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Single type-2 astrocytes show multiple independent sites of Ca^{2+} signaling in response to histamine

(H_1 receptors/inositol 1,4,5-trisphosphate/Ca^{2+} signal compartmentalization)

NAOYUKI INAGAKI*†, HIROYUKI FUKUI*, SEIJI ITO‡, ATSUSHI YAMATODANI*, AND HIROSHI WADA*

*Department of Pharmacology II, Faculty of Medicine, Osaka University, Yamadaoka 2-2, Suita 565, Japan; and ‡Department of Cell Biology, Osaka Bioscience Institute, Furuedai 6-2-4, Suita 565, Japan

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ABSTRACT **Intracellular Ca^{2+} plays an important role in signal transduction as a second messenger. In various types of cells, inositol 1,4,5-trisphosphate-induced elevations of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) have been reported to be uniform in single cells or originate at discrete sites from which they then propagate throughout the cells. These observations so far imply that a single cell functions as a minimal unit for inositol 1,4,5-trisphosphate-induced Ca^{2+} signaling. In this study, we examined the effects of histamine on $[Ca^{2+}]_i$ of type-2 astrocytes using fura-2-based digital imaging fluorescence microscopy and found an unusual type of Ca^{2+} signaling in these cells. Histamine induced $[Ca^{2+}]_i$ elevation in type-2 astrocytes by means of histamine H_1 receptors. Submaximal concentrations of histamine (10^{-7}–10^{-6} M) evoked multiple sites of oscillatory $[Ca^{2+}]_i$ elevation in single type-2 astrocytes. These Ca^{2+} "hot spots" were localized in the processes of the astrocytes but not in the cell bodies. The time courses of $[Ca^{2+}]_i$ oscillations in different hot spots were not synchronized, indicating that each of them formed an independent compartment of Ca^{2+} signaling. When higher concentrations (10^{-5}–10^{-4} M) of histamine were added, $[Ca^{2+}]_i$ in the processes remained elevated at high levels and $[Ca^{2+}]_i$ elevations propagated from the processes to the cell bodies. These results suggest that individual processes of type-2 astrocytes can form minimal units for Ca^{2+} signaling in response to submaximal concentrations of histamine and that single type-2 astrocytes may function as multiple units for Ca^{2+} signaling.**

The activity of the central nervous system can be attributed to signal transmission among its constituent cells, and Ca^{2+} plays an important role in signal transduction as an intracellular second messenger. One of the major pathways for the elevation of intracellular free calcium concentration ($[Ca^{2+}]_i$) is triggered by receptor-mediated formation of inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3], which induces intracellular Ca^{2+} mobilization and extracellular Ca^{2+} influx (1–3). Recent progress in measuring $[Ca^{2+}]_i$ using calcium-sensitive indicators and digital imaging fluorescence microscopy enabled us to study the spatio-temporal dynamics of Ca^{2+} signals within single cells (4, 5). Previous studies with this technique have shown that Ins(1,4,5)P_3-induced $[Ca^{2+}]_i$ elevations in single cells are uniform (2), indicating that a single cell functions as a minimum unit for Ins(1,4,5)P_3-induced Ca^{2+} signaling. In some cases, Ins(1,4,5)P_3-induced $[Ca^{2+}]_i$ elevations originate at discrete regions in the cell and then propagate throughout the cell in the form of waves (2, 6). Spatial heterogeneity in the amplitude of Ins(1,4,5)P_3-induced $[Ca^{2+}]_i$ elevation within cells has also been reported (7). However, these temporal and spatial gradients of $[Ca^{2+}]_i$ elevation have been attributed to unequal distribution of either receptors, enzymes responsible for metabolizing Ins(1,4,5)P_3, or Ins(1,4,5)P_3-sensitive Ca^{2+} pools (1, 2) but not to multiple sites of Ca^{2+} signaling within the cells.

Type-2 astrocytes are a recently identified cell population in the central nervous system that exhibit a characteristic morphology *in vitro*—namely, small cell bodies with long radial processes (8–10). Recently, type-2 astrocytes have been shown to express glutamate receptors (11, 12), suggesting that they are also involved in cell-to-cell communication in the central nervous system. Histamine acts as one of the signal messengers released from neurons (13–15), and previous studies have shown that mixed cultures of type-2 and type-1 astrocytes express histamine H_1 receptors (16, 17), activation of which produces Ins(1,4,5)P_3 (14). In the present study, we examined the effects of histamine on $[Ca^{2+}]_i$ of type-2 astrocytes by fura-2-based digital imaging fluorescence microscopy (4, 5). Histamine induced $[Ca^{2+}]_i$ elevation in type-2 astrocytes by means of H_1 receptors. Furthermore, we observed multiple independent sites of Ca^{2+} signaling within single astrocytes. These phenomena may be related to the morphology and functions of these cells.

MATERIALS AND METHODS

Preparation of Cell Cultures. Primary cultures of type-2 astrocytes were prepared from the cerebrum, where dense histaminergic fibers distribute in the brain (18, 19), as reported previously (20). Newborn Wistar rats were killed by decapitation. The cerebral hemispheres were collected in Joklik's modified minimum essential medium (MEM) under sterile conditions and the meninges were carefully removed using an operation microscope. The cerebral hemispheres were triturated mechanically with a pipette and then dissociated enzymatically with dispase (neutral protease, dispase grade II) solution (3 mg/ml) in Joklik's modified MEM as reported (17). The dissociated cells in the culture medium (Eagle's MEM containing 10% fetal calf serum, 100 units of penicillin G per ml, and 60 μg of kanamycin per ml) were seeded in culture flasks (75 cm^2) at a density of 1×10^5 cells per cm^2. The cultures were incubated at 37°C in a humid atmosphere of 95% air/5% CO_2; the medium was changed the next day and twice a week thereafter. After 2 weeks *in vitro*, the small process-bearing O-2A progenitor cells (21) grown on the top of the type-1 astrocyte monolayer were removed mechanically by shaking the culture flasks overnight, seeded onto glass coverslips attached to silicon walls (Heraeus Flexiperm Disc), and subcultured in the same medium containing fetal calf serum for differentiation into type-2 astrocytes (21, 22). After 7 days, >70% of the cultured cells exhibited the characteristics of type-2 astrocytes—i.e., they were stellate cells immunoreactive to antiglial fibrillary acid protein (GFAP) and A2B5 (23) antibodies (8). Type-2 astro-

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Abbreviations: $[Ca^{2+}]_i$, intracellular free calcium concentration; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate.

†To whom reprint requests should be addressed.

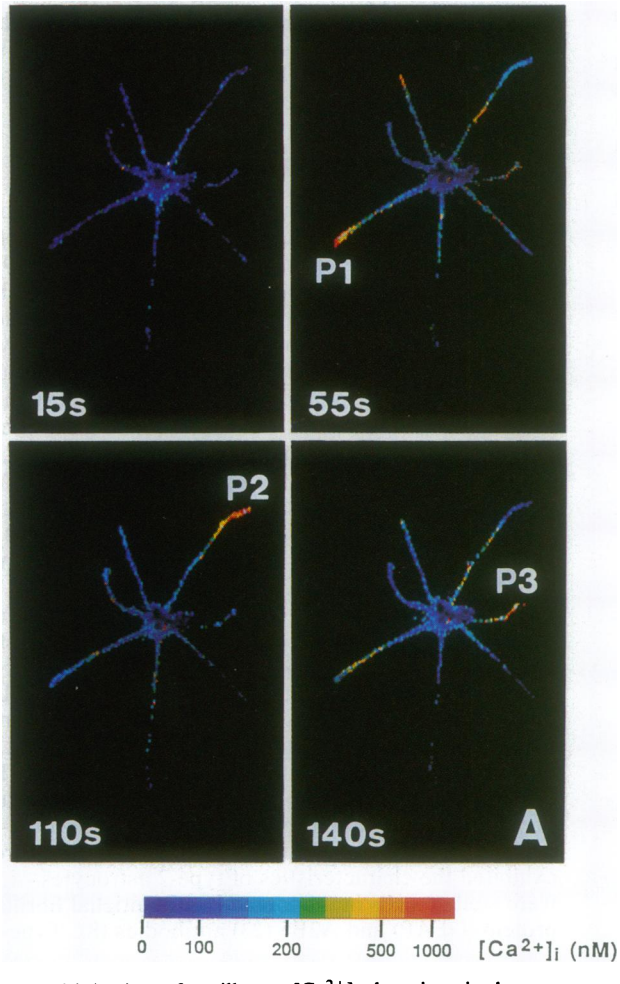
cytes were distinguished morphologically as stellate cells under a phase-contrast microscope; minor cells with fine networks of processes (9, 10) were discarded as oligodendrocytes. About 96% of these stellate cells were GFAP positive and 90% were A2B5 positive when subsequently checked immunocytochemically.

Measurement of $[Ca^{2+}]_i$ in Single Cells. The $[Ca^{2+}]_i$ of single cells was measured as reported (4, 5). The cells were incubated with fura-2 AM (1 μM) diluted in the culture medium for 30 min and washed with the medium for 10 min. A coverslip was placed on a thermostat-regulated stage (37°C) of an Olympus IMT-2 inverted microscope. Drugs were dissolved in Hepes-buffered Krebs–Ringer solution [consisting of the following (in mM): NaCl, 115; KCl, 5.4; $CaCl_2$, 2; $MgCl_2$, 0.8; glucose, 13.8; Hepes, 20 (pH 7.4)] and applied to the cells on glass coverslips. Fluorescence excitation was provided from a Hamamatsu 75-W Xe lamp, and excitation wavelengths of 340 nm and 380 nm were selected by computer-controlled movement of filters in the light path. Paired recordings were made every 2–10 s, and fluorescence images were obtained using a Hamamatsu SIT camera C2400-08h and stored in a digital image processor Argus-100. $[Ca^{2+}]_i$ was calculated from the ratio of the fluorescence intensities obtained with excitations at 340 nm and 380 nm on a pixel basis (4).

Estimations of the Potencies of Histamine Agonists and Antagonists. As described in *Results*, not all cell bodies responded to histamine, and the temporal patterns and amplitudes of the $[Ca^{2+}]_i$ elevation varied in individual cells. So, for exact analysis of the potencies of histamine agonists, we calculated the mean $[Ca^{2+}]_i$ during stimulations with ligands instead of simple calculations of peak $[Ca^{2+}]_i$. Furthermore, we stimulated the astrocytes with 10^{-4} M histamine as a control 250 s after the first stimulation with agonists (see Fig. 3 *A* and *B*) and obtained the potencies of the agonists relative to the potency of histamine in the second stimulation by dividing the mean $[Ca^{2+}]_i$ during the first agonist stimulation by the mean $[Ca^{2+}]_i$ during the control stimulation. Similarly, we estimated the potency of histamine antagonists by calculating the relative $[Ca^{2+}]_i$. The relative $[Ca^{2+}]_i$ was obtained by dividing the mean $[Ca^{2+}]_i$ during the second stimulation by 10^{-4} M histamine in the presence of antagonists by the mean $[Ca^{2+}]_i$ during the first control stimulation with 10^{-4} M histamine (see Fig. 3*C*).

RESULTS

Histamine induced $[Ca^{2+}]_i$ elevation in type-2 astrocytes. First, we examined the spatial distribution of the $[Ca^{2+}]_i$ elevation in the processes and cell bodies of the cells by raising the spatial resolution of image analysis at the expense of temporal resolution. The basal $[Ca^{2+}]_i$ in the astrocytes was about 100 nM and was uniform throughout the cells (Fig. 1*A*). When they were stimulated with submaximal concentrations (10^{-7}–10^{-6} M) of histamine, the majority of the cells showed $[Ca^{2+}]_i$ elevation in the processes but not in the cell bodies. Histamine at 10^{-6} M induced $[Ca^{2+}]_i$ elevation in 62% of type-2 astrocytes (n = 42). Of 26 astrocytes that responded to 10^{-6} M histamine, 18 cells (70%) showed $[Ca^{2+}]_i$ elevations only in the processes and 8 cells (30%) showed $[Ca^{2+}]_i$



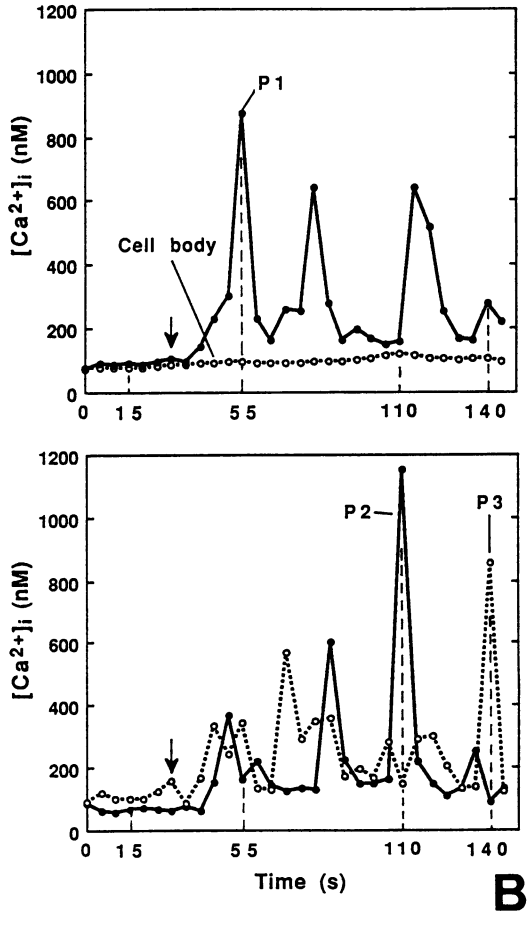


FIG. 1. Multiple sites of oscillatory $[Ca^{2+}]_i$ elevations in the processes of a single type-2 astrocyte induced by 10^{-6} M histamine. (*A*) Serial images of $[Ca^{2+}]_i$ of a type-2 astrocyte stimulated by 10^{-6} M histamine. (*B*) Time courses of $[Ca^{2+}]_i$ elevations of hot spots in the processes (P1, P2, P3) and the cell body of the cell in *A*. The arrows indicate the application time of histamine. (×350.)

elevations in the processes and cell bodies. In typical cases, multiple sites of $[Ca^{2+}]_i$ elevation were observed in the processes of single type-2 astrocytes (Fig. 1*A*), and these "hot spots" showed oscillatory $[Ca^{2+}]_i$ elevations (Fig. 1*B*). Peak $[Ca^{2+}]_i$ of the hot spots usually reached >500 nM and sometimes >1000 nM (Fig. 1*B*). The time courses of $[Ca^{2+}]_i$ in different Ca^{2+} hot spots were not synchronized, indicating that each of them formed an independent compartment of Ca^{2+} signaling (Fig. 1*B*). The hot spots were usually localized in the terminals of the processes (Fig. 2*A*). However, in some cases, they were also observed in more proximal regions of long processes, and occasionally two independent hot spots occurred within a single process.

When higher concentrations (10^{-5}–10^{-4} M) of histamine were added to type-2 astrocytes, the temporal pattern of $[Ca^{2+}]_i$ elevation in the processes of most cells changed from an oscillatory pattern to a sustained one (Fig. 2*B*), and $[Ca^{2+}]_i$ elevations propagated from the processes to the cell bodies (Fig. 2*A*). About 73% of type-2 astrocytes responded to 10^{-5} M histamine (n = 56). In 35 (85%) of the 41 cells that responded to 10^{-5} M histamine, Ca^{2+} signals were first observed in the processes and were then propagated to the cell bodies. The time lag between $[Ca^{2+}]_i$ elevations in the processes and the cell bodies ranged from 2 s to 14 s, with a mean value of 5.2 ± 0.7 s. In 4 cells (10%) of the 41 cells, the time lag was <2 s and therefore could not be detected. In 2 cells (5%), $[Ca^{2+}]_i$ elevations occurred only in the processes.

We further examined the histamine receptor subtypes involved in the $[Ca^{2+}]_i$ elevation. Because measurement of $[Ca^{2+}]_i$ in the processes required high spatial resolution of image processing at the expense of temporal resolution, pharmacological characterization of $[Ca^{2+}]_i$ in the processes was difficult. Therefore, we instead pharmacologically characterized the $[Ca^{2+}]_i$ elevation in the cell bodies. Because not all cell bodies responded to histamine and the amplitudes of the $[Ca^{2+}]_i$ elevation varied in individual cells, we stimulated the astrocytes with 10^{-4} M histamine as a control 250 s after the first stimulation with agonists (Fig. 3 *A* and *B*) and examined the potencies of the agonists relative to that of histamine in the second stimulation (see *Materials and Methods*). Repeated addition of 10^{-4} M histamine gave similar temporal patterns of $[Ca^{2+}]_i$ elevation, but the mean concentration of the second $[Ca^{2+}]_i$ elevation was about 70% of that of the first, possibly due to partial desensitization (Fig. 3*A*). Therefore, the relative potency of 10^{-4} M histamine was 145% ± 10% (Fig. 3*D*). Histamine induced a dose-dependent $[Ca^{2+}]_i$ elevation (Fig. 3*D*). The H_1 agonists 2-methylhistamine (10^{-3} M), 2-(2-thiazolyl)ethylamine (10^{-3} M) (Fig. 3*B*), and 2-(2-pyridyl)ethylamine (10^{-3} M) also induced $[Ca^{2+}]_i$ elevation. However, the H_2 agonists dimaprit (10^{-3} M) and impromidine (10^{-4} M) and the H_3 agonist α-methylhistamine (10^{-6} M) did not induce it (Fig. 3*D*).

As in the agonist study, we estimated the potency of histamine antagonists by calculating the relative $[Ca^{2+}]_i$. All H_1 antagonists examined—namely, mepyramine, D-chlorpheniramine, and L-chlorpheniramine—inhibited the histamine-induced $[Ca^{2+}]_i$ elevation dose dependently (Fig. 3 *C*, *E*, and *F*). Furthermore, D- and L-chlorpheniramine showed stereoselectivity in the inhibitions (Fig. 3*F*). The H_2 antagonist famotidine (10^{-4} M) and the H_3 antagonist thioperamide did not inhibit the $[Ca^{2+}]_i$ elevation (Fig. 3*E*). These results indicate that the $[Ca^{2+}]_i$ elevation observed here was induced by Ins(1,4,5)P_3 through activation of H_1 receptors (14).

We also examined whether histamine-induced $[Ca^{2+}]_i$ elevation occurred through another major pathway for $[Ca^{2+}]_i$ elevation, voltage-sensitive Ca^{2+} channels, because voltage-sensitive Ca^{2+} currents have been demonstrated in type-2 astrocytes (24, 25). The L-type voltage-sensitive Ca^{2+} channel blockers nifedipine (10^{-5} M) and nilvadipine (10^{-5} M) and the N-type voltage-sensitive Ca^{2+} channel blocker ω-cono-

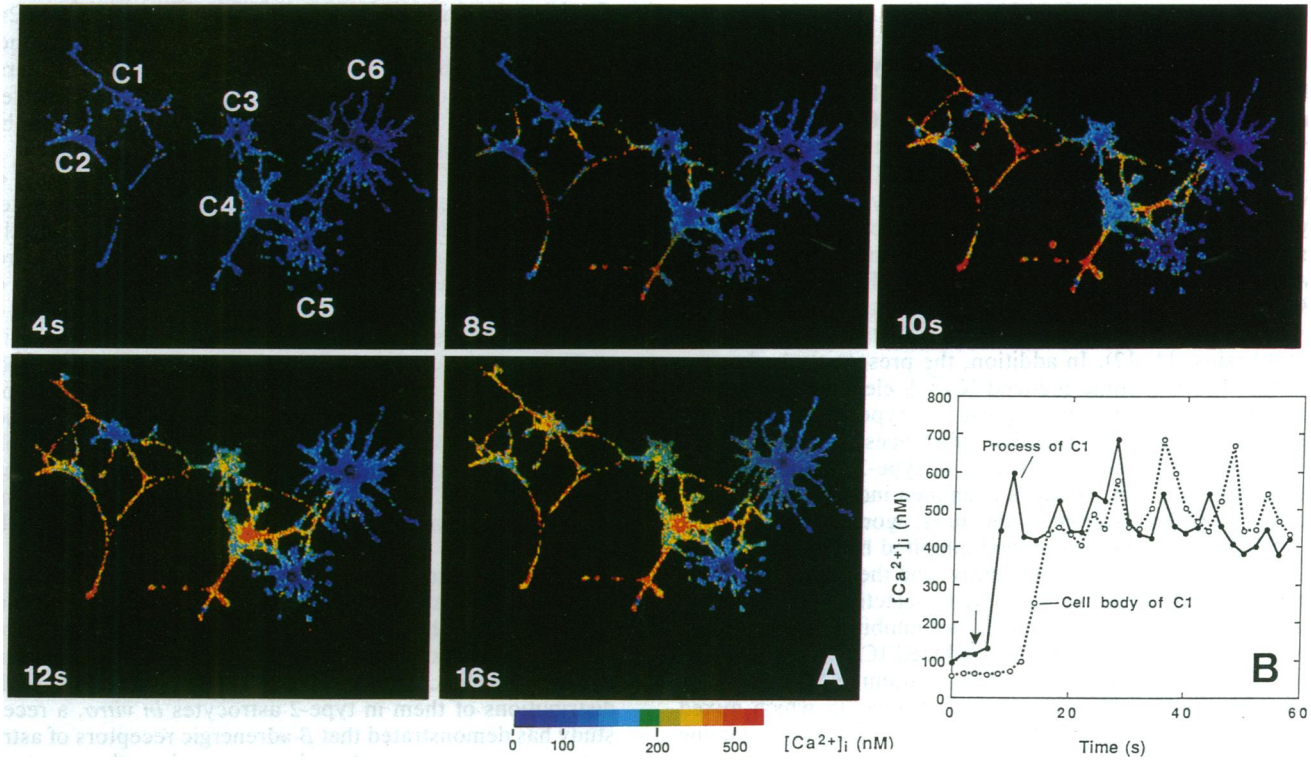


FIG. 2. Propagation of Ca^{2+} waves from the processes to the cell bodies of type-2 astrocytes induced by 10^{-5} M histamine. (*A*) Serial images of $[Ca^{2+}]_i$ in type-2 astrocytes stimulated by 10^{-5} M histamine. In four cells (C1–C4), $[Ca^{2+}]_i$ elevation first occurred in the processes and Ca^{2+} waves were propagated to the cell bodies in several seconds. In one cell (C5), $[Ca^{2+}]_i$ elevation occurred only in some processes. One cell (C6) showed a slight increase of $[Ca^{2+}]_i$. (*B*) Time courses of $[Ca^{2+}]_i$ elevations in a process and the cell body of C1. The arrow indicates the application time of histamine. (×280.)

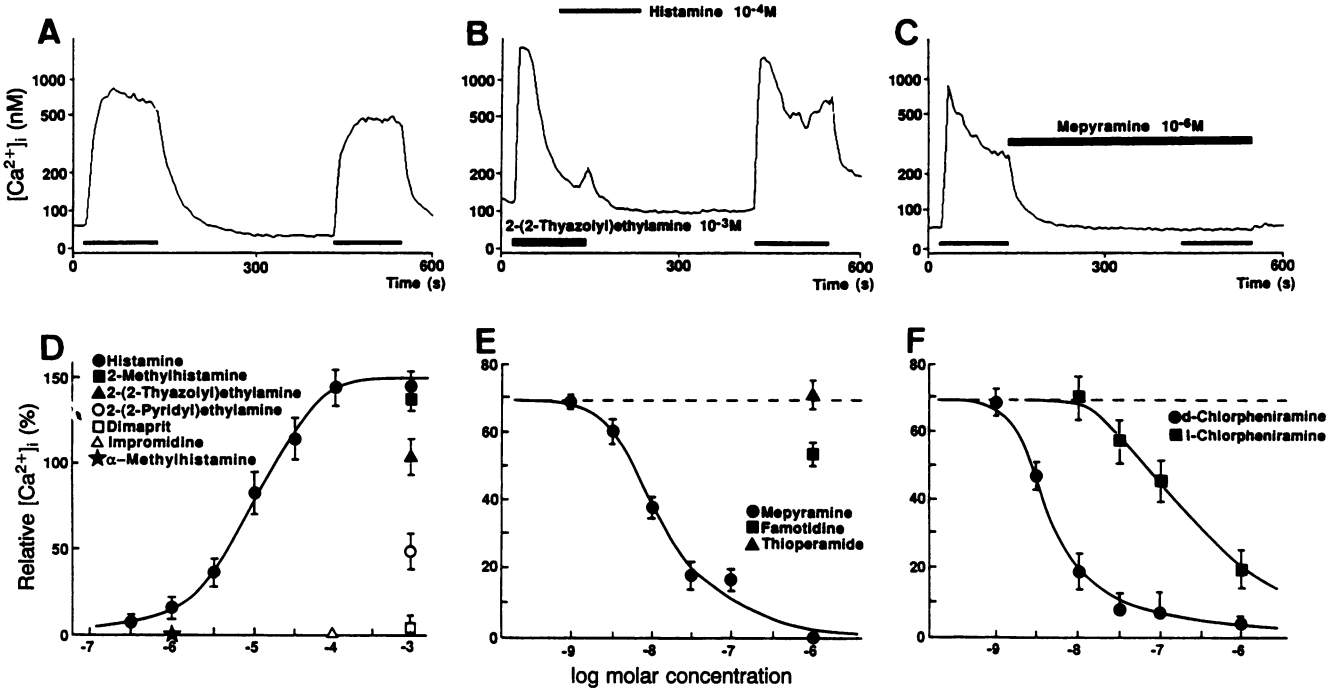


FIG. 3. Pharmacological characterization of histamine-induced $[Ca^{2+}]_i$ elevation in type-2 astrocytes. (*A*) Similar temporal patterns of Ca^{2+} elevations induced by repeated additions of 10^{-4} M histamine with an interval of 250 s. (*B*) $[Ca^{2+}]_i$ elevation induced by 2-(2-thyazolyl)ethylamine (10^{-3} M). (*C*) Inhibition of histamine-induced $[Ca^{2+}]_i$ elevation by mepyramine (10^{-6} M). (*D*) Relative potencies of histamine and histamine agonists for the elevation of $[Ca^{2+}]_i$ in type-2 astrocytes. (*E* and *F*) Relative potencies of histamine antagonists for the inhibition of histamine (10^{-4} M)-induced $[Ca^{2+}]_i$ elevation in type-2 astrocytes. All $[Ca^{2+}]_i$ values were measured from the cell bodies of the astrocytes. Values (*D–F*) are means ± SEM obtained from at least six independent experiments.

toxin (10^{-6} M) had no effect on the $[Ca^{2+}]_i$ elevation induced by 10^{-4} M histamine (data not shown).

DISCUSSION

Histamine Induces $[Ca^{2+}]_i$ Elevation in Type-2 Astrocytes by Means of H_1 Receptors by Producing Ins(1,4,5)P_3. Accumulating reports using primary cultures of astrocytes have shown that astrocytes are potential targets for various neuroactive substances including histamine (17, 26, 27). However, these studies have not shown clearly that type-2 astrocytes are targets for neurotransmitters, because conventional preparations of astrocytes consist of a small population of type-2 astrocytes and a large population of type-1 astrocytes (8, 21). Recently, the existence of excitatory amino acid receptors was demonstrated on type-2 astrocytes from neonatal brains (11, 12). In addition, the present study demonstrated that histamine induced $[Ca^{2+}]_i$ elevations in type-2 astrocytes. These results suggest that type-2 astrocytes are targets for various neuroactive substances.

Histamine elevated the $[Ca^{2+}]_i$ of type-2 astrocytes dose dependently. All H_1 agonists examined induced $[Ca^{2+}]_i$ elevation, whereas H_2 agonists and an H_3 agonist did not induce it. All H_1 antagonists examined inhibited histamine-induced $[Ca^{2+}]_i$ elevation dose dependently, and the inhibitions by D- and L-chlorpheniramine were stereoselective. On the other hand, H_2 and H_3 antagonists did not inhibit it. These results clearly indicate that histamine-induced $[Ca^{2+}]_i$ elevation in type-2 astrocytes is mediated by histamine H_1 receptors, being consistent with the previous studies in which mixed cultures of type-2 and type-1 astrocytes expressed histamine H_1 receptors at high density (16, 17).

Histamine H_1 receptors are regarded to be coupled to phospholipase C, which catalyzes the formation of Ins(1,4,5)P_3 and diacylglycerol (14). We have confirmed this coupling of H_1 receptors in type-2 astrocytes by demonstrating that histamine induces Ins(1,4,5)P_3 formation in type-2 astrocyte-enriched cultures by means of H_1 receptors (H. Kondou, N.I., H.F., Y. Koyama, A. Kanamura, and H.W., unpublished data). Recent studies have shown the existence of voltage-sensitive Ca^{2+} currents in type-2 astrocytes (24, 25). However, the $[Ca^{2+}]_i$ elevation was affected by neither L-type nor N-type voltage-sensitive Ca^{2+} channel blockers. These results indicate that the $[Ca^{2+}]_i$ elevation observed here was caused by the formation of Ins(1,4,5)P_3 but not by Ca^{2+} influx through the voltage-sensitive Ca^{2+} channels.

Ca^{2+} Signal Compartmentalization in the Processes of Type-2 Astrocytes. The most remarkable and unexpected results of the present study were obtained by examining the spatial distribution of the $[Ca^{2+}]_i$ elevation. When the astrocytes were stimulated with submaximal concentrations of histamine, which are probably physiological, multiple sites of $[Ca^{2+}]_i$ elevations occurred in single type-2 astrocytes (Fig. 1). These sites were localized in the processes but not in the cell bodies. The time courses of $[Ca^{2+}]_i$ in these hot spots showed oscillatory patterns, and those in different processes were not synchronized even within the same cell. These results indicate that each hot spot formed an independent compartment of Ca^{2+} signaling. Several possible mechanisms of Ca^{2+} signal compartmentalization in the processes may be considered.

(*i*) The processes are more sensitive than the cell bodies to histamine stimulation. This may be because either histamine H_1 receptors, phospholipase C, or Ins(1,4,5)P_3-sensitive Ca^{2+} pools are distributed more densely in the processes than in the cell bodies. Although little is known about the spatial distributions of them in type-2 astrocytes *in vitro*, a recent study has demonstrated that β-adrenergic receptors of astrocytes *in vivo* are more densely expressed on the processes than on the cell bodies (28). Alternatively, the cell bodies may have more efficient $[Ca^{2+}]_i$ buffering activities than the processes due to a higher volume/cell surface ratio.

(*ii*) The compartmentalization phenomenon may also be due to factors limiting diffusion of potential mediators of Ca^{2+}

waves, such as Ca^{2+} and Ins(1,4,5)P_3 (2). The diameters of the processes are very small. Therefore, a long time will be required for the mediators to diffuse to the cell bodies, in contrast to the case of spherical cells, in which the mediators can rapidly diffuse throughout the cell (2, 6). In addition, Ca^{2+} oscillation enables signaling, maintaining an average $[Ca^{2+}]_i$, and possibly the concentration of Ins(1,4,5)P_3, at low levels (29, 30). Therefore, the low average concentrations of the mediators in Ca^{2+} hot spots may prevent $[Ca^{2+}]_i$ elevations from spreading to neighboring regions. When type-2 astrocytes were stimulated by higher doses of histamine, the temporal pattern of $[Ca^{2+}]_i$ elevation in the processes changed from an oscillatory pattern to a sustained one. A similar dose-dependent shift of $[Ca^{2+}]_i$ elevation from an oscillatory to a sustained pattern has been reported in H_1 receptor-mediated $[Ca^{2+}]_i$ elevation in endothelial cells (31). During the sustained $[Ca^{2+}]_i$ elevation, average $[Ca^{2+}]_i$ in the processes was maintained at high levels and $[Ca^{2+}]_i$ elevation propagated from the processes to the cell bodies within several seconds (Fig. 2). Thus oscillatory $[Ca^{2+}]_i$ elevation may be one of key mechanisms for Ca^{2+} signal compartmentalization.

Functional Implications. Is neuron type-2 astrocyte signal transmission important for maintaining the activities of the central nervous system? Does Ca^{2+} signal compartmentalization in the processes have an advantage for the functions of type-2 astrocytes? Little is known about the functions of type-2 astrocytes in the brain. However, their processes are reported to form endfeet on nodes of Ranvier in the optic nerve (9, 10). Some histaminergic axons are myelinated (32) and type-2 astrocytes are mainly distributed in the white matter of the brain (8). Therefore, the processes of type-2 astrocytes may terminate on nodes of Ranvier of axons containing histamine and other neurotransmitters in the brain. At least it is conceivable that the processes of type-2 astrocytes make contacts with some neuronal elements in the brain. If so, these processes may be important for maintaining or regulating the activities of neurons. The processes may take up and metabolize neurotransmitters (33–35), regulate local ionic concentrations (36, 37), or supply nutrients to neurons (38). It is noteworthy in this respect that the endfeet of retinal glial cells are reported to have higher densities of ion channels that mediate potassium buffering than the cell bodies have (37). A possibility is that the activities of the processes of type-2 astrocytes are regulated by neurotransmitters released from the axons on which they form endfeet (12, 25). When axons get excited, their energy demand and local ionic concentrations will change and demands for inactivating transmitters will emerge. Then the activities of the processes may change by receptor-mediated Ins(1,4,5)P_3-induced Ca^{2+} signaling (1) and possibly by the activation of the diacylglycerol/protein kinase C pathway (39). If individual type-2 astrocytes have endfeet on different axons with different activities, each process must function differently according to the activity of its associated axon. Ca^{2+} signal compartmentalization has a great advantage for maintaining the independence of the Ca^{2+} signaling in individual processes and may enable a single cell to function as multiple Ca^{2+} signal units.

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