equipped with a xenon arc lamp (75 W) and interference filters (340 ± 5 and 380 ± 5 nm). The cultured cells were continuously perfused with BSS at a rate of 2 ml/min. The images of fluorescent Ca^{2+} indicator-loaded cells were obtained through a sharp cut filter (>480 nm), detected by a digital cooled CCD camera (HiSCA; Hamamatsu Photonics Co., Shizuoka). Ratio images (F340/F380) were obtained by dividing a fluorescent image induced by 340 nm excitation with one obtained by 380 nm excitation at minimum intervals of once every 0.45 s using an image analysis system (ORCA, Hamamatsu Photonics Co.).

Determination of ATP released from the cells in culture

The release of ATP from the cells in culture was studied with the ATP determination kit (Molecular Probes, Inc., Eugene, OR, USA) and a luminometer (Lumat LB9501; Berthold GmbH & Co., Bad Wildbad, Germany). The cells were cultured for 4 days in a 24-well culture dish at a density of 1.0×10^4 cells per well. Cells grown in each cell were incubated for 15 min in 300 μl of drug-free or test drug containing BSS at 28°C. Then the incubation medium was collected in a test tube and heated for 15 min at 90°C and stored as a sample medium on ice until the ATP assay. The ATP content in each sample was analyzed according to the conventional method. ATP assay standard medium [0.5 mM D-luciferin, 1.25 μg/ml luciferase, 25 mM Tricine buffer, 5.0 mM $MgSO_4$, 0.1 mM EGTA, 1.0 mM DTT] (100 μl) was combined in a reaction tube with sample medium (100 μl) and put it immediately into the luminometer. The luminescence was measured for 10 s. We measured known concentrations of ATP in each series of experiments to confirm the reliability of the assay system and to obtain a calibration curve.

Since the relative light units of luminescence varied among the series of cultures, we normalized each value obtained in each series of experiment by dividing the luminescence light units obtained from drug-free sample medium.

Drugs used

Bifemelane hydrochloride (4-(*O*-benzylphenoxy)-*N*-methylbutylamine hydorochloride) was a gift from Mitsubishi Welpharma Co. (Tokyo). The ATP assay kit was purchased from Molecular Probes, Inc. Fura-2/AM was purchased from Dojido Co. Carbachol, histamine, ATP, ethylene glycol bis (2-aminoethyl ether)-*N,N,N'N'*-tetraacetic acid (EGTA), atropine sulfate, mepiramine maleate, pyridoxal phosphate-6-azophenyl-2',4' disulfonic acid (PPAD), and 2-aminoethoxydiphenyl borate (2-APB) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA).

Results

Pharmacological profiles of Kings-1 cells, a human origin astrocyte clonal cell

We examined the pharmacological profiles of Kings-

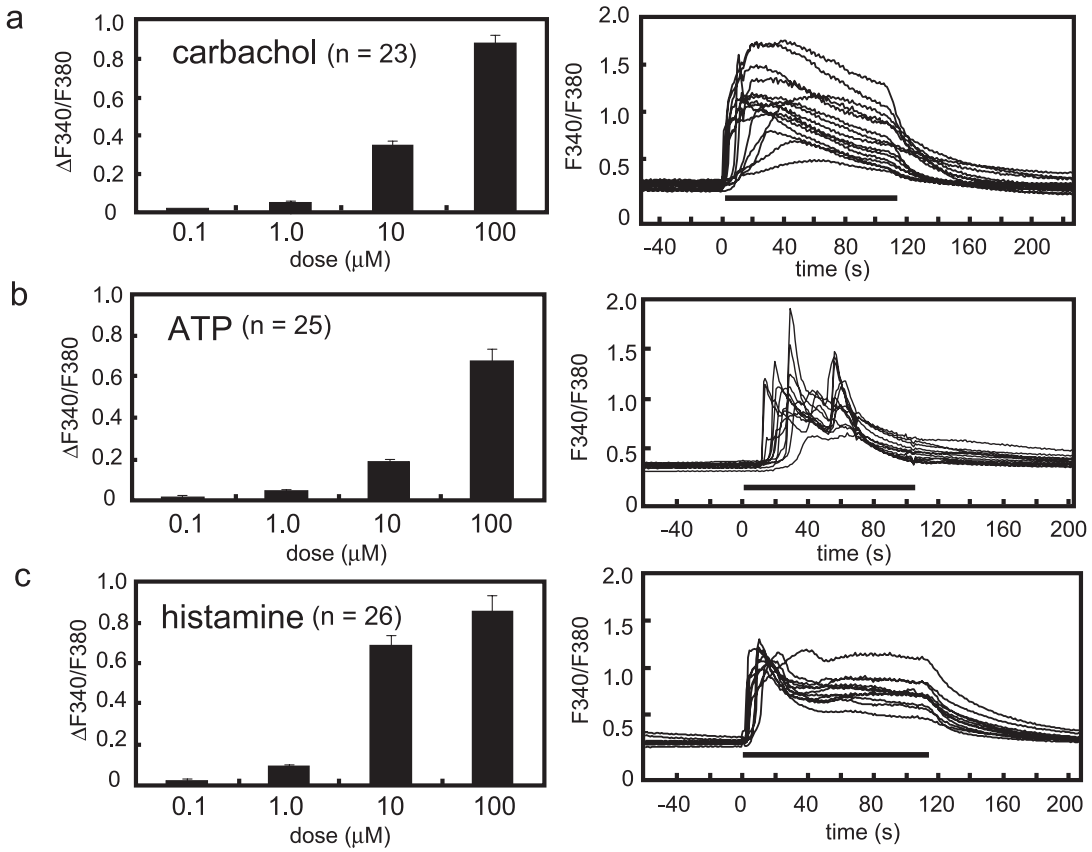

Fig. 1. Effects of carbachol, ATP, and histamine on the $[Ca^{2+}]_i$ of Kings-1, human origin astrocyte clone cells. Left: The dose-response relationships for carbachol (a), ATP (b), and histamine (c) were indicated. Ordinates: The increase in $[Ca^{2+}]_i$ expressed as differences between the fura-2 fluorescence ratio (F340/F380) in the resting and the peak level obtained after drug administration. Each column indicated the mean with standard error that was calculated based upon the difference of the base line and peak ratio during drug administration. Abscissa: drug dose in μM. Right: Recordings of $[Ca^{2+}]_i$ during the administration of drugs (100 μM). Ordinates: The fura-2 fluorescence ratio (F340/F380). Abscissa: Time in s. The examples of effects of test drugs are shown as superimposed traces obtained from 12 cells.

1 cells under calcium imaging by using neurotransmitters and their antagonists to confirm the availability of these cells on pharmacological profiling of the drugs. We found that the cell expressed several receptors for neurotransmitters, which were important for studying the physiological and pharmacological properties of astrocytes in the brain.

Carbachol, a cholinergic receptor agonist (0.1 – 100 μM), induced a dose-dependent increase in $[Ca^{2+}]_i$ that was characterized by a fast onset increase followed by a sustained increase during 120-s administration (Fig. 1a). ATP (0.1 – 100 μM) caused a transient dose-dependent increase in $[Ca^{2+}]_i$, which was occasionally accompanied by a slow onset second peak in $[Ca^{2+}]_i$ during the administration (Fig. 1b). Histamine (0.1 – 100 μM) induced an increase in $[Ca^{2+}]_i$ in a similar pattern to carbachol (Fig. 1c). These effects of carbachol, histamine, and ATP were blocked by atropine sulfate (1 μM), a muscarinic receptor antagonist; mepiramine maleate (10 μM), a histamine H_1-receptor antagonist; and PPAD (10 μM), a P2 purinergic receptor antagonist, respectively (data not shown).

Although we examined the effects of many other agonists for neurotransmitter receptors, such as glutamate, serotonin, noradrenalin, and dopamine, these agonists had little or no effects on the $[Ca^{2+}]_i$ of the cell even in high concentrations (100 μM).

Effects of bifemelane on Kings-1 cells

The effects of bifemelane on the $[Ca^{2+}]_i$ were examined in Kings-1 cells. The drug caused only a slight increase in $[Ca^{2+}]_i$ in a concentration of 125 μM. However, at a concentration greater than 250 μM, the drug induced an obvious dose-dependent increase in $[Ca^{2+}]_i$ (Fig. 2b). As had been observed in rat astrocytes (6), lower concentration of bifemelane induced a slow onset increase in the $[Ca^{2+}]_i$ of human origin astrocytes, but at a concentration of 1000 μM, the drug induced a

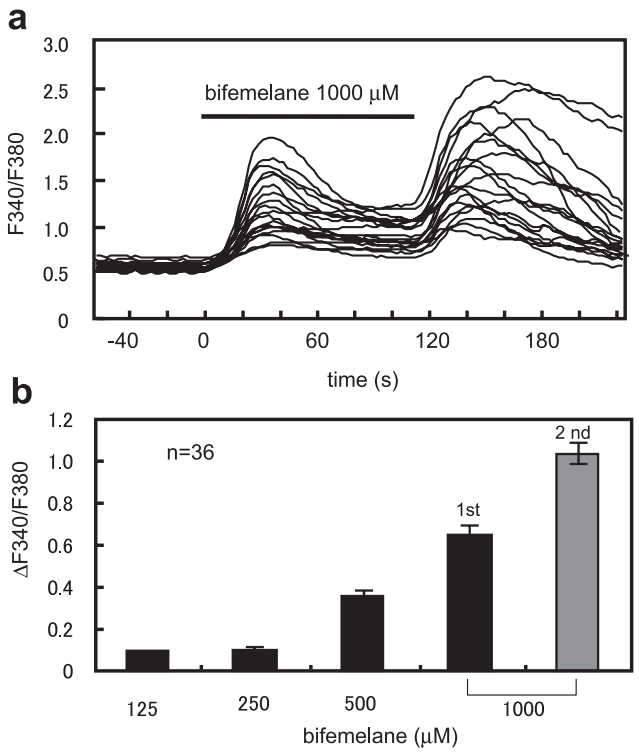

Fig. 2. Dose-response relationship to bifemelane in Kings-1. a: Effects of bifemelane on the cells. The traces obtained from 19 cells during the administration of bifemelane (1000 μM) were superimposed. Note second larger responses were induced during washing. Abscissa: Time in s. Ordinate: Fluorescence ratio as indicated in Fig. 1 graphs on the right. b: Dose-response relationship to bifemelane. Each column indicated the mean with standard error that was calculated based upon the difference of the base line and peak ratio during drug administration. In concentrations of 1000 μM, we could observe the second peak during washing. Abscissa: dose in μM. Ordinate: as indicated in the Fig. 1 graphs on the left.

faster onset, a transient increase in $[Ca^{2+}]_i$ during the administration, and a second increase during washing of the drug (Fig. 2: a and b).

The peak of the increase in $[Ca^{2+}]_i$ during bifemelane administration remained in the Ca^{2+}-free medium (containing 1 mM EGTA), but the second peak was abolished by washing with the Ca^{2+}-free medium (Fig. 3a). The second peak was observed when the washing was performed with Ca^{2+} (2.0 mM) containing medium (Fig. 3b). The first peak was dependent upon the intracellular store and the second peak was dependent upon the extracellular Ca^{2+}.

To confirm the origin of the first $[Ca^{2+}]_i$ peak during bifemelane administration, we treated the cells with 2-APB, an IP_3 receptor blocker. As shown in Fig. 4, a and b, 2-APB (100 μM) blocked the effects on $[Ca^{2+}]_i$, demonstrating that the endoplasmic reticulum was the source of the first peak of $[Ca^{2+}]_i$ during bifemelane administration.

Effects of bifemelane on ATP release from Kings-1 cells

To examine the physiological role of the increase in $[Ca^{2+}]_i$ during the administration of neurotransmitter agonists, we measured the release of ATP from the cell during stimulation. After a 15-min incubation in normal BSS, we could detect obvious levels of ATP in the extracellular incubation medium (resting level: 60.3 ± 20.0 nM, n = 10). In the extracellular medium containing 1000 μM of bifemelane, the ATP level increased to about 8 – 15 times higher than the resting level. As shown in Fig. 5, bifemelane (0 – 1000 μM) induced the release of ATP in a dose-dependent manner.

Although the results demonstrated the participation of Ca^{2+} in ATP release from the cells, histamine and carbachol, which caused almost the same degree of increase in $[Ca^{2+}]_i$ as bifemelane, had no effects on the ATP release at concentrations of 100 and 1000 μM (Fig. 5).

The effect of PPAD, a P2-receptor blocker, on the $[Ca^{2+}]_i$ increase induced by bifemelane

The effects of bifemelane, carbachol, and histamine on $[Ca^{2+}]_i$ and ATP release suggested that the $[Ca^{2+}]_i$ increase did not necessarily participate in the release of ATP from the cell, but the increase in $[Ca^{2+}]_i$ induced by bifemelane was the result of activation of the P2 receptor by released ATP. To prove this possibility, we examined the effect of PPAD, a P2 purinergic receptor antagonist, on the effect of bifemelane on $[Ca^{2+}]_i$. As shown in Fig. 6, the effect of the drug was significantly blocked by 60-s pretreatment with PPAD (10 μM). Furthermore we confirmed that a high concentration of ATP (10 mM) induced the transient increase in $[Ca^{2+}]_i$ during the administration and the second increase during washing, which was similar to that induced by bifemelane (Fig. 6d).

The Ca^{2+}-dependency of bifemelane-induced ATP release from Kings-1 cells

The above results suggested that the increase in $[Ca^{2+}]_i$ induced by bifemelane was the secondary effect of released ATP. However, pretreatment with BAPTA/AM (10 μM), an intra-cellularly loadable Ca^{2+} chelator, for 60 min before bifemelane administration significantly blocked the ATP release (Fig. 7) This result suggested that bifemelane-induced ATP release was at least partly Ca^{2+} dependent.

Since the release of glutamate from the asytrocytes is facilitated in the divalent-cation-free medium (10, 11), we tested the effects of Ca^{2+}- and Mg^{2+}-free medium on the ATP release from the cell. There was no significant increase in ATP release during the exposure, but the release was significantly reduced in this condition. On

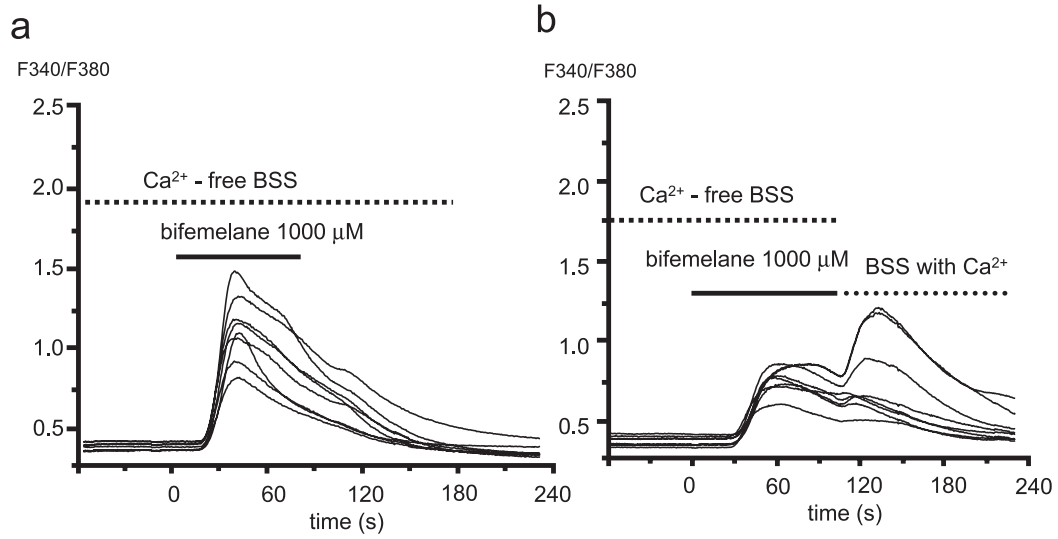

Fig. 3. Extracellular Ca^{2+} dependent- and independent-$[Ca^{2+}]_i$ increase induced by bifemelane. Effects of bifemelane on $[Ca^{2+}]_i$ of Kings-1 in the Ca^{2+}-free EGTA (1 mM) containing BSS perfused for 3 min before the bifemelane administration. In the Ca^{2+}-free condition, there was no second increase in $[Ca^{2+}]_i$ during washing (a), but this increase did occur during washes with Ca^{2+}-containing BSS (b). In each figure, traces obtained from 8 cells were superimposed. Ordinates: Fura-2 fluorescence ratio. Abscissas: Time in s.

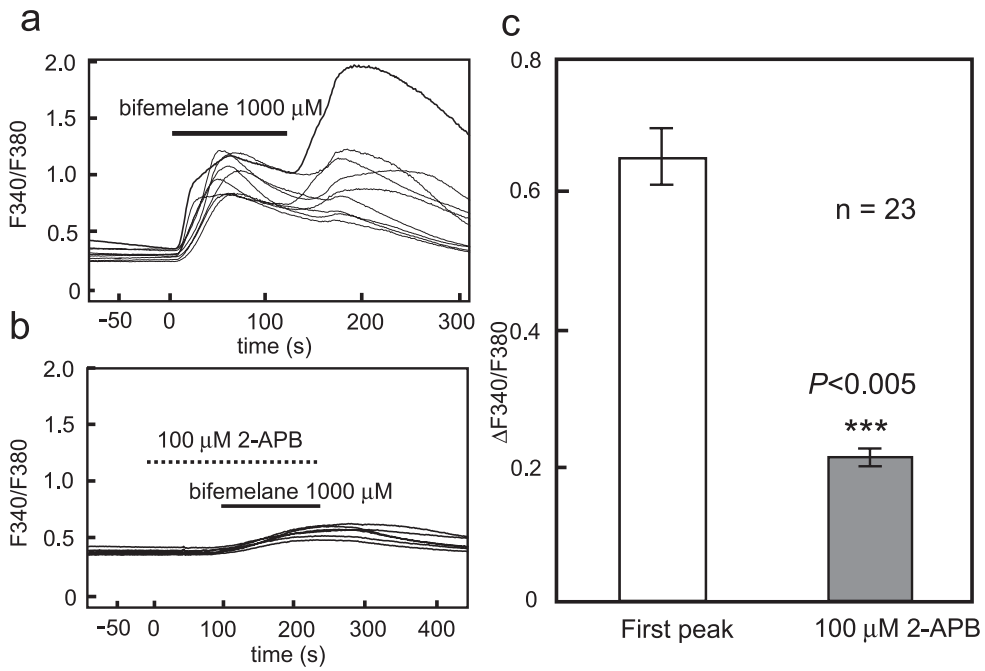

Fig. 4. Effects of 2-APB, an IP_3 receptor antagonist, on bifemelane (1000 μM)-induced increase in $[Ca^{2+}]_i$. a: Example of control responses shown as 9 superimposed traces. b: The effect of 2-APB on bifemelane-induced increase in $[Ca^{2+}]_i$ (9 superimposed traces). Ordinate and abscissa are the same as Fig. 3. c: Comparison between the control and 2-APB treated mean with standard error (n = 23). Statistically significant by the Student *t*-test (***P<0.005).

the other hand, bifemelane-induced ATP release was significantly facilitated in the divalent-cation-free medium (Fig. 8). Those results indicated that ATP release from the cell and the effects of bifemelane on the process might involve Ca^{2+}-dependent processes. Further studies on these processes are now under investigation.

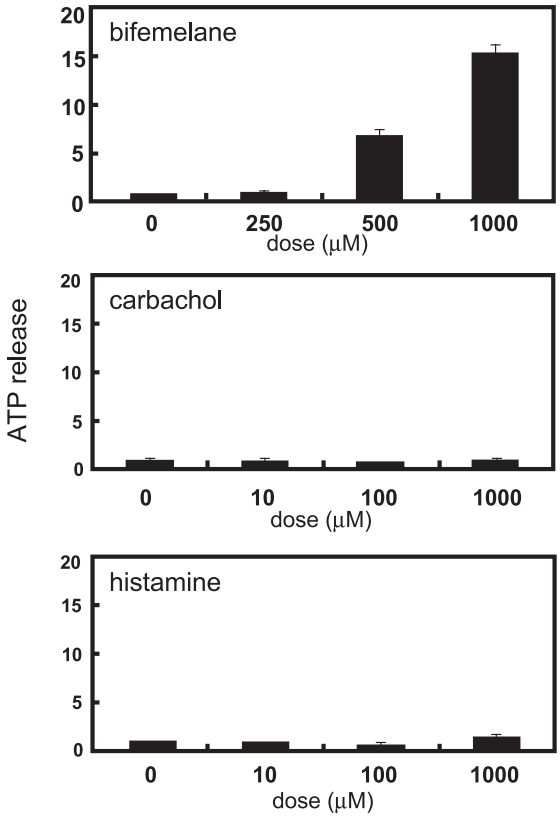

Fig. 5. Dose-response relationship for ATP release during administration of bifemelane, carbachol, and histamine. The ordinate in each graph indicates the relative amount of ATP release to the resting level with standard error (in this series of experiments, resting ATP level was 134.7 ± 8.4 nM) (n = 4). Abscissa in each graph indicates the dose of the drugs in μM.

Discussion

We examined the pharmacological profiles of Kings-1 cells, human origin astrocytes, by administering neurotransmitters and their specific antagonists on Ca^{2+} imaging. We found that carbachol, histamine, and ATP caused dose-dependent increase in $[Ca^{2+}]_i$. Specific receptor antagonists revealed that the receptors expressed on the cell were muscarinic acetylcholine receptors, H_1-histamine receptors, and P2-purinergic receptors, which were primarily coupled with the Gq-protein, as seen in rat astrocytes (8, 12). However, we did not detect any effects of glutamate and monoamines, such as noradrenalin, dopamine and serotonin, on the $[Ca^{2+}]_i$ level of Kings-1 cells. Those results, however, do not necessarily indicate the absence of the other subtypes of those receptors.

Although the sensitivity of the human origin astrocytes to bifemelane seemed to be lower than that of rat

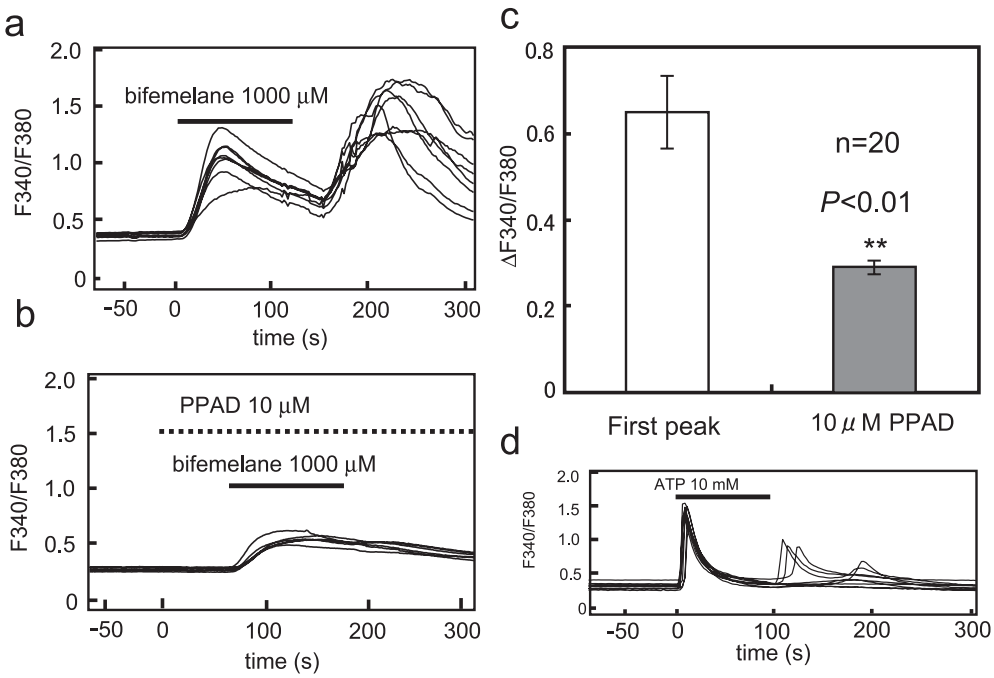

Fig. 6. Effects of PPAD, a P2 receptor antagonist, on bifemelane (1000 μM)-induced increase in $[Ca^{2+}]_i$. a: Example of control responses shown as 8 overlayed traces. b: Example of the effect of PPAD (10 mM) on the effect of biffemelane (6 traces overlayed). c: Comparison between the control and PPAD-treated mean with standard error (n = 20). Statistically significant in the Student *t*-test (**P<0.01). d: Example of the effect of ATP in a high concentration.

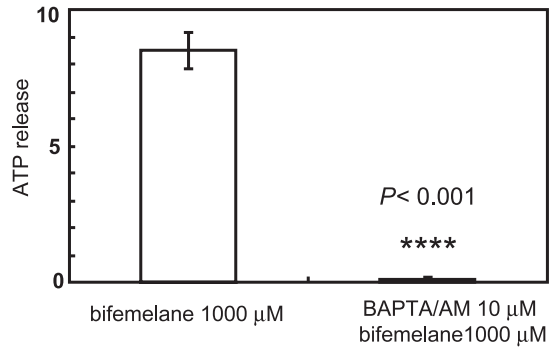

Fig. 7. Effects of BAPTA/AM on the bifemelane-induced ATP-release. Cells were treated with BAPTA/AM (10 μM) for 60 min before administration of bifemelane (1000 μM). Mean with standard error indicated. The ATP release was blocked by the treatment (****P<0.001, Student t-test). Ordinate in each graph indicates the relative amount of ATP release to the resting level (in this series of experiments, the resting ATP level was 25.3 ± 11.5 nM) (n = 4).

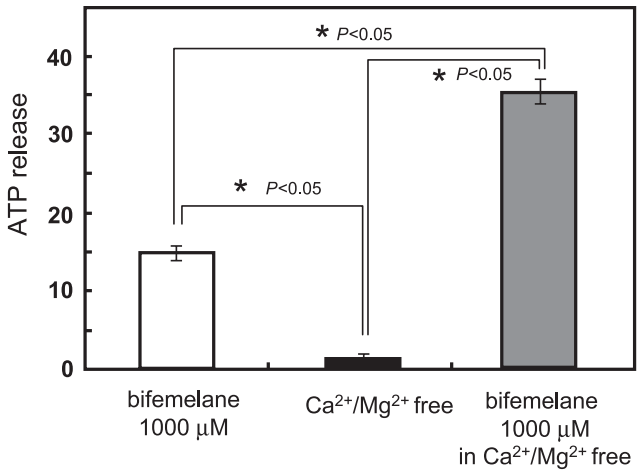

Fig. 8. Effects of Ca^{2+}/Mg^{2+}-free medium on resting and bifemelane-induced ATP-release. Effects of Ca^{2+}/Mg^{2+}-free medium and bifemelane (1000 μM) containing Ca^{2+}/Mg^{2+}-free medium on ATP release from the cells was examined. Mean with standard error indicated. This set of experiments was performed as in Fig. 5. The were statistically analyzed by a post-hoc test (Tukey method). The difference between each set of comparison was statistically significant (*P<0.05, n = 4).

astrocytes, characteristics of the effects on $[Ca^{2+}]_i$ were almost the same as those found in rat astrocytes (5). The effects of bifemelane were dose-dependent. At the highest concentration, the drug induced a second peak during washing out of the drug as had been observed in rat astrocytes. Since the first peak partially remained in the Ca^{2+}-free medium and was blocked by 2-APB, an IP_3-receptor blocker, the source for the increase was the endoplasmic reticulum, the intracellular Ca^{2+} store site. However, since the second peak during washing out of the drug was found to be abolished in Ca^{2+}-free medium and could be induced by exchanging the washing medium with normal Ca^{2+} medium, it can be estimated that Ca^{2+} entered the cells through store-operated Ca^{2+} channels (5, 13).

Since our data demonstrated that carbachol and histamine, which caused a strong increase in $[Ca^{2+}]_i$, had no effects on the ATP release, the increase in $[Ca^{2+}]_i$ level did not always participate in ATP release from astrocytes. These results suggested that the potential mechanism of action is the indirect effect of the drug on the cells through the release of ATP. The possibility was confirmed by the antagonizing effect of PPAD, a P2-purinergic receptor antagonist.

Next we examined the mechanisms for ATP release from the cells. Recent studies demonstrated the diversity of the mechanisms for release of neurotransmitters and trophic factors from glial cells. Some are dependent upon the same Ca^{2+}-dependent mechanisms as those for neurotransmitter release from the presynaptic nerve terminal (14, 15). However, some mechanisms other than the Ca^{2+} dependent vesicular release have been reported, including ATP receptor channels (16), gap junction hemi-channels (17, 18), volume sensitive anion channels (19 – 21), and also the reverse activation of transporters (22).

Strong inhibitory effects of BAPTA, an intracellularly loadable Ca^{2+} chelator, on the bifemelane induced Ca^{2+} release and also the significant inhibitory effects of divalent-cation-free medium on resting Ca^{2+} release suggest the participation of a Ca^{2+}-dependent process on the ATP release from the cells. However, the significant stimulating effects of bifemelane on the cells treated with divalent cation-free medium suggested other processes that may be dependent upon the extracellular divalent cations, such as gap-junction hemi-channels, which also participate in the release. There may be some mechanism for releasing ATP that is partially Ca^{2+}-examined the role of anion channels and the $P2X_7$ receptor as a channel for ATP release, we had not obtained conclusive results yet. The mechanism of transmitter release from astrocytes seemed to be much more complicated than that of neurotransmitter release from the nerve terminal.

Spontaneous ATP release from astrocytes participates in brain function as one of the broad inhibitory factors (23). In the present study, we found small, but obvious ATP release into the extracellular medium without stimulation. The amount of ATP in the extracellular space of the normal brain may alter from time to time dependent upon the environmental condition that will be determined by synaptic and also astorcytic activities. The released ATP will further modulate the synaptic activities. Such dynamic and broad regulatory systems

that exist in the brain may be important to establish stable brain functions.

As an ATP release accelerator, bifemelane will cause the astrocyte to open some of channels for ATP release and then the released ATP may activate ATP receptors such as the P2 purinergic receptors, which will regulate the synaptic functions and subsequent brain functions widely. Since the effective doses were far higher than those reported as therapeutic, it is difficult to ascribe the observed effect to its therapeutic effects. However, bifemelane may be a lead-compound in the search for new therapeutic drugs for controlling brain function through the activation of astrocytes. Furthermore, since our previous study demonstrated the novel inhibitory effect of bifemelane on the capacitive Ca^{2+} entry in astrocytes, the drug could be used as one of the tools for elucidating the roles of astrocytes in the brain.

Acknowledgments

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