Available online at www.sciencedirect.com
SCIENCE DIRECT
European Journal of Pharmacology 518 (2005) 107 – 110
www.elsevier.com/locate/ejphar

Short communication

Purification and aqueous phase atomic force microscopic observation of recombinant $P2X_2$ receptor

Ken Nakazawa [a,*], Yoko Yamakoshi [b,1], Toshie Tsuchiya [c], Yasuo Ohno [a]

[a] Division of Pharmacology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan
[b] Division of Organic Chemistry, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan
[c] Division of Medical Devices, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan

Received 4 April 2005; received in revised form 14 June 2005; accepted 20 June 2005
Available online 28 July 2005

Abstract

Recombinant $P2X_2$ receptor was observed by atomic force microscope in the aqueous phase. The $P2X_2$ receptor was expressed in an insect cell line, and recombinant proteins were prepared under native conditions. The membrane fractions were extracted, and histidine-tagged receptor protein was purified from the fractions by column chromatography. When the purified protein fraction was diluted with water and served for atomic force microscopy, dispersed particles of about 3 nm in height were observed. In the presence of 1 mM ATP, the assembly-like images of the particles were obtained. More densely assembled images of the particles were achieved when the protein was dissolved in a Tris buffer containing 1 mM ATP. Under this condition, imaging of the surface of the particles exhibited a circular structure with a diameter of about 10 nm having a pore-like structure. These results suggest that atomic force microscopy provides structural information about $P2X_2$ receptor in aqueous phase.

Keywords: P2X receptor; Atomic force microscopy; Protein structure; ATP

1. Introduction

P2X receptors are ion channel forming membrane proteins that are activated by extracellular ATP, and their physiological roles have been shown in various tissues including the central nervous system (see reviews, Khakh, 2001; North, 2002; Vial et al., 2004). This ion channel/receptor family consists of 7 subclasses ($P2X_1$ to $P2X_7$), and is believed to have molecular structures distinct from so-called "ligand-gated channel super family" including nicotinic acetylcholine receptor/channels and ionotropic glutamate receptor channels. Structural analyses such as the X-ray crystal analysis have not been made for P2X receptor/channel family. In addition, because of their distinct structures, estimation from homology modeling based on known three-dimensional structures of other proteins is difficult. Thus, information concerning the structure and morphology of P2X receptor is lacking. Atomic force microscopy is an approach for structural analysis that allows the analysis of a small amount (nanogram to microgram) of uncrystalized protein. Atomic microscopy enables the observation of both individual and assembled protein molecules in the aqueous phase, which may reveal dynamic forms of biologically active proteins (Müller and Engel, 2002). Recently, Barrera et al. (2005) reported atomic force microscopy imaging of dried P2X receptor protein. In the present study, we have prepared $P2X_2$ receptor protein from an insect cell line expression system, and made atomic force microscopy imaging in aqueous phase. The imaging has revealed that $P2X_2$ receptor is a pore-forming protein for the first time.

* Corresponding author. Tel.: +81 3 3700 9704; fax: +81 3 3707 6950.
E-mail address: nakazawa@nihs.go.jp (K. Nakazawa).
[1] Present address: Center for Polymers and Organic Solids, Department of Chemistry and Biochemistry, University of California, Santa Barbara, CA 93106-9510, USA.

0014-2999/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2005.06.018

2. Materials and methods

2.1. Preparation of recombinant $P2X_2$ receptor protein

N-terminal hexahistidine-tagged recombinant rat $P2X_2$ receptor was expressed using baculovirus-Sf9 system, which has been used for the expression of membrane receptor proteins (e.g., Boundy et al., 1993; Ng et al., 1993). cDNA encoding rat $P2X_2$ receptor (Brake et al., 1994) was subcloned into pFast BAC HTc vector (BD Bioscience Clonetech, Palo Alto, CA, USA). The recombinant virus was transfected to insect-derived clonal Sf9 cells. After culturing at a volume of 500 ml at room temperature, the culture medium was centrifuged at 130 $\times g$ for 5 min, and the precipitated cells were washed with Ca^{2+}, Mg^{2+}-free phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4; PBS(−)) twice. The cells were then suspended in a Tris–HCl (pH 7.4) lysis buffer containing Triton X-100, and homogenized. NaCl was added such that its final concentration became 100 mM. This solution was centrifuged at 30 000 $\times g$ for 20 min, and the supernatant was then centrifuged at 380 000 $\times g$ for 10 min. Polyacrylamide gel electrophoresis followed by immunoblotting analysis with anti-hexahistidine antibody showed that hexahistidine-tagged proteins of an expected size (56 kD) were found in this supernatant. Further purification was made using Chelating Sepharose FF colums. Ni^{2+}-bound columns were equilibrated with a buffer containing 20 mM Tris–HCl and 0.5 M NaCl (pH 8.0), and samples were applied. The bound receptor proteins were eluted by stepwise increase of imidazole (10, 20, 50, 100, 200 and 500 mM). The concentrations of the receptor protein in the eluted solutions were estimated by measuring absorbance at 595 nm. The most purified $P2X_2$ receptor protein (>90% of total protein) was found in the fraction eluted by 10 mM imidazole, and this fraction was served for atomic force microscopy imaging. The purified $P2X_2$ receptor exhibited the ability to bind ATP when photoaffinity labeling with [α-^{32}P]ATP was performed according to Kim et al. (1997). In this experiment, the binding of [α-^{32}P]ATP was markedly reduced by 100 μM nonradiolabeled ATP.

2.2. Atomic force microscopy imaging

The protein solution (about 1.5 μM) was diluted to appropriate concentrations (0.1 to 10 nM) with water, and the diluted solution was placed on freshly cleaved mica. After 30 min, unbound proteins were washed away with water, and served for atomic force microscopy imaging. When ATP (disodium salt; Sigma, St. Louis, MO, USA) was added to water, 1 mM solution was neutralized to pH 7.4 with 2 N NaOH (final Na^+ concentration was about 16 mM). In part of the experiments, the protein solution was diluted with a Tris buffer of the following composition (in millimolar): Tris 50, KCl 150, $MgCl_2$ 10, dithiothreirol 1 (pH 7.0). This buffer composition was similar to that utilized for atomic force microscopy imaging of *Escherichia coli* GroES (Cheung et al., 2000). Imaging was made in an aqueous tapping mode using MFP-3D (Asylum Research, Santa Barbara, CA, USA) equipped with OMCL-TR800PSA (Olympus, Tokyo, Japan) as a probe.

3. Results

Fig. 1A shows atomic force microscopy images of purified $P2X_2$ receptor proteins in water. A larger part of the proteins were found as dispersed particles. The height of single $P2X_2$ receptor proteins was about 3 nm (Fig. 1B). In the presence of 1 mM ATP, larger particles, presumably clots of several receptor proteins, were observed in addition to dispersed particles (Fig. 1C). A flatly and densely assembled image was obtained when the proteins were dissolved in a Tris buffer containing 1 mM ATP (Fig. 2A). Densely packed assembly is advantageous for atomic force microscopy imaging because resolution is improved due to smaller movement of probes along *Z*-axis (Müller and Engel, 2002). When the protein assembly shown in Fig. 2A was imaged at higher magnification, a circular structure with a pore was observed (Fig. 2B). The diameter of the circular structure was about 10 nm, and that of the pore was several nanometers. Without ATP, the protein was not densely assembled and did not exhibit uniform direction (not shown).

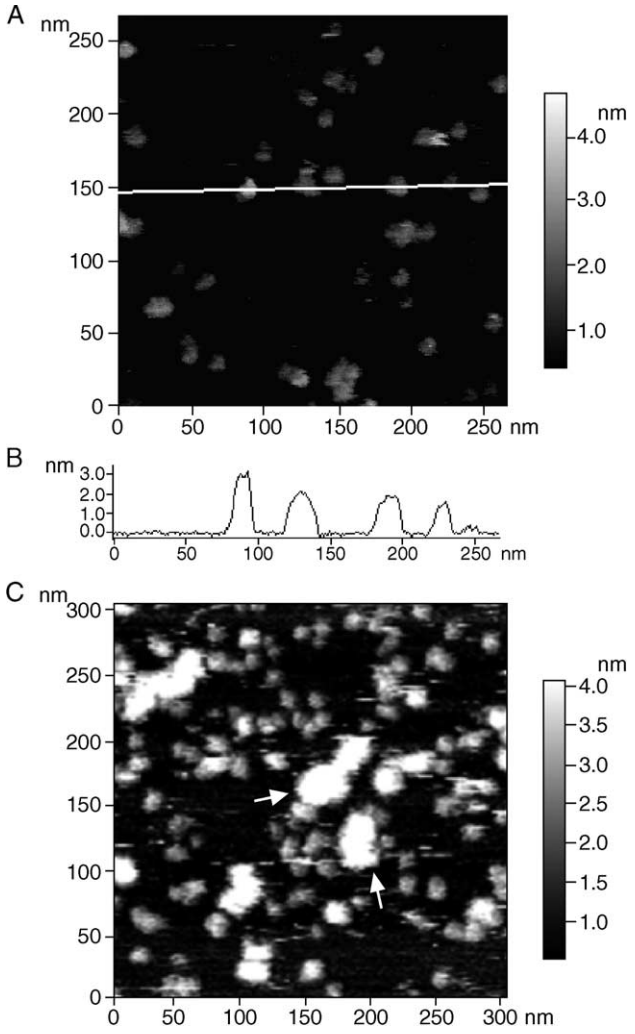

Fig. 1. (A) An atomic force microscopy image of $P2X_2$ receptor proteins in water. Isolated single receptor proteins and their small assemblies are seen. (B) A section of the image shown in (A). The section was made along with the line. The height of receptor proteins is about 3 nm or less. (C) An image of $P2X_2$ receptor proteins in the presence of 1 mM ATP. In addition to single receptor proteins, clots of the proteins (indicated by arrows) were also seen.

4. Discussion

For atomic force microscopy imaging of membrane proteins, densely expressed proteins in particular cells have

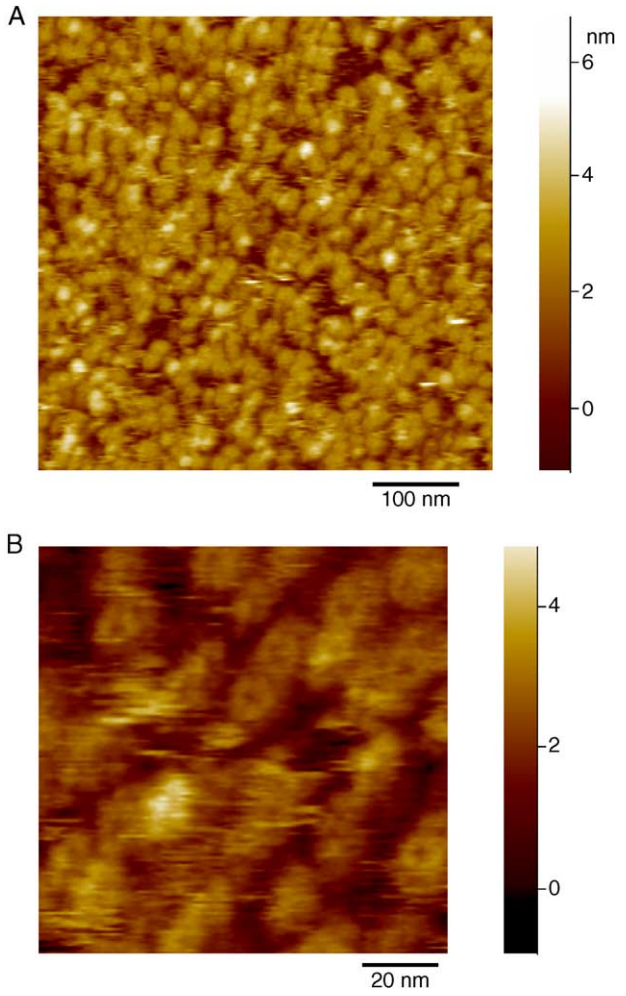

Fig. 2. (A) An image of $P2X_2$ receptor proteins in a Tris buffer containing 1 mM ATP. The proteins were flatly and densely assembled. (B) An expanded image. The upper surfaces of individual proteins exhibited a circular structure having a pore in its center.

been served in the presence of lipid bilayers (Müller and Engel, 2002; Müller et al., 2002). This two-dimensional (2D) protein crystal can provide high resolution of images, especially when combined with image processing including averaging. However, preparation of 2D crystals requires skilled techniques or special equipment. The present study has shown that recombinant $P2X_2$ can be imaged by atomic force microscopy without special techniques, but by simply adding agonist molecule, ATP. The role of ATP in promoting the densely packed assembly is unclear at present. It is speculated that receptor protein molecules without ATP freely move and exhibit various conformations, whereas ATP-bound receptor molecules exhibit only one or a restricted number of conformations in aqueous phase. The pore identified in the center of the protein may be the ion channel involved in $P2X_2$ receptor. A similar pore has been observed in connexin that also forms ion channels (Müller et al., 2002). It is unclear that the pore corresponds to the inner mouth or the outer mouth of the channel. Nevertheless, it is interesting that a number of proteins appear to exhibit similar surface structure (Fig. 2B). P2X receptor possesses a large extracellular domain, and, thus, it is possible that this domain is orientated upward to increase contact with the aqueous phase. Atomic force microscopy imaging of isolated membrane proteins may be less advantageous to elucidate biological functions compared to those embedded in lipid bilayer. However, isolated proteins are more readily observed than membrane preparations, and the imaging of these proteins may provide insights into the intrinsic properties of proteins and useful information to clarify the interactions between proteins and the membrane.

Barrera et al. (2005) observed dried $P2X_2$ receptor protein as a simple particle. We have revealed the outer structure of the protein, suggesting that resolution was better in the present study. However, our observation has not resolved trimeric assembly of $P2X_2$ receptor protein, which has been shown using antibodies specific for the protein (Barrera et al., 2005). Trimeric assembly of P2X receptor has been also demonstrated by electrophysiological and biochemical studies, and two transmembrane regions of each subunit are believed to contribute to the forming of channel pore (North, 2002; Vial et al., 2004). If $P2X_2$ receptor forms a six-barrel channel like connexin, this may account for a similar pore size (about several nanometers). Further improvement will be necessary to identify individual subunit proteins that form $P2X_2$ receptor and clarify more detailed structure by atomic force microscopy.

Acknowledgments

We are grateful to Dr. Jeffrey W. Bode of Department of Chemistry and Biochemistry, University of California, Santa Barbara for improving our manuscript. This work was partly supported by a Health and Labour Science Research Grant for Research on Advanced Medical Technology from the Ministry of Health, Labour and Welfare, Japan awarded to K.N., Y.Y. and T.T., and a grant-in-aid for scientific research from the Ministry of Education, Science, Sports and Culture, Japan (KAKENHI 13672319) awarded to K.N.

References

Barrera, N.P., Ormond, S.J., Henderson, R.M., Murrell-Langnado, R.D., Edwardson, J.M., 2005. AFM imaging demonstrates that $P2X_2$ receptors are trimers, but that $P2X_6$ receptor subunits do not oligomerize. J. Biol. Chem. 280, 10759–10765.

Boundy, V.A., Luedtke, R.R., Gallitano, A.L., Smith, J.E., Filtz, T.M., Kallen, R.G., Molinoff, P.B., 1993. Expression and characterization of the rat D3 dopamine receptor: properties and development of antibodies. J. Pharmacol. Exp. Ther. 264, 1002–1011.

Brake, A.J., Wagenbach, M.J., Julius, D., 1994. New structural motif for ligand-gated ion channels defined by an ionotropic ATP receptor. Nature 371, 519–523.

Cheung, C.L., Hafner, J.H., Lieber, C.M., 2000. Carbone nanotube atomic force microscopy tips: direct growth by chemical vapor deposition and application to high-resolution imaging. Proc. Natl. Acad. Sci., U. S. A. 97, 3809–3813.

Khakh, B.S., 2001. Molecular physiology of P2X receptors and ATP signalling at synapses. Nat. Rev. 2, 165–174.

Kim, M., Yoo, O.J., Choe, S., 1997. Molecular assembly of the extracellular domain of $P2X_2$, an ATP-gated ion channel. Biochem. Biophys. Res. Commun. 240, 618–622.

Müller, D.J., Engel, A., 2002. Conformations, flexibility, and interactions observed on individual membrane proteins by atomic force microscopy. Methods Cell Biol. 68, 257–298.

Müller, D.J., Hand, G.M., Engel, A., Soslinsky, G., 2002. Conformational changes in surface structures of isolated connexin 26 gap junctions. EMBO J. 21, 3598–3607.

Ng, G.Y., George, S.R., Zastawny, R.L., Caron, M., Bouvier, M., Dennis, M., O'Dowd, B.F., 1993. Human serotonin 1B receptor expression in Sf9 cells: phosphorylation, palmitoylation, and adenylyl cyclase inhibition. Biochemistry 32, 11727–11733.

North, R.A., 2002. Molecular physiology of P2X receptors. Physiol. Rev. 82, 1013–1067.

Vial, C., Roberts, J.A., Evans, R.J., 2004. Molecular properties of ATP-gated P2X receptor ion channels. Trends Pharmacol. Sci. 25, 487–493.