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Digital fluorescence imaging of elementary steps of neurosteroid synthesis in rat brain glial cells[1]

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Abstract

With fluorescence microscopic imaging, we have examined Ca^{2+} signaling, LDL uptake and distribution of cytochrome *P*450 scc in individual rat brain glial cells in order to investigate the molecular mechanisms of neurosteroid synthesis. Astrocytes and oligodendrocytes were cultured from newborn rat brain. Ca^{2+} signaling was observed in Calcium Green-1 loaded astrocytes upon neurotransmitter stimulations using video-enhanced microscopy. Upon stimulation of serotonin and glutamate, we observed typically three types of Ca^{2+} signaling which were Ca^{2+} oscillations, a transient increase in Ca^{2+} concentration and Ca^{2+} oscillations superimposed on a transient Ca^{2+} increase. On the other hand, histamine and ATP induced only a transient increase in Ca^{2+} without oscillatory response. Uptake of octadecyl rhodamine (R18) labeled LDL by astrocytes and oligodendrocytes was observed in the time scale of 30 min with confocal laser scanning microscopy. Some localization of LDL in the cytoplasm was observed for astrocytes. For oligodendrocytes, incorporated LDL was distributed over the entire cytoplasmic region of both cell body and multiple branched cell processes. The presence of a significant amount of cytochrome *P*450 scc was demonstrated with immunofluorescence staining in both astrocytes and oligodendrocytes. The density of *P*450 scc in both glial cells was suggested to be around 1% of that in bovine adrenocortical fasciculata cells. The results lead to an improved quantitative picture of neurosteroid synthesis in glial cells. © 1997 Elsevier Science B.V.

Keywords: Fluorescence microscopic imaging; Calcium imaging; LDL uptake; Cytochrome *P*450 scc; Glial cells

1. Introductions

Astrocytes guide neuronal development, metabolize ions and neurotransmitters, and regulate central nervous system vasculature [1]. Although astrocytes have been considered to supportive, rather than transmissive, in the adult nervous

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system, recent studies have challenged this assumption by demonstrating that astrocytes possess functional neurotransmitter receptors. Ca^{2+} signaling in astrocytes was observed to be induced upon stimulation by neurotransmitters such as glutamate, norepinephrine and serotonin [2,3]. These observations imply a primary role of Ca^{2+} signals in neuron-glia communication [4,5]. In fact, when a Ca^{2+} signal was evoked in astrocytes, the signal propagated to induce Ca^{2+} elevation in neurons [6,7]. Oligodendrocytes form myelin sheaths around the larger axons and speed up conduction of the nerve impulse [1]. There are only a few studies on Ca^{2+} signaling in oligodendrocytes. Neuron-glia communication also occurs with neurosteroids, pregnenolone derivatives, which are synthesized in glial cells [8]. Neurosteroids modulate neuron–neuron communication by changing GABA receptor conductivity of Cl^- and are therefore essential for neuronal plasticity [8]. Neurosteroid synthesis was observed in neuroglial cells such as astrocytes and oligodendrocytes [9–12]. Possible candidates of stimuli for neurosteroid synthesis are serotonin, glutamate, histamine and ATP. For these stimulations, Ca^{2+} might play an important role in analogy to steroid hormone synthesis in adrenocortical cells. Upon neurotransmitter stimulation, we speculate that the signal transduction may occur sequentially [11] through hormone receptor activation in the plasma membrane → increase in concentration of Ca^{2+} in the cytoplasm → steroid hydroxylation by cytochrome *P*450 scc in mitochondria. Cholesterol, a substrate for steroidogenesis, may be incorporated into glial cells with lipoprotein via receptor-mediated endocytosis. Cholesterol would then be transported to mitochondira where cytochrome *P*450 scc cleaves a side-chain of cholesterol, resulting in pregnenolone production. Pregnenolone would be finally transformed into pregnenolonesulfate by sulfotransferase in the cytoplasm.

So far, there have only been a few qualitative studies of the elementary steps in neurosteroid synthesis. The mechanism for cholesterol uptake is unknown. The receptor of low density lipoprotein (LDL) was suggested to participate in cholesterol uptake of glial cells [13]. However, there are no reports of direct LDL incorporation into individual glial cells. The biochemical examination of cytochrome *P*450 scc was inconclusive, and only the presence of *P*450 scc was suggested with immunofluorescence staining in oligodendrocytes [11,13].

2. Experimental procedures

2.1. Chemicals

Calcium Green-1/AM and R18 were purchased from Molecular Probes (OR, USA). FITC-conjugated goat anti-rabbit IgG was purchased from Jackson Immunoresearch Lab. (USA). Column purified rabbit anti-*P*450 scc antibody was prepared according to the method described elsewhere [14]. Powdered basal medium Eagle (BME) and fetal calf serum were obtained from GIBCO (USA). L-glutamine from BioWittaker (USA). Glass-bottom dishes were obtained from MatTek (USA). All other chemicals were of highest purity commercially available.

2.2. Preparation of glial cell cultures

Primary brain cell cultures were prepared as previously described [15,16]. Briefly, the cerebral hemispheres were dissected and the meninges were removed under microscope. The cerebral tissue was treated with 0.25% trypsin solution for 10 min at 37°C and then dissociated by extrusion through a stainless steel mesh (320 μm poresize, 40-mesh screen) into a tissue culture dish with BME containing 5% calf serum and 0.2% glucose.

The cell suspension was then centrifuged at 800 rpm for 5 min. The cell pellet was resuspended, dispersed by pipetting, and centrifuged again. This procedure was repeated three times. After a final centrifugation, the cell pellet was resuspended (1×10^6 cells ml^{-1}) in BME solution containing 5% calf serum, 0.4% glucose, 0.3% $NaHCO_3$, 292 μg ml^{-1} glutamine, and 100 μg ml^{-1} kanamycin in a tissue culture dish. After 2–4 weeks of culture, when confluence had been attained, the cultured cells were trypsinized, dispersed in BME solution, and replated at a density

of 1×10^6 cells ml^{-1}. Parallel cultures were used for immunofluorescence staining for the identification of astrocytes and oligodendocytes as previously described [15,16]. The culture medium was renewed on the second day of the culture. For microscopic observation, cells were plated onto 35 mm ϕ glass-bottom dishes previously coated with poly-L-lysine. The cells were cultured for 10–30 days at a cell density of 0.8×10^4 cells cm^{-2}.

2.3. Cell loading with Calcium Green-1

Cells were loaded for 10 min at 37°C with 3 μM Calcium Green-1/AM (from 1 mM stock solution in dimethyl sulfoxide) in the presence of 0.02% Pluronic F-127 in 1 ml of Hepes buffer medium pH 7.4, containing 120 mM NaCl, 4.0 mM KCl, 1.25 mM $CaCl_2$, 1.0 mM NaH_2PO_4, 5.0 mM $MgSO_4$, 20 mM Hepes supplemented with 0.1% glucose and 0.1% BSA.

2.4. LDL isolation

LDL was isolated from fresh bovine blood by the modified method of Havel et al. [17] using sequential differential floatation in sodium chloride and sodium bromide solutions. The resultant suspension of LDL was dialyzed to remove sodium bromide against 20 mM Hepes with 150 mM NaCl, 0.01% EDTA, pH 7.4 at 4°C overnight. The sample was stored at 4°C under nitrogen. All the LDL sample was used within 7 days after isolation to avoid denaturation.

2.5. Fluorescence labeling of LDL with R18

R18 was solubilized in dimethyl sulfoxide and 160 μl of the solution (1 mM) was incubated with 2.2 mg ml^{-1} of LDL in a total volume of 1.2 ml at 37°C for 1 h under nitrogen. Unbound R18 was removed by centrifugation gel filtration with Sephadex G-25 column. For each column, 200 μl of the suspension was applied and centrifuged at $3000 \times g$ for 3 min. The elution buffer was 20 mM Hepes pH 7.4 containing 150 mM NaCl.

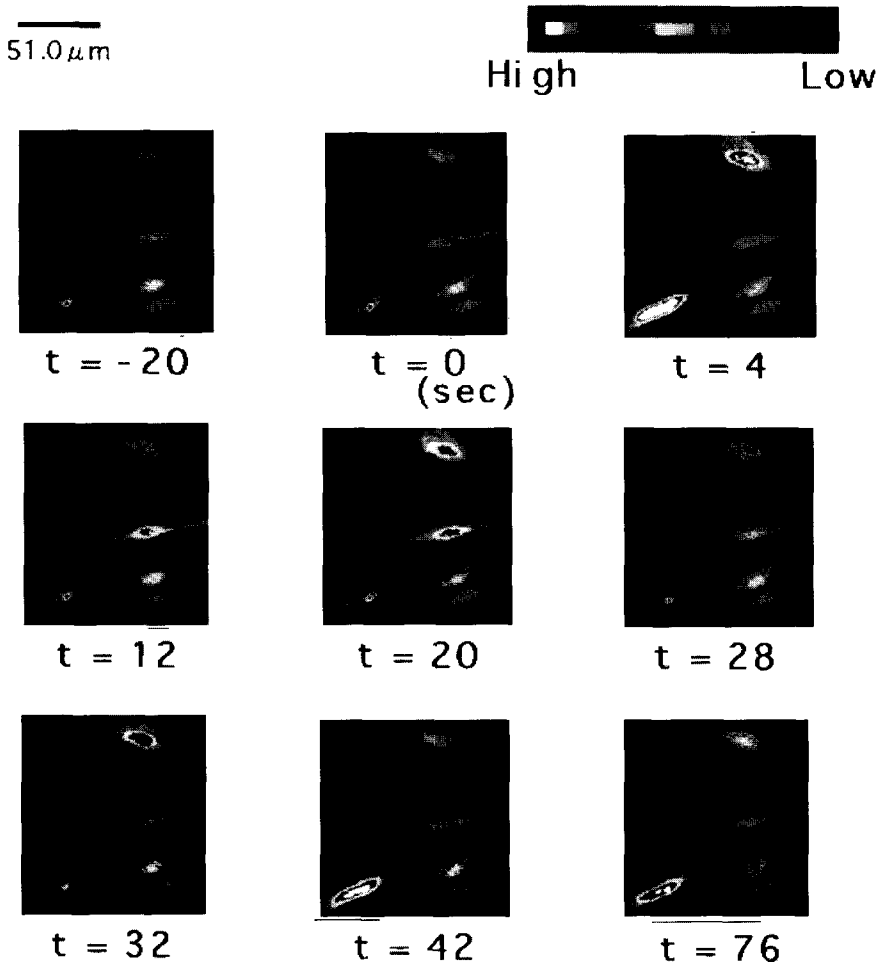
2.6. Fluorescence imaging and analysis

We used a video-enhanced fluorescence microscope which consisted of an inverted microscope (Nikon TMD-300, Japan) equipped with a xenon lamp for excitation and a SIT camera (Hamamatsu Photonics C-1145, Japan). The glass-bottom dishes were mounted on an inverted Nikon microscope equipped with the temperature chamber which maintained the air atmosphere at 37°C with high humidity, using a warm air-supplying system. We used a 40 × special Nikon objective with very low fluorescence background. For Calcium Green-1 and fluorescein-isothiocyanate (FITC), we selected the excitation wavelength of 450–490 nm and fluorescence above 520 nm with IF excitation filter, DM500 dichroic mirror and a BA 520 emission filter. Image analysis was performed with an ARGUS-50 system (Hamamatsu Photonics, Japan). The video output was digitized and the images were stored in frame memory. For generation of time-course traces of fluorescence intensity as shown in Fig. 1, averaging was performed over an area of 10×10 pixels and 15 successive video frames were averaged, resulting in a 0.5 s time resolution.

We used a confocal laser scanning microscope equipped with an argon ion laser (Spectra Physics Stabilite 2016, USA) for excitation and a confocal scan head (Bio-Rad MRC-600 UV, England). This confocal microscope shared the same Nikon inverted microscope as the video-enhanced microscopy. The fluorescence distribution of R18 labeled LDL was measured with the excitation at 514 nm and fluorescence above 550 nm selected with 514DF10 excitation filter, DR540LP dichroic reflector and 550LP emission filter.

2.7. Incorporation of R18 labeled LDL in glial cells

The observation medium contains 20 mM Hepes pH 7.4, 120 mM NaCl, 4 mM KCl, 1 mM NaH_2PO_4, 1.25 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.1% BSA, 0.1% glucose. Glial cells in glass-bottom dishes were rinsed twice with the observation medium, then 20 μl of R18 labeled LDL was


51.0 μm
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Time (sec)

Fig. 1.

applied to glial cells in 1 ml buffer. After incubation of cells with LDL for around 10 min at 37°C in the glass-bottom dish fixed on the microscope stage, we recorded the images successively with the confocal microscope.

2.8. Immunofluorescence and cytochemical staining

All the staining treatments were performed for cell cultures at room temperature on a glass-bottom dish. Cytochrome *P*450 scc was stained with antibodies after fixation of cells with 3% formalin in neutral buffer solution (pH 7.4) for 30 min and followed by membrane permeabilization with 1% Triton X-100 for 4 min. To avoid nonspecific absorption to cells, antibodies to *P*450 scc were pretreated with 0.5% bovine liver powder at 4°C. After application of 30 μg IgG in 1 ml of phosphate buffered saline, cell cultures were incubated for 30 min. Cells were washed 5 times with phosphate buffered saline. Then FITC-conjugated goat anti-rabbit IgG was applied at 1:200 dilution.

For the demonstration of free cholesterol, glial cells were stained for 15 min with 1 μg ml^{-1} filipin after fixation of cells for 10 min with 10% formalin [18]. The distribution of filipin complexed with free cholesterol was measured with a video-enhanced microscope by observing the fluorescence which was excited at 360 nm and collected above 420 nm.

3. Results and discussions

3.1. Neurotransmitter-induced Ca^{2+} signaling

We have observed that the application of serotonin, glutamate, histamine and ATP with concentration of 1–100 μM induced both oscillatory and nonoscillatory responses of the intracellular calcium concentration $[Ca^{2+}]_i$ in rat astrocytes using single cell imaging of Calcium Green-1 loaded cells. As shown in Fig. 1, 1 μM of serotonin induced Ca^{2+} oscillations for about 18% of Ca^{2+}-signaling cells. The oscillations consisted of repetitive Ca^{2+} spikes with frequency around 0.04 Hz. Typical Ca^{2+} oscillations lasted for several minutes. The half width was around 10 s for each repetitive Ca^{2+} spike. The phase of Ca^{2+} oscillations was different between individual cells. Not only stable Ca^{2+} oscillations, but also damped Ca^{2+} oscillations were observed. For about 64% of Ca^{2+}-signaling cells, we observed a transient elevation in Ca^{2+} concentration which consisted of about 10 s rise phase and a gradually decreasing phase for around 30–300 s which varied significantly from one cell to another. For about 18% of Ca^{2+}-signaling cells, we observed Ca^{2+} oscillations superimposed on a transient elevation in Ca^{2+}. The percentage of cells exhibiting Ca^{2+} oscillations was smaller for 100 μM of glutamate than that for serotonin. The relative proportion of cells, which showed different Ca^{2+} signals, was around 7% (oscillations), 70% (transient elevation) and 23% (oscillation plus transient elevation). On the other hand, histamine and ATP induced only a transient elevation of Ca^{2+}, except for only a few % of cells showing Ca^{2+} oscillations for 1 μM histamine stimulation [19,20]. Around half of astrocytes showed Ca^{2+} signaling upon neurotransmitter stimulation with serotonin, glutamate and histamine, while ATP induced Ca^{2+} signaling for almost all astrocytes [21]. For each neurotransmitter stimulation, we performed several independent experiments each of which contained around 20 cells in a microscopic field. There have been extensive investigations for glutamate-induced Ca^{2+} signaling in astrocytes and astrocyte-neuron communication [2,5,6]. On the other hand, the quantitative classification of sero-

Fig. 1. Imaging and time course of Ca^{2+} signaling in astrocytes induced by serotonin stimulation. Panel A, pseudocoloured images demonstrate that the astrocytes show Ca^{2+} signaling upon 1 μM serotonin stimulation at $t = 0$ s. The fluorescence intensity of Calcium Green-1 is indicated with a colour bar from blue (low intensity) to red/white (high intensity). Time t is indicated in seconds. The spatial scale is indicated with a horizontal black bar of 51.0 μm. Panel B, time course of typical Ca^{2+} signals for astrocytes induced by 1 μM serotonin. Curve 1, Ca^{2+} oscillations; curve 2, transient Ca^{2+} elevation; curve 3, Ca^{2+} oscillations superimposed on transient Ca^{2+} elevation. The vertical scale ($\Delta F/F_0$) is the ratio of the fluorescence intensity change ($F-F_0$) to the basal fluorescence F_0 for Calcium Green-1 and the horizontal axis is time in seconds. The arrow indicates the addition of serotonin. Curves 1 and 3 are vertically displaced for clarity in order to avoid overlapping each other.

tonin-induced Ca^{2+} signaling was first demonstrated in the present study, though serotonin was previously shown to induce oscillatory response in astrocytes [4]. We do not report Ca^{2+} signaling measurements for oligodendrocytes, since the number of oligodendrocytes in culture was not sufficient to obtain quantitative results. There are also some previous reports which indicate that oligodendrocytes show Ca^{2+} signaling by glutamate, histamine, norepinephrine and ATP [22–24].

3.2. Uptake of R18 labeled LDL in rat glial cells

Glial cells actively took up R18-LDL. Fig. 2 shows astrocytes and an oligodendrocyte which took up R18-LDL for 20 min. Some localization of LDL in the cytoplasm was observed for astrocytes. On the other hand, in oligodendrocytes, LDL was distributed over an entire cytoplasmic region of both cell body and multiple branched cell processes. The area of cells which were occupied by the fluorescence of LDL in endosomes increased progressively depending on the time after the LDL-application over 30 min. After around 30 min, LDL internalization by cells reached saturation as judged from no further increase of the fluorescent area in cells.

3.3. Distribution of cytochrome P450 scc and free cholesterol

The presence of a significant amount of cytochrome *P*450 scc was demonstrated with immunofluorescence staining with column purified anti-*P*450 scc IgG in both astrocytes and oligodendrocytes (Fig. 3). Some localization of *P*450 scc was observed within the cytoplasmic spaces of astrocytes. On the other hand, for oligodendrocytes, *P*450 scc distributed over the entire cytoplasm of not only cell body but also multiple branched cell processes.

As shown in Fig. 3, we obtained almost the same fluorescence intensity of FITC conjugated with secondary IgG between astrocytes and oligodendrocytes which coexisted in the same preparation, when we applied 30 μg anti-*P*450 scc IgG per 1 ml of phosphate buffered saline. Adrenocortical fasciculata cells showed about 100 times stronger fluorescence of FITC than that of astrocytes, when the same amount of 30 μg anti-*P*450 scc IgG ml^{-1} of phosphate buffered saline was used. It is, therefore, suggested that the relative density of *P*450 scc in astrocytes, oligodendrocytes and adrenocortical fasciculata cells may be around 1:1:100. Localization of free cholesterol was observed mainly in plasma membranes with fluorescence imaging of filipin complexed with free cholesterol using video microscopy (Fig. 3). No strong filipin fluorescence was observed in the cytosol.

3.4. Comparison with steroidogenesis in adrenocortical cells

Rat brain glial cells synthesize neurosteoids, but the mechanisms are poorly investigated as compared with steroidogenesis in adrenal cortex [25–27]. Since both cells show Ca^{2+} signaling, LDL incorporation and *P*450 scc activity, there may be some common mechanisms for steroidogenesis in glial and adrenocortical cells. Although we did not obtain sufficient results to conclude the significance of Ca^{2+} signaling in neurosteroidogenesis of astrocytes, Ca^{2+} signaling was indicated to be a second messenger for adrenocorticotropic hormone (ACTH) action in steroidogenesis of adrenocortical cells [28]. For ACTH-induced Ca^{2+} signaling in adrenocortical fasciculata cells, three types of Ca^{2+} signaling were also observed which were similar to those in astrocytes. They were Ca^{2+} oscillations (33%), a step increase in Ca^{2+} concentration (10%), and Ca^{2+} oscillations superimposed on a step increase in Ca^{2+} (57%). Ca^{2+} oscillations were generated by both the release of Ca^{2+} from ER and influx of extracellular Ca^{2+}, since Ca^{2+} oscillations were abolished not only by chelating extracellular Ca^{2+} with EGTA but also with inhibitors of Ca^{2+} pumps in endoplasmic reticulum such as thapsigargin. The Ca^{2+} signaling was required for corticoid production, because the corticoid producion was considerably reduced by the suppression of Ca^{2+} signaling with EGTA.

In this study, both astrocytes and oligodendrocytes were first showns to take up LDL by means

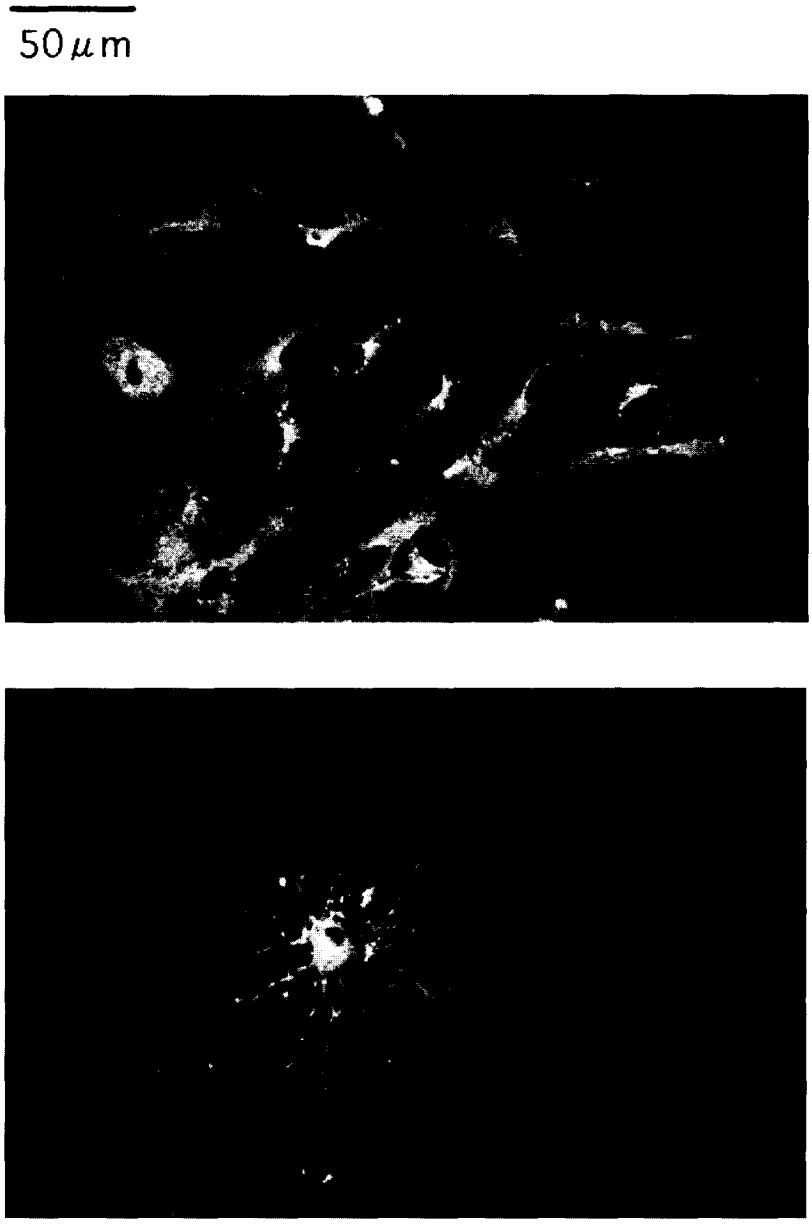

Fig. 2. Time-dependent uptake of R18-LDL by rat glial cells. The confocal fluorescence images are taken at 20 min after the addition of R18-LDL at 37°C. The white area and spots indicate R18-LDL incorporated in glial cells. Upper, astrocytes; Lower, oligodendrocyte. The optical section is set to 0.7 μm. The spatial scale is indicated with a horizontal black bar.

of endocytosis, though the presence of LDL receptors was previously indicated by immunostaining [13].These results strongly suggest that glial cells utilize LDL as a primary source of cholesterol. The rate of LDL-incorporation by glial cells was almost the same as adrenocortical fasciculata

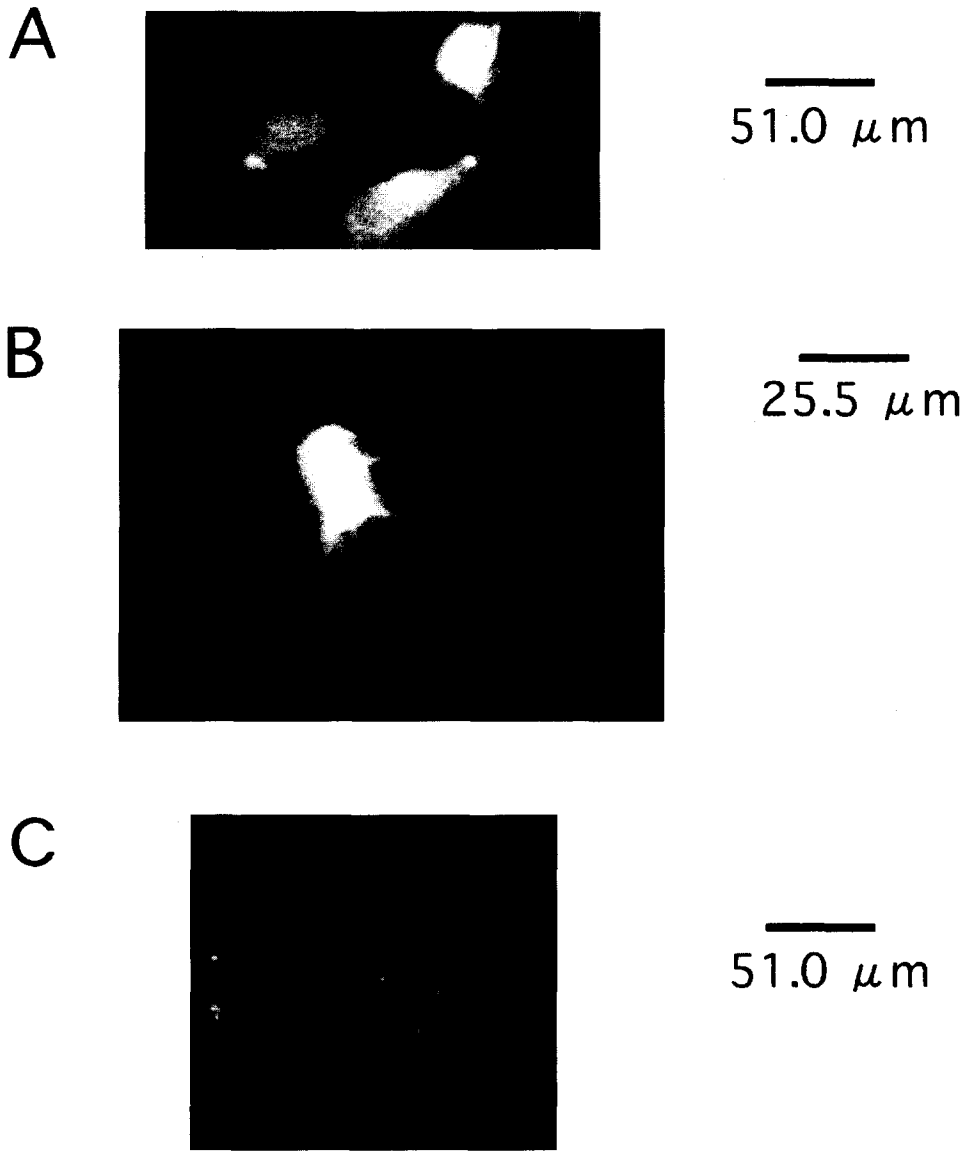

Fig. 3. Fluorescence images of cytochrome *P*450 scc and free cholesterol. Cyotchrome *P*450 scc and free cholesterol were stained and images were taken with the video-enhanced microscope as described under Experimental procedures. A, *P*450 scc in astrocytes; B, *P*450 scc in oligodendrocyte; C, cholesterol in astrocytes. The spatial scale is indicated with black bars.

cells. In both cells, the uptake of R18-LDL occurred in the time scale of 30 min. The distribution of LDL in early endosomes was not very localized but rather delocalized over the entire cytoplasm as judged from cross section images using confocal microscopy. Possible movement of LDL from early endosomes to late endosomes and then to lysosomes should be a subject of further investigations, as well as participation of coated pits and coated vesicles.

Cholesterol distribution was very different between astrocytes and adrenocortical cells. Though a significant amount of free cholesterol was observed in the cytoplasm as well as in plasma membranes for adrenocoritcal fasciculata cells (Kimoto et al., unpublished data), only plasma membranes contained free cholesterol for astrocytes.

The presence of cytochrome *P*450 scc in neuroglial cells was determined with immunofluorescence staining and/or mRNA determination [11–13]. The absolute amount of *P*450 scc was indicated to be considerably smaller in brain glial cells than in adrenocortical cells. The amount of glial *P*450 scc was reported to be 0.01% of that found in adrenal cells from mRNA expression [12]. This number is much smaller than 1% from our determination of *P*450 scc amount. Such a small amount of glial *P*450 scc prevents biochemical analysis of the cytochrome such as pregnenolone production from cholesterol. Large scale primary cultures of glial cells will be necessary for quantitative enzymatic assay of *P*450 scc which will require purified mitochondria or even purified *P*450 scc. Assay of neurosteroid production was, so far, performed with primary cultured glial cells. The enzymatic production was indicated for pregnenolone, pregnenolone, pregn-5-ene-3 beta, 20 alpha-diol [13]. These and other pregnenolone derivatives positively or negatively modulate the GABA-gating of Cl^- channels. However, the detailed characterization of the biosynthetic apparatus in glial cells is far from complete and needs extensive further investigations, when compared with steroidogensis in adrenocortical cells.

4. Conclusion

Essential steps in neurosteroid synthesis were intensively analyzed with real-time digitized microscopy in individual glial cells. Possible second messenger Ca^{2+} signaling was observed to be oscillatory and nonoscillatory upon stimulation with neurotransmitters. Rapid cholesterol incorporation by means of receptor-mediated endocytosis of LDL was indicated by the active uptake of LDL. The presence of cytochrome *P*450 scc in glial cells suggests the enzymatic conversion of cholesterol to pregnenolone.

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References

[1] W. Walz, in H. Ketterman and B.R. Ranson (Eds.), Receptors of Physiology in Neuroglia, Oxford University Press, NY, 1995, pp. 185–319.
[2] J. De Barry, A. Ogura and Y. Kudo, Eur. J. Neurosci., 3 (1991) 1146–1154.
[3] N. Inagaki, H. Fukui, S. Ito, A. Yamatodani and H. Wada, Proc. Natl. Acad. Sci. USA, 88 (1991) 4215–4219 H.
[4] A.N. van den Pol, S.M. Finkbeiner and A.H. Cornell-Bell, J. Neurosci., 12 (1992) 2648–2664.
[5] L. Pasti, T. Pozzan and G. Carmignoto, J. Biol. Chem., 270 (1995) 15203–15210.
[6] M. Nedergaard, Science, 263 (1994) 1768–1771.
[7] V. Parpura, T.A. Basarsky, F. Liu, K. Jeftinija, S. Jeftinija and P.G. Haydon, Nature 369 (1994) 744–747.
[8] V. Papadopoulos, Endocrine Rev., 14 (1993) 222–240.
[9] Z.Y. Hu, E. Bourreau, I. Jung-Testas, P. Robel and E.E. Baulieu, Proc. Natl. Acad. Sci. USA, 84 (1987) 8215–8219.
[10] I. Jung-Testas, Z.Y. Hu, E.E. Baulieu and P. Robel, Endocrinol., 125 (1989) 2083–2091.
[11] E.E. Baulieu and P. Robel, J. Steroid Biochem. Mol. Biol., 37 (1990) 395–403.
[12] S.H. Mellon and C.F. Deschepper, Brain Res., 629 (1993) 283–292.
[13] I. Jung-Testas, H. Weintraub, D. Dupuis, B. Eychenne, E.E. Baulieu and P. Robel, J. Steroid. Biochem. Mol. Biol., 42 (1992) 597–605.
[14] S.A. Usanov, A.A. Chernogolov and V.L. Chashchin, FEBS Letts., 255 (1989) 125–128.
[15] H. Asou, S. Hirano and K. Uyemura, Cell Struct. Func., 14 (1989) 561–568.
[16] H. Asou, K. Hamada, Y. Takeda and K. Uyemura, J. Neurosci. Res., 40 (1995) 519–534.
[17] R.J. Havel, H.A. Eder and J.H. Bragdon, J. Clin. Invest., 34 (1955) 1345–1353.

[18] E.H.E. Yandouzi, P. Zlatkine, G. Moll and C.L. Grimellec, Biochem., 33 (1994) 2329–2334.
[19] S.M. Finkbeiner, Neuron, 8 (1992) 1101–1108.
[20] H. Fukui, N. Inagaki and S. Ito, Agents. Actions. (Suppl), 33 (1991) 161–180.
[21] M.W. Salter and J.L. Hicks, J. Neurosci., 14 (1994) 1563–1575.
[22] S. Kirischuk, J. Scherer, H. Kettenmann and A. Verkhratsky, J. Physiol. (London), 483 (1995) 41–57.
[23] M. He and K.D. McCarthy, J. Neurochem., 63 (1994) 501–508.
[24] M. Takeda, D.J. Nelson and B. Soliven, Glia, 14 (1995) 225–236.
[25] T. Kimura, Mol. Cell. Biochem., 36 (1981) 105–122.
[26] E.R. Simpson and M.R. Waterman, Can. J. Biochem. Cell. Biol., 61 (1983) 692–707.
[27] Cytochrome-*P*450, T. Omura, Y. Ishimura and Y. Fujii-Kuriyama (Eds.), 2nd ed., Kodansha, Tokyo, 1993.
[28] T. Kimoto, Y. Ohta and S. Kawato, Biochem. Biophys. Res. Commun., 221 (1996) 25–30.