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Pharmacological detection of AMPA receptor heterogeneity by use of two allosteric potentiators in rat hippocampal cultures

[1,5]Masayuki Sekiguchi, [3]Jiro Takeo, [1]Takayuki Harada, [2]Takako Morimoto, [4]Yoshihisa Kudo, [3]Shinya Yamashita, [2]Shinichi Kohsaka & [1]Keiji Wada

[1]Department of Degenerative Neurological Diseases, and [2]Department of Neurochemistry, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187; [3]Central Research Laboratory, Nippon Suisan Kaisha Ltd, 559–6 Kitano-cho, Hachioji, Tokyo 192 and [4]Tokyo University of Pharmacy and Life Science, School of Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan

1 In order to examine whether a recently developed allosteric potentiator for AMPA receptors, 4-[2-(phenylsulphonylamino)ethylthio]-2,6-difluoro-phenoxyacetamide (PEPA), can be utilized as an indicator of AMPA receptor heterogeneity, the action of PEPA upon the increase of intracellular free calcium ion concentration ($[Ca^{2+}]_i$) elicited by AMPA was investigated in rat hippocampal cultures, and the action was compared with that of cyclothiazide, a well characterized allosteric modulator of AMPA receptors.

2 PEPA dose-dependently potentiated AMPA-induced increase of $[Ca^{2+}]_i$. In 90% (72 out of 80) of the cells in which cyclothiazide acts, PEPA potentiated the increased $[Ca^{2+}]_i$ induced by AMPA with pronounced cell-to-cell variation in rat hippocampal cultures.

3 The ratio of the potentiation by PEPA to the potentiation by cyclothiazide (P/C ratio) also varied with cells between 0 and 2.15. It was found that the cultured hippocampal cells consisted of multiple populations with different P/C ratios. Among them two populations exhibited characteristic P/C ratios; low (0 to 0.15; 27 out of 80 cells, 34%) and high ($\geqslant$2.00; 1 out of 80 cells, 1%) P/C ratios. The P/C ratios of the other populations were between 0.25 and 1.20, and these cells constituted 65% (52 out of 80 cells) of the cells tested.

4 Reverse transcriptase-polymerase chain reaction analysis suggested that GluR2-flip, GluR1-flip, GluR2-flop, and GluR1-flop were abundantly expressed (in this rank order) in the cultures used.

5 In *Xenopus* oocytes expressing GluR1, GluR3, or these subunits plus GluR2, the potentiation of AMPA response by PEPA and by cyclothiazide varied with subunit and splice-variant combinations, and the P/C ratio was between 0.19 and 2.20. Oocytes with low P/C ratios (0.19 to 0.50) and low sensitivity to PEPA potentiation (1.9 fold to 6.41 fold) were those expressing flip variants predominantly, and oocytes with high P/C ratios (1.8 to 2.2) were those expressing flop variants predominantly. Oocytes with intermediate P/C ratios (0.51 to 1.20) were those expressing various combinations of flip and flop variants, and it was impossible to specify the relative abundance of flip and flop variants in these cells. Therefore, the P/C ratio can be used to infer subunit/splice variant expression only when the ratio is low or high.

6 These results suggest that the potentiation by PEPA alone reveals cell-to-cell heterogeneity of AMPA receptors, but a comparison of the actions of PEPA and cyclothiazide further facilitates the detection of the heterogeneity.

Keywords: AMPA receptor; heterogeneity; PEPA; cyclothiazide; allosteric potentiator; splice variant; flip; flop; hippocampal culture

Introduction

Functional α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors are thought to be pentamers (Wenthold *et al*., 1992; Ferrer-Montiel & Montal, 1996) formed from distinct subunits encoded by four different genes, GluR1, GluR2, GluR3 and GluR4 (Hollman *et al*., 1989; Keinanen *et al*., 1990). Each subunit is known to exist as two isoforms, flip and flop, which are produced by alternative mRNA splicing (Sommer *et al*., 1990). It is likely that there are no fixed stoichiometries in the receptors made up of more than one type of subunit. Rather, receptor stoichiometry is thought to be determined by the amount of subunit protein expressed in a given cell. AMPA receptors consisting of different subunit and/or splice-variant combinations show different properties. For example, it has been found that incorporation of GluR2 into receptor complexes dramatically reduces calcium ion permeability of the receptor channel (Hollmann *et al*., 1991; Hume *et al*., 1991). This reduction is attributed to amino acid changes resulting from RNA editing at the Q/R site in GluR2 RNA (Sommer *et al*., 1991). Incorporation of differentially spliced isoforms into receptor complexes produces AMPA receptors with different desensitization profiles (Sommer *et al*., 1990; Mosbacher *et al*., 1994). Editing at R/G sites within RNAs encoding GluR2, GluR3 and GluR4 further increases the multiplicity of AMPA receptors, and this editing affects the rates of recovery from desensitization (Lomeli *et al*., 1994). RNA editing at Q/R and R/G sites (Sommer *et al*., 1990; Mosbacher *et al*., 1994), as well as the relative levels of expression of subunits (Hollman *et al*., 1989; Keinanen *et al*., 1990; Monyer *et al*., 1991) and flip-flop alternative splicing (Sommer *et al*., 1990; Monyer *et al*., 1991) are under developmental control and occur in a cell-specific manner. The cell-specific developmental modulation of the stoichiometry of AMPA receptors may be important for the determination of excitability and vulnerability of neurones to toxicity (Geiger *et al*., 1995; Lambolez *et al*., 1996).

[5] Author for correspondence.

Pharmacological detection of cell-to-cell heterogeneity of AMPA receptors can provide useful information for the interpretation of physiological events. Toxins such as JSTX-3 (Herlitze *et al*., 1993) and argiotoxin-636 (Blaschke *et al*., 1993) have been used to demonstrate the absence of GluR2 in AMPA receptor complexes, in that these toxins selectively block receptors lacking GluR2. Evans blue also acts as an AMPA receptor antagonist but shows different subunit selectivity than JSTX-3 and argiotoxin-636 (Keller *et al*., 1993). The cell-to-cell heterogeneity of AMPA receptors is also demonstrated by use of cyclothiazide, an allosteric potentiator for the receptors (Fleck *et al*., 1996).

Cyclothiazide differentially reduces desensitization of AMPA receptor flip and flop variants and the flip variants are much more sensitive to this drug (Partin *et al*., 1994). Comparing the action of cyclothiazide with the action of drugs which preferentially and potently act on the flop variant could be an effective means for detecting heterogeneity among receptors.

Until quite recently, aniracetam was the only drug known to act preferentially on flop splice variants of AMPA receptors (Johansen *et al*., 1995). However, aniracetam is much less potent for flop than is cyclothiazide for flip (Johansen *et al*., 1995; Partin *et al*., 1996) and must be used at significantly higher concentrations than cyclothiazide to produce an equivalent effect (Johansen *et al*., 1995; Partin *et al*., 1996). We recently found (Sekiguchi *et al*., 1997) a novel allosteric potentiator of AMPA receptors, 4-[2-(phenylsulphonylamino)ethylthio]-2,6-difluoro-phenoxyacetamide (PEPA). PEPA, as well as cyclothiazide (Patneau *et al*., 1993; Yamada & Tang, 1993) and aniracetam (Isaacson & Nicoll, 1991), suppress desensitization of AMPA receptors (Sekiguchi *et al*., 1997). PEPA acts preferentially on flop variants and is 100 times more potent than aniracetam, and is therefore comparable to the action of cyclothiazide on flip (Sekiguchi *et al*., 1997). Also, PEPA is a more potent suppressor of desensitization of receptors containing GluR3 and GluR4 as opposed to those containing GluR1 (Sekiguchi *et al*., 1997).

In the present study, we hypothesized that cell-to-cell heterogeneity of functional AMPA receptors could be detected by comparing the effects of PEPA and cyclothiazide on the AMPA-induced increase of intracellular free calcium ion concentration ($[Ca^{2+}]_i$). We found pronounced cell-to-cell variations in the potentiation by PEPA alone as well as in the ratio of the potentiation by PEPA to the potentiation by cyclothiazide (P/C ratio). It seems that the cell-to-cell variations suggest the cell-to-cell heterogeneity of AMPA receptors. In order to investigate the origin of the detected heterogeneity, cloned AMPA receptor subunits were expressed in *Xenopus* oocytes, and the potentiation by PEPA and the P/C ratio was monitored. The results suggested that expression of different combinations of subunits and splice-isoforms causes variation in the potentiation by PEPA and the P/C ratio in the oocytes.

Methods

Cell culture

Primary cultures of dissociated hippocampal neuronal cells were prepared from foetal rats at 18 days gestation. Tissue fragments of the hippocampal areas were dissected from embryonic rat brains (Wistar rats of either sex) under a stereomicroscope (Olympus). Tissue fragments were added to 10 ml of Ca^{2+}- and Mg^{2+}-free HEPES buffer (pH7.4) containing 0.25% trypsin (Life Technologies) and DNase I (Boehringer), and incubated for 15 min at 37°C. After mechanical dissociation by pipetting, the cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal bovine serum and 2% B-27 supplement (Life Technologies). Dissociated cells were plated onto poly-D-lysine-precoated coverslips (about 8×10^4 cells/well) fixed onto the bottom of 60 mm plastic dishes in which the plastic under the coverslip was cut out. The plated cells were incubated for 2 days in a humidified, 5% CO_2 incubator at 37°C. The medium was then changed to serum-free DMEM containing 2% B-27 supplement. The medium was changed every 2 to 3 days thereafter. Some of the cultures were stained with antibodies for microtuble-associated protein 2 (MAP2) and for glial fibrillary acidic protein (GFAP) after 8 days incubation. About 60% to 95% of the cells in culture were immunopositive for MAP2 (data not shown).

Measurements of cytosolic free calcium ion concentration

Rat hippocampal cultures (8 days *in vitro)* in 60 mm dishes were loaded with 10 μM Fura-2 AM (a calcium-sensitive fluorescent indicator dye) in a control medium consisting of in mM: HEPES (pH 7.4) 5, NaCl 135, KCl 5.4, $MgCl_2$ 0.8, $CaCl_2$ 1.8 and D-glucose 10, for 30 to 40 min at 37°C. The cultures were transferred to the stage of a Nikon Diaphot inverted microscope (magnification 200× or 400×). Throughout the experiments, cells were perfused with the control medium containing 0.5 μM tetrodotoxin (TTX) at a constant flow rate (2.0 ml min^{-1}). TTX was added to block release of glutamate from presynaptic terminals in response to depolarization caused by AMPA receptor activation. PEPA and cyclothiazide (Sigma) were dissolved in DMSO at 100 mM, and AMPA (RBI) was dissolved in the control medium at 1 mM. These solutions were diluted into the control medium to prepare working solutions. The maximal concentration of DMSO in the medium was 0.15% (150 μM PEPA solution). Cells were examined under epifluorescence illumination with a 75-W xenon source with 340 and 380 nm excitation filters alternated under computer control to monitir Ca^{2+}-free and Ca^{2+}-bound forms of Fura-2, respectively (dichroic mirror, 400 nm; emission filter, 510 nm). The emitted images obtained every 2.9 s (200×) or 5 s (400×) were acquired with a CCD camera and the data were digitized with an image-processing system (ARGUS-50/CA, Hamamatsu Photonics). Ratios of excitation at 340 and 380 nm (340/380 ratios) were calculated after background subtraction for each region. The 340/380 ratios were also measured for several concentrations of a standard $CaCl_2$ solution imaged in glass microslides (without cells) for preparing calibration lines for Ca^{2+} concentration ranging from 0.01 to 0.60 μM (200×) or 0.01 to 0.35 μM (400×). The calibration lines in the inset of Figure 2a are regression lines of the 340/380 ratio on $[Ca^{2+}]_i$, which were calculated by the method of least squares.

RT–PCR

After 7 days incubation *in vitro*, hippocampal cells were collected with a cell scraper. Total RNA was isolated from the cells (about 1×10^7 cells) by the Isogen total RNA extraction kit (Nippon Gene). The RNA was converted to cDNA by reverse transcriptase (Superscript II, Life Technologies) and oligo(dT) primers according to the manufacturer's instructions. The cDNA product was used as the template for PCR amplification. Pairs of PCR primers were designed to amplify

specific flip or flop variants based on the sequence of probes used by Sommer *et al*. (1990) for *in situ* hybridization (F = forward primers, R = reverse primer). 1F: 5′-AGAGGGACGAGACCAGACAACCAG-3′ (1713-1736, from the initial ATG codon of GluR1). 1Ro: 5′-GCTGGTCTTGTCCTTGGAGTCACC-3′ (2397-2374 of GluR1-flop). 1Ri: 5′GCTGGTCTTGTCCTTACTTCCGGA-3′ (2397-2374 of GluR1-flip). 2F: 5′-AGATGGAAGAGAAACACAAAGTAG-3′ (1734-1757 of GluR2). 2Ro: 5′-ACTGGTCTTTTCCTTGGAATCACC-3′ (2418-2395 of GluR2-flop). 2Ri: 5′-ACTGGTCTTTTCCTTACTTCCCGA-3′ (2418-2395 of GluR2-flip). 3F: 5′ACCTCGTGACCCACAAAGCCCTCC-3′ (1749-1772 of GluR3). 3Ro: 5′-ACTGGTCTTGTCCTTGGAGTCACC-3′ (2433-2410 of GluR3-flop). 3Ri: 5′-ACTGGTCTTGTCCTTACTCCCGGA-3′ (2433-2410 of GluR3-flip). 4F: 5′-GGATGGGAAGGAAGGACCCAGTGA-3′ (1737-1760 of GluR4). 4Ro: 5′-ACTCGTCTTGTCCTTGGAGTCACC-3′ (2422-2339 of GluR4-flop). 4Ri: 5′-ACTCGTCTTGTCCTTGCTTCCCGA-3′ (2422-2339 of GluR4-flip). G3PDHF: 5′-TGAAGGTCGGTGTCAACGGATTTGGC-3′ (35-60 of glyceraldehyde-3-phosphate dehydrogenase, G3PDH). G3PDHR: 5′-CATGTAGGCCATGAGGTCCACCAC-3′ (1017-994 of G3PDH). G3PDHF and G3PDHR (CLONTECH) were used to amplify a 983 base pair (bp) fragment from G3PDH cDNA to serve as an internal standard. PCR reactions contained the following: primers for GluR (0.2 μM each), primers G3PDHF and G3PDHR (0.2 μM each), template cDNA (15 ng), Tris-HCl (10 mM, pH 8.3), KCl (50 mM), $MgCl_2$ (1.5 mM), dNTPs (200 μM) and Taq DNA polymerase (2.5 u). The volume of the reaction was adjusted to 100 μl with water. Eight tubes were prepared to correspond to the eight sets of primer combinations and the same template cDNA solution was used in all reactions. Reactions were assembled on ice, initiated by addition of polymerase, heated at 94°C for 3 min and then loaded into the thermalcycler. Each amplification cycle consisted of a heat-denaturation step at 94°C for 15 s, annealing of primers at 58°C for 2 min, and polymerization at 72°C for 2 min. A portion of each reaction mixture was electrophoresed on an agarose gel (2%) containing ethidium bromide (10 μg ml^{-1}) and then visualized under u.v. illumination. The amplification of sequences encoding specific GluR(1-4) splice-variants produced was confirmed by digestion of the PCR products with restriction endonuclease. *MaeI* produces 572 and 113 bp fragments from GluR1-flip but does not cut GluR1-flop. *HincII* produces 566 and 119 bp fragments from GluR2-flop but does not cut GluR2-flip. *HincII* also produces 447, 121 and 117 bp fragments from GluR3-flop, and 447 and 238 bp fragments from GluR3-flip. *StuI* produces 598 and 90 bp fragments from GluR4-flop but does not cut GluR4-flip. Because the number of PCR cycles and the intensity of the 983 bp G3PDH band (measured by densitometry) exhibited a linear correlation between PCR cycles 25 and 29 (data not shown), 27 cycles were used in all experiments. Furthermore, since the intensity also linearly increased according to the amount of RNA used from 3.75 to 30 ng at 27 cycles (data not shown), 15 ng RNA were used.

Xenopus *oocyte expression system*

Expression of AMPA receptors in *Xenopus* oocytes and subsequent two-electrode voltage clamp recordings were performed as described previously (Sekiguchi *et al*., 1997). Briefly, cRNA was *in vitro* transcribed from plasmids encoding AMPA receptor subunits. The concentration of cRNA solutions for GluR1-flip, GluR1-flop, GluR2-flip, GluR2-flop, GluR3-flip and GluR3-flop was adjusted to 1 mg ml^{-1}, and the solutions were electrophoresed on a formaldehyde-denaturing agarose gel to verify the size and density of the RNA bands. Each of the six transcription reactions produced a single band of the expected size, and all six bands showed similar u.v. intensities when stained with ethidium bromide. Solutions of GluR1 or GluR3 cRNA were mixed with GluR2 cRNA in various proportions before injection into oocytes, and 50 nl of the solution was injected per oocyte. Before the injection, the oocytes were defolliculated by treatment with collagenase (3 mg ml^{-1}) for 2 h at room temperature. Electrophysiological recording was performed under constant perfusion of frog Ringer solution (2 ml min^{-1}). AMPA, PEPA and cyclothiazide were applied by perfusion in the frog Ringer solution. These solutions were prepared by the method described above, except frog Ringer solution was used instead of control medium.

Results

Effects of PEPA and cyclothiazide on the AMPA-induced increase of intracellular free calcium ion concentration in hippocampal cultures

Figure 1 shows intracellular free calcium ion-imaging in hippocampal cultures preloaded with Fura-2 AM, a fluorescent calcium-sensitive indicator. This system is suitable for investigating the effects of PEPA in many cells at a time. It is well established that activation of AMPA receptors indirectly activates voltage-dependent calcium channels if depolarization induced by AMPA receptors reaches the threshold of the calcium channels. AMPA (1 to 100 μM) applied to the hippocampal culture induced an increase of the 340/380 ratio in a dose-dependent manner (data not shown). In a large number of cells, 1 μM AMPA very weakly increased the ratio, but in some cells a moderate increase in the ratio was evident (see panel A1 in Figure 1a). This difference is most likely due in part to differences in the expression of GluR2 (Hollmann *et al*., 1991; Hume *et al*., 1991; Geiger *et al*., 1995). PEPA (100 μM) applied alone did not elicit an increase in the 340/380 ratio in any of the 11 cells in which AMPA increased the ratio (data not shown). PEPA (25 μM) potentiated the increase of 340/380 ratio induced by AMPA in a small number of cells (Figure 1a, panel A1 + P25). The potentiation was observed in a much larger number of cells when 150 μM PEPA was applied (Figure 1a, panel A1 + P150). Figure 1b shows a comparison of the potentiation of AMPA response by PEPA and cyclothiazide. There are ten visible cells in the field of each photograph. When the 340/380 ratio was simply judged by quasi-color, the outstanding increase of 340/380 ratio was observed in cells 1, 2, 5 and 10 in response to co-application of AMPA (10 μM) and PEPA (100 μM). On the other hand, the clear increase of the ratio was observed in all of these cells in response to co-application of AMPA (10 μM) and cyclothiazide (100 μM). These results suggest that there were pronounced cell-to-cell variations in the extent of action of PEPA. An outstanding example is seen between cells 4 and 5; cyclothiazide potently increased the 340/380 ratio in both cells, but PEPA increased the ratio more potently in cell 5 than in cell 4.

Figure 2a also shows an example of the between-cell variation of PEPA action, in which the 340/380 ratio is continuously monitored in single hippocampal cells. Co-application of AMPA and PEPA slightly increased the 340/380 ratio, but co-application of AMPA and cyclothiazide strongly increased the ratio. However, in a different cell,

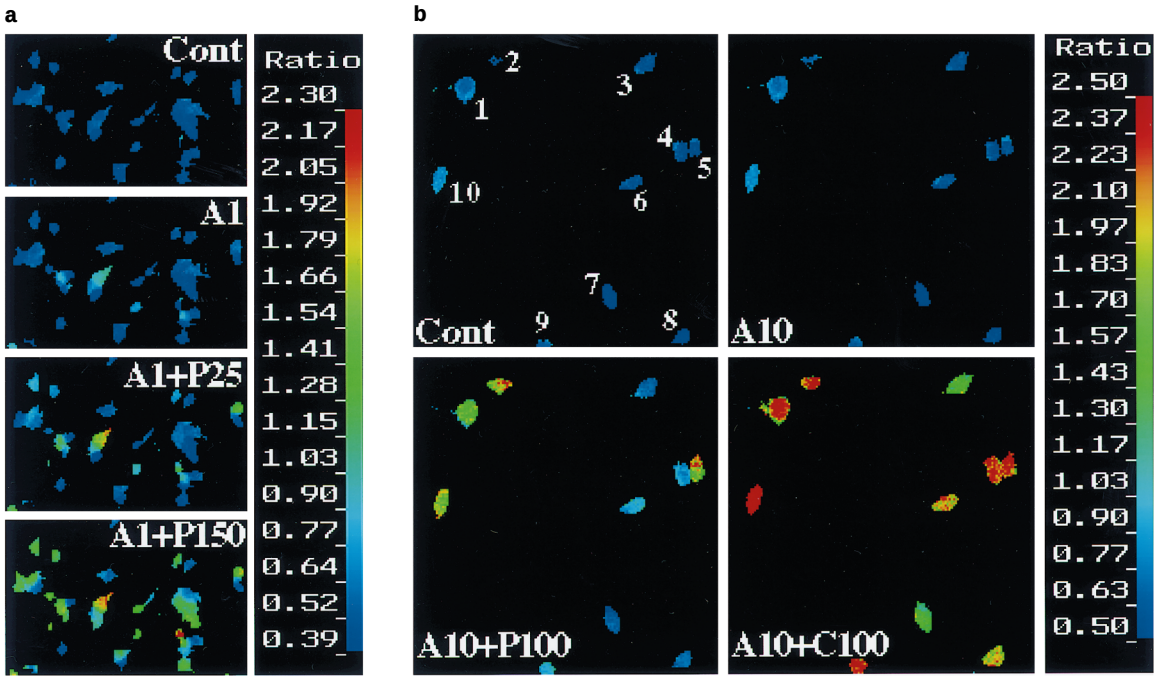

Figure 1 Intracellular free calcium ion-imaging in hippocampal cultures preloaded with Fura-2 AM showing that PEPA potentiates AMPA-induced increase of 340/380 ratio in cultured hippocampal cells. (a) Dose-dependent potentiation by PEPA of the 340/380 ratio. Cont: control before application of drugs. A1, A1 + P25 and A1 + P150: records at the data points when maximum response was obtained after application of 1 μM AMPA, 1 μM AMPA + 25 μM PEPA and 1 μM AMPA + 150 μM PEPA, respectively. The bar on the right shows the correlation between the 340/380 ratio and quasi-color. Magnification 200×. (b) Comparison of the potentiation by PEPA (100 μM) and cyclothiazide (100 μM). Cont: control before application of drugs. A10, A10 + P100, and A10 + P100; records at the point when maximum response was obtained after application of 10 μM AMPA, 10 μM AMPA + 100 μM PEPA and 10 μM AMPA + 100 μM PEPA, respectively. The numbers in the control panel identify each cell. Magnification 400×.

PEPA potentiated the AMPA response more effectively than cyclothiazide in this cell (Figure 2b). We applied the drugs in order of AMPA, AMPA + PEPA and AMPA + cyclothiazide throughout experiments. The inset of Figure 2a shows the relation between 340/380 ratio and concentration of Ca^{2+} which is linear up to 2.5 magnification 200×, $r^2 = 0.984$, magnification 400×, $r^2 = 0.972$, where r is the correlation coefficient). There were two cells in which the cyclothiazide response was over 2.5 in the 340/380 ratio, and these were excluded from data analysis. The scatter plot in Figure 3a shows potentiation of the AMPA-induced increase of $[Ca^{2+}]_i$ by PEPA versus that potentiated $[Ca^{2+}]_i$ by cyclothiazide in each of the culture cells tested (a total of 80 cells from five different culture dishes, magnification 200×). The potentiation by PEPA and potentiation by cyclothiazide showed pronounced cell-to-cell variations which were greater in cyclothiazide- than in PEPA-treated cells. The potentiation by PEPA varied between 1.0 fold (no potentiation) and 27.7 fold, and the potentiation by cyclothiazide varied between 1.1 fold and 101.0 fold (note the difference in the scale between the abscissae and the ordinates of Figure 3a). The histogram in Figure 3b, which was plotted from the data shown in Figure 3a, shows the number of cells falling into each range of the P/C ratio. The hippocampal cultures we used also showed pronounced cell-to-cell variations with respect to the P/C ratio, and it was found that the cells consisted of multiple populations with respect to P/C ratio. Among them, the characteristic populations were those which exhibited low P/C ratios <0.15 (27/80 cells, 34%) and high P/C ratios >2.00 (1/80 cells, 1%). The P/C ratios of other populations were between 0.25 and 1.20, and these cells constituted 65% (52 out of 80 cells) of the cells tested. No morphological differences were observed between these cells.

Effects of PEPA and cyclothiazide on the AMPA-induced current responses in Xenopus *oocytes*

To investigate the origin of heterogeneity detected in hippocampal cells, we expressed AMPA receptor subunits in *Xenopus* oocytes by injecting RNA transcripts prepared from cDNA encoding the subunits. The oocytes were then electrophysiologically characterized. In order to select subunits that are used in the oocyte experiments, we first analysed AMPA receptor subunits and their splice variants expressed in our hippocampal culture cells by RT–PCR. Total RNA was isolated from the cultures for this purpose. After conversion to cDNA, PCR was performed with two sets of primers. One set amplified specific GluR variants while the other set, added as an internal control, specifically amplified glyceraldehyde-3-phosphate dehydrogenase (G3PDH) to produce a band of 983 bp. Figure 4 shows a photograph of an agarose gel of the PCR products. A band corresponding to GluR variants exhibited slightly lower mobility than the 658 bp size marker, consistent with its expected size of 685 bp. The GluR2-flip variant was amplified to a greater extent than the other GluR variants. There were also GluR bands in the GluR1-flip, GluR1-flop, GluR2-flop, GluR3-flip, or GluR4-flip PCR reactions, but the densities of these bands were much weaker than that of GluR2-flip. However, the GluR bands in GluR1-flip and GluR2-flop reactions were more dense than the others. In this gel, we could not detect a clear band in the case of GluR3-flop and GluR4-flop. However, this does not mean that these RNAs were not expressed, because a trace band appeared upon increasing either the number of PCR cycles or the amount of reaction mixture applied to the gel. Therefore, in the cultures we used, GluR mRNAs were expressed with the following rank order: GluR2-flip≫

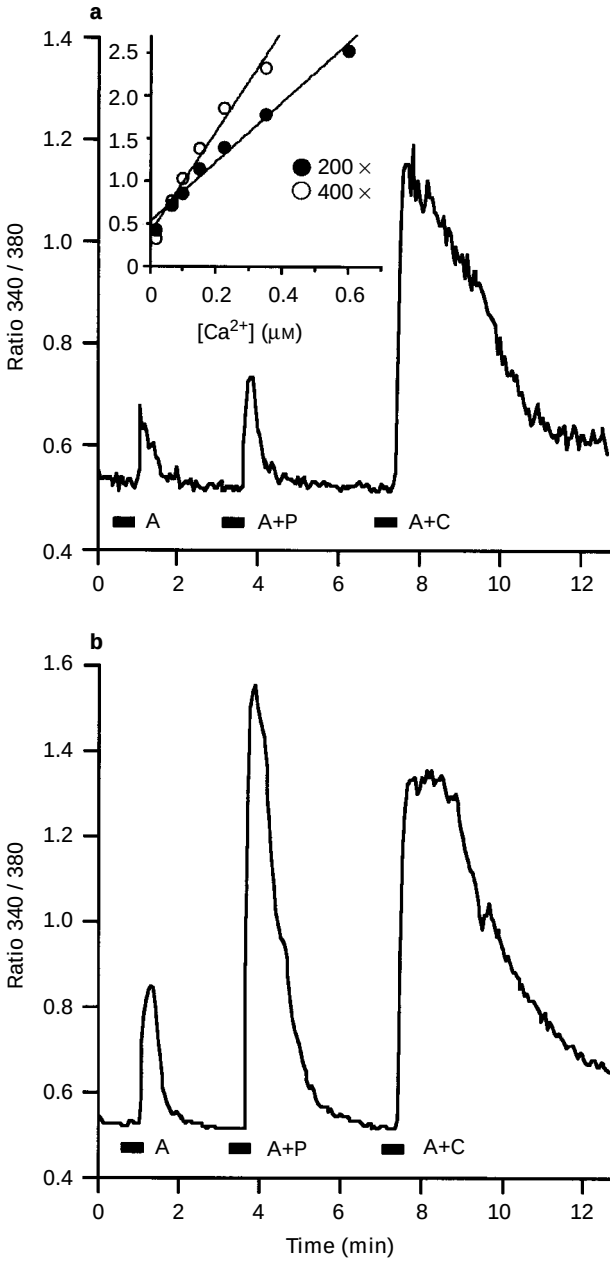

Figure 2 (a and b) Continuous recording of the 340/380 ratio in two hippocampal cells. Drugs were applied during the periods indicated by bars. A: 10 μM AMPA; A + P: 10 μM AMPA + 100 μM PEPA; A + C: 10 μM AMPA + 100 μM cyclothiazide. Inset: the linear relationship between calcium ion concentration and 340/380 ratio at magnification 200 × and 400 ×.

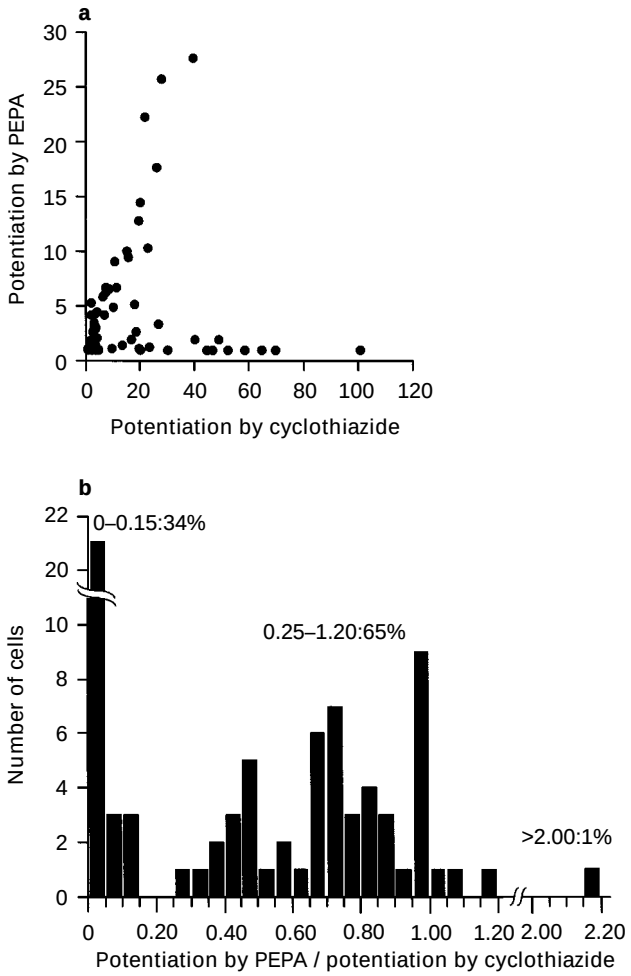

Figure 3 (a) A scatter plot illustrating potentiation by PEPA of AMPA-induced increase of $[Ca^{2+}]_i$ versus potentiation by cyclothiazide of AMPA-induced increase of $[Ca^{2+}]_i$ in each of the cultured hippocampal cells tested ($n = 80$). The potentiation by PEPA was calculated as follows: maximal increase of 340/380 ratio induced by AMPA (10 μM) plus 100 μM PEPA/maximal increase of the 340/380 ratio induced by AMPA (10 μM) alone. The potentiation by cyclothiazide was calculated as follows: maximal increase of 340/380 ratio induced by AMPA (10 μM) plus 100 μM cyclothiazide/maximal increase of 340/380 ratio induced by AMPA (10 μM) alone. (b) The distribution of P/C ratio values between rat cultured hippocampal cells. Abscissa scale: P/C ratio, which was calculated as follows: potentiation by 100 μM PEPA (as calculated above)/potentiation by 100 μM cyclothiazide (as calculated above). Ordinate scale: number of cells.

GluR1-flip ⩾ GluR2-flop > GluR1-flop > GluR3-flip and GluR4flip > GluR3-flop and GluR4-flop.

Based on the results of RT–PCR analysis, we expressed GluR1, GluR3, GluR1 plus GluR2 or GluR2 plus GluR3 in *Xenopus* oocytes. We excluded a combination of GluR1 and GluR3, because complexes consisting of GluR1 and GluR3 have not been detected in hippocampal tissue (Wenthold *et al*., 1996). Figure 5 shows representative current recordings from three oocytes expressing, respectively, GluR1-flip + GluR2-flip (1 : 4), GluR1-flop + GluR2-flip (1 : 1), and GluR2-flop + GluR3-flop (1 : 1). The values in parentheses represent the relative amounts of splice-variant-specific cRNAs in the oocyte injection solution. In Figure 5 the concentration, duration and order of application of drugs were similar to those used in the experiments shown in Figure 3. Both PEPA and cyclothiazide potentiated AMPA response in each of the three combinations of subunits expressed. However, there were clear differences in the relative activities of PEPA and cyclothiazide. PEPA was less potent than cyclothiazide in the oocyte expressing GluR1-flip + GluR2-flip. In contrast, PEPA was more potent than cyclothiazide in the oocyte expressing GluR2-flop + GluR3-flop. PEPA and cyclothiazide potentiated AMPA response similarly in the oocyte expressing GluR1-flop + GluR2-flip.

The results of similar experiments performed in oocytes expressing various AMPA receptor subunits are summarized in Table 1. The P/C ratio varied with subunit and splice-variant compositions and the ratio of their compositions. The highest P/C value (2.20) was obtained in oocytes expressing GluR1-flop alone, and the lowest value (0.19) was obtained in oocytes expressing GluR1-flip + GluR2-flip (1 : 4). From Table

1, we found that the s.e.mean of the P/C ratio was smaller than that of the potentiation by PEPA or cyclothiazide. Namely, the s.e.mean of the P/C ratio was within 10% of the mean value in all 20 kinds of oocytes tested (oocytes expressing GluR3-flop plus GluR2-flip (9 : 1) showed the largest s.e.mean when compared with the mean value, 10.0% of the mean values). On the other hand, the s.e.mean of the potentiation by PEPA or cyclothiazide was larger than 10% of the mean value in 9 (PEPA) or 12 (cyclothiazide) kinds of oocytes. Oocytes expressing GluR3-flop plus GluR2-flip (1 : 1) showed the largest s.e.mean when compared with the mean value in both the potentiations by PEPA (30%) and by cyclothiazide (22%). These results suggest that the P/C ratio is more consistent than the potentiation by PEPA or cyclothiazide alone as a measure of AMPA receptor heterogeneity.

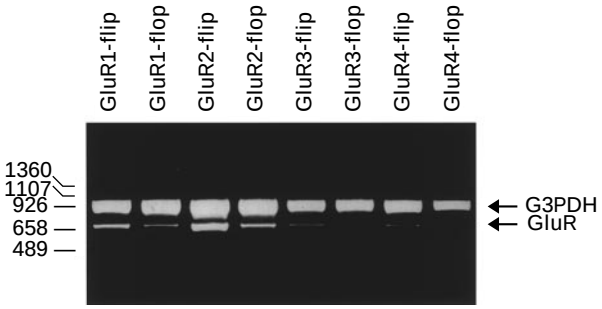

Figure 4 RT–PCR amplification of total RNA isolated from hippocampal cells cultured for 8 days *in vitro*. An ethidium bromide-stained agarose gel is shown. Eight sets of primers were used to amplify specific fragments of GluR(1-4/flip and flop). All the primers amplified fragments of approximately 685 bp (indicated by GluR). The numbers on the left correspond to DNA size markers (bp). The band migrating at approximately 926 bp represents the amplified product G3PDH which was used as an internal standard in each PCR reaction.

Figure 6 shows a scatterplot correlating the potentiation by cyclothiazide versus the potentiation by PEPA in oocytes. AMPA receptors consisting of flip variants alone showed higher sensitivity to cyclothiazide, and those consisting flop variants alone showed higher sensitivity to PEPA. AMPA receptors consisting of both flip and flop variants showed nearly equivalent sensitivity to PEPA and cyclothiazide. The slope of linear regressions fit through the data for each group were 0.15, 1.30, and 1.97 for the flip, flip + flop and flop variants respectively.

Comparison between results from culture cells and oocytes

Figure 7a shows the cellular variation of the P/C ratio versus potentiation by PEPA in oocytes. From these data, it was suggested that oocytes expressing only flip variants (GluR1-flip alone, GluR1-flip + GluR2-flip, or GluR3-flip + GluR2-flip), except for those expressing GluR3-flip alone, exhibit a low P/C ratio (<0.5) and low sensitivity for potentiation by PEPA ($\leqslant 6$ fold). In our experiments oocytes expressing GluR3-flip showed a relatively high P/C ratio (0.70) (see Discussion). In contrast, the oocytes with high P/C ratios (>1.8) were those expressing GluR1-flop or GluR3-flop alone or complexes of these flop variants and GluR2-flop. In this group, oocytes expressing GluR3-flop or GluR3-flop + GluR2-flop showed greater sensitivity to potentiation by PEPA than oocytes expressing GluR1-flop or GluR1-flop + GluR2-flop. In a previous study, we suggested that PEPA preferentially affected GluR3 and GluR4 subunits rather than GluR1 subunits (Sekiguchi *et al*., 1997). This subunit preference was maintained when AMPA was used as an agonist.

On the other hand, oocytes expressing various splice-variant combinations of GluR1 + GluR2 or GluR2 + GluR3 had P/C ratios between 0.5 and 1.2. In these cells, the effect of co-expression of GluR2 upon the P/C ratio was complex.

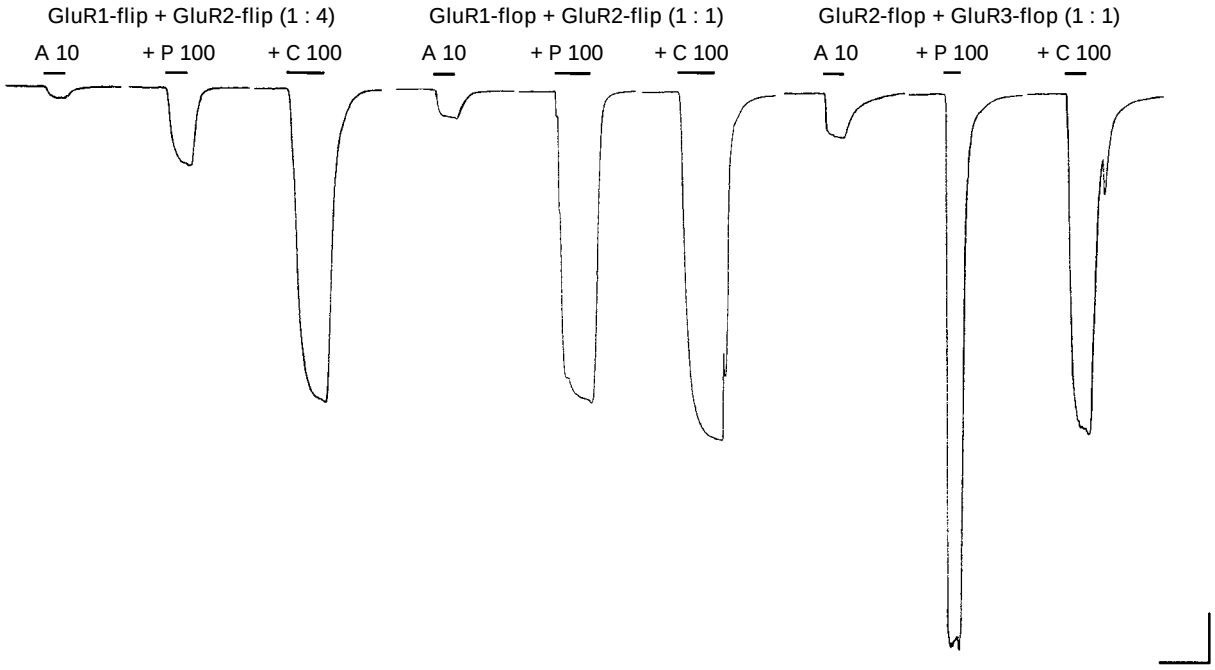

Figure 5 Electrophysiological current records in three *Xenopus* oocytes injected with cRNAs specific for the AMPA receptor subunits as denoted (relative cRNA ratios in parentheses). A 10: 10 μM AMPA; + P 100: 10 μM AMPA + 100 μM PEPA; + C 100: 10 μM AMPA + 100 μM cyclothiazide. A solution containing AMPA alone was applied first, and a solution containing both AMPA and PEPA was applied after $\geqslant 1$ min wash. A solution containing both AMPA and cyclothiazide was finally applied after at least a 3 min wash. Calibration bars: 40 s and 100 nA. Holding potential = −100 mV.

Table 1 Potentiation of AMPA response by PEPA and cyclothiazide in *Xenopus* oocytes injected with cRNA specific for AMPA receptors

cRNA injected	*Potentiation by PEPA*	*Potentiation by CYZ*	*P/C ratio*	n
R1i	1.90±0.17	5.31±0.97	0.39±0.03	7
R1i : R2i = 1 : 4	6.41±0.55	34.06±4.19	0.19±0.01	5
= 1 : 12	5.94±0.62	31.15±4.18	0.20±0.02	5
R1i : R2o = 9 : 1	5.06±0.68	8.50±1.45	0.61±0.03	5
= 3 : 1	18.76±1.95	21.5±2.17	0.87±0.03	5
= 1 : 1	20.08±1.93	19.92±1.54	1.00±0.04	5
R3i	2.51±0.09	3.59±0.22	0.70±0.03	5
R3i : R2i = 1 : 4	4.39±0.25	8.61±1.14	0.47±0.03	5
R3i : R2o = 1 : 1	4.78±0.35	6.83±0.71	0.71±0.02	4
R1o	2.90±0.24	1.34±0.13	2.20±0.20	5
R1o : R2i = 4 : 1	7.43±1.07	8.78±1.55	0.86±0.03	5
= 1 : 1	9.49±0.54	11.57±0.85	0.83±0.03	4
= 1 : 9	9.20±2.38	17.86±3.42	0.48±0.04	5
R1o : R2o = 1 : 1	7.44±0.47	3.53±0.09	2.11±0.12	4
R3o	11.14±1.17	5.95±0.23	1.86±0.14	6
R3o : R2i = 9 : 1	14.38±1.76	7.49±0.89	1.94±0.02	4
= 1 : 1	8.85±1.26	7.18±0.99	1.23±0.05	5
= 1 : 4	12.27±1.01	11.75±0.91	1.04±0.02	5
= 1 : 9	10.10±0.45	11.94±0.69	0.85±0.02	5
R3o : R2o = 1 : 1	21.07±6.35	10.51±2.28	1.88±0.11	5

Xenopus oocytes were injected with cRNA denoted (i = flip, o = flop). After 3 days incubation, the oocytes were perfused with 10 μM AMPA, 10 μM AMPA + 100 μM PEPA, or 10 μM AMPA + 100 μM cyclothiazide (CYZ) for about 30 s at a holding potential of −100 mV. Potentiation by PEPA or by CYZ was calculated as the current amplitude of the response induced by (AMPA + PEPA) or (AMPA + CYZ) divided by the current amplitude of the response induced by AMPA alone. P/C ratio = potentiation by PEPA divided by the potentiation by CYZ. Values are expressed as mean ± s.e.mean of the values from 4 to 7 oocytes (*n*).

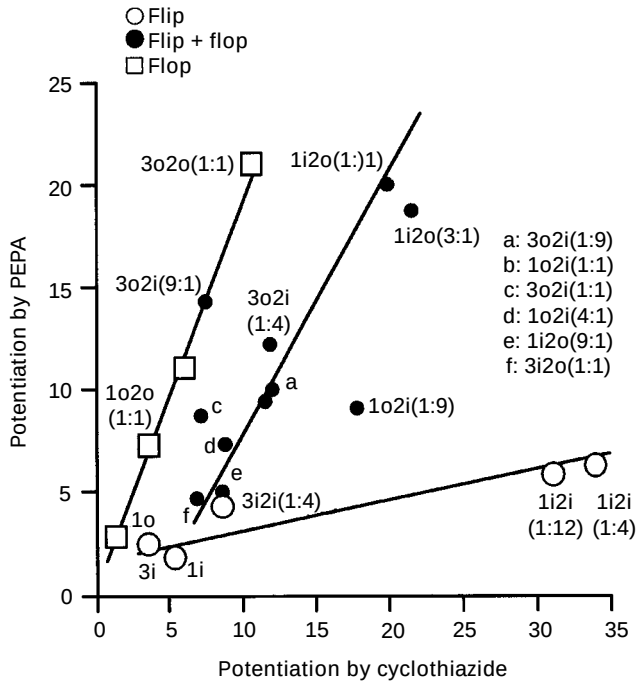

Figure 6 Scatterplot correlating the potentiation by cyclothiazide versus the potentiation by PEPA in oocytes. Oocytes were injected with mixtures of cRNA in the relative levels denoted in parentheses. For example, 1i2o (1 : 1) means that cRNAs for GluR1-flip and GluR2-flop were combined 1 : 1 and then the mixture was injected into oocytes. The potentiation was calculated as follows: [current amplitude of response induced by AMPA (10 μM plus PEPA or cyclothiazide (100 μM)]/[current amplitude of response induced by AMPA (10 μM) alone]. Holding potential = −100 mV. Oocytes were injected with cRNA for flip-variant subunits alone; with cRNAs for a mixture of flip- and flop-variant subunits and with cRNA for flop-variant subunits alone. Data for each group are fitted separately with linear regression (r^2 = 0.863, 0.536, and 0.996 for flip, flip + flop and flop, respectively).

Namely, co-expression of GluR2-flop with GluR1-flip potentiated the action of PEPA more strongly than the action of cyclothiazide (see R1i versus R1i + R2o of Table 1), which caused an increase of the P/C ratio (0.39 in R1i versus 1.00 in R1i : R2o = 1 : 1). However, co-expression of GluR2-flop with GluR3-flip potentiated the actions of PEPA and cyclothiazide nearly equally (see R3i versus R3i + R2o in Table 1), which did not induce changes in the P/C ratio (0.70 in R3i versus 0.71 in R3i + R2o = 1 : 1). Furthermore, co-expression of GluR2-flip with GluR1-flop potentiated the action of cyclothiazide more strongly than the action of PEPA (see R1o versus R1o + R2i in Table 1), which caused a decrease in the P/C ratio (2.20 in R3o versus 0.83 in R1o : R2i = 1 : 1). However, co-expression of GluR2-flip with GluR3-flop did not significantly affect the action of PEPA but potentiated the action of cyclothiazide (see R3o versus R3o + R2i in Table 1), which induced a decrease in the P/C ratio (1.86 in R3o versus 1.23 in R3o : R2i = 1 : 1).

Figure 7b shows the cellular variation of P/C ratios and potentiation by PEPA in hippocampal cells (constructed from data in Figure 3). When Figure 7b was compared with Figure 7a, three points became evident concerning the expression of AMPA receptor subunits and variants in the cultured hippocampal cells we used. (1) There was a group of cells (27/80, 34%) with low P/C ratios (<0.15) and low sensitivity for potentiation by PEPA (<6 fold). (2) Relatively few cells exhibited high P/C ratios. Only one cell with a high P/C ratio (2.2) was observed and this cell showed low sensitivity for PEPA. Similarities in the P/C ratio and in the potentiation by PEPA seem to suggest the possibility that these cells express flop variants of GluR1 and GluR2 abundantly.

Therefore, it is likely that only a minor population of cells abundantly expressed GluR1-flop or GluR1-flop + GluR2-flop in our cultures. Since we could not find cells with high P/C ratios and high sensitivity for PEPA, cells which abundantly express GluR3-flop or its complexes with GluR2-flop seemed

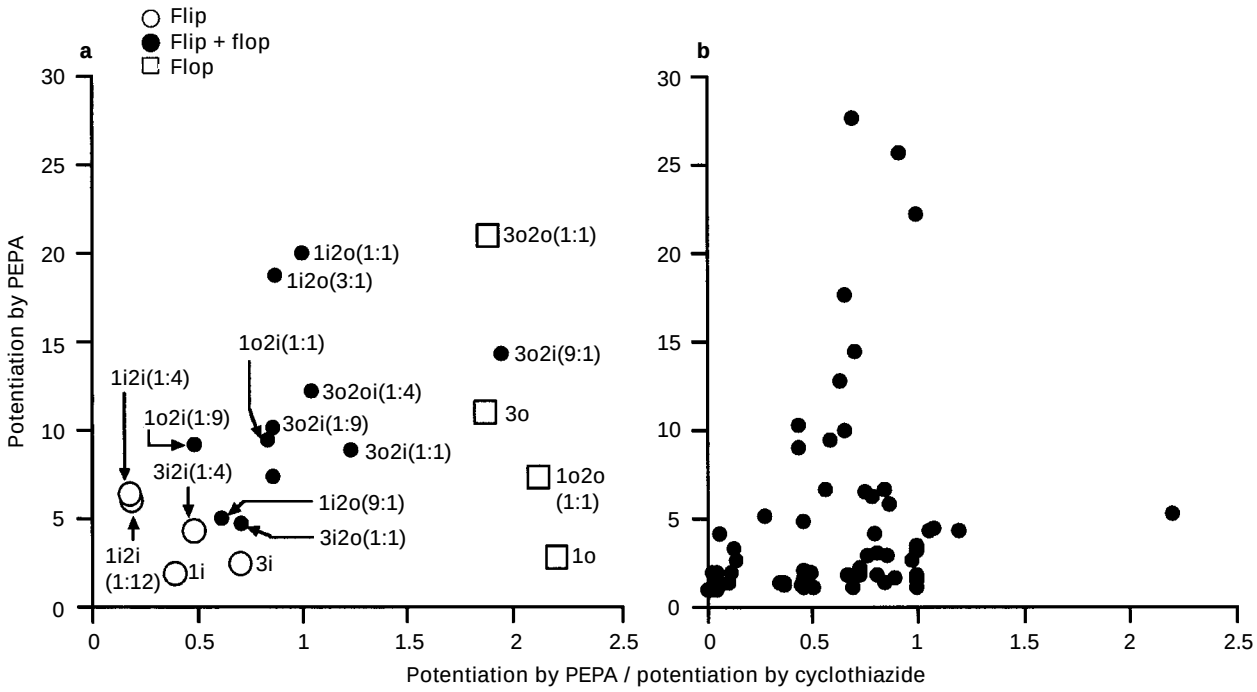

Figure 7 Cellular variation of P/C ratios and potentiation by PEPA in *Xenopus* oocytes expressing AMPA receptor subunits and splice variants (a) and in rat hippocampal cultures (b). Each cell was plotted according to values of the P/C ratio and the potentiation by PEPA. The P/C ratio in oocytes was calculated as follows: {[current amplitude of response induced by AMPA (10 μM) plus 100 μM PEPA]/[current amplitude of response induced by AMPA (10 μM) alone]}/{[current amplitude of response induced by AMPA (10 μM) plus 100 μM cyclothiazide]/[current amplitude of response induced by AMPA (10 μM) alone]}. The same data in Figure 6 were used for oocytes. P/C ratios in hippocampal cells were calculated as in Figure 3 and potentiation by PEPA was calculated as follows: [maximal increase of 340/380 ratio induced by AMPA (10 μM) plus 100 μM PEPA]/[maximal increase of 340/380 ratio induced by AMPA (10 μM) alone].

to be absent or very rare in our cultures. (3) A large number of cells (52/80, 65%) exhibited intermediate P/C ratios (0.5 to 1.2). In these cells, the potentiation by PEPA varied widely, and it was impossible to specify the relative abundance of two splice variants in these cells.

Discussion

Our results from hippocampal cultures revealed that hippocampal cells consist of multiple populations with respect to P/C ratio, which suggests that multiple AMPA receptor subtypes are expressed in the hippocampal cultures we used. In the hippocampus, CA1 pyramidal and dentate granule cells show late postnatal onset of expression of flop mRNA resulting in mosaic expression of flip and flop variants at postnatal days 12 to 17 (Geiger *et al*., 1995). On the other hand, flip-flop alternative splicing does not significantly occur until postnatal days 12 to 17 in CA3 pyramidal cells and expression of flip variants predominates during this time period (Monyer *et al*., 1991; Geiger *et al*., 1995). In contrast, dentate gyrus interneurones appear to express flop variants initially at postnatal day 8 (Monyer *et al*., 1991) and this expression predominates at postnatal days 12 to 17 (Geiger *et al*., 1995). It seems that the cell type- and developmental stage-dependent expression of AMPA receptor splice variants is one of the mechanisms underlying the detection of multiple populations in hippocampal cells by a P/C ratio.

Fleck *et al*. (1996) recently suggested, by use of cyclothiazide alone, that there are at least three populations of AMPA receptor subtypes in hippocampal cultures. Their criterion was a time constant of recovery from modulation by cyclothiazide either fast (<400 ms), intermediate (2 to 4), or slow (>20 s), and they found that, of 73 cells tested, 19 had fast time constants for the recovery (Fleck *et al*., 1996). They also showed, in human embryonic kidney 293 cells expressing recombinant AMPA receptor subunits, that the cells expressing GluR1 alone or GluR1-flop and GluR2-flop had a fast time constant (Fleck *et al*., 1997). Therefore, it appears that about 26% of the cells they tested expressed flop variants predominantly. However, in our work, only 1 out of 80 cells tested had a high P/C ratio, suggesting that only about 1% of the cells we tested expressed flop variants predominantly. This difference probably results from a difference in the culture used. In the experiments by Fleck *et al*. (1996) primary cultures of neurones were grown on a glial cell feeder layer, whereas in our experiments, neurone-rich primary cultures were maintained in serum-free media. It is well known that the presence of glial cells in culture facilitates the growth of neurones, which may facilitate flip-flop alternative splicing.

Our results from *Xenopus* oocytes confirm that both potentiation by PEPA and P/C ratio varies with subunits and splice variants expressed in oocytes. Cells exhibiting both a low P/C ratio (<0.5) and a low sensitivity for potentiation by PEPA (<6 fold) expressed flip variants predominantly. The low P/C ratio results from a combination of the weak potentiation by PEPA and potent action by cyclothiazide. On the other hand, cells exhibiting high P/C ratios (1.8 to 2.2) expressed flop variants predominantly. The high P/C ratio results from a combination of the potent action of PEPA and the weak action by cyclothiazide. In the cells with high P/C ratios, cells with high sensitivity for PEPA expressed GluR3-flop and those with low sensitivity for PEPA expressed GluR1-flop.

However, it was impossible to specify the relative abundance of flip and flop variants in the cells with intermediate P/C ratios (0.5 to 1.2). For example, a P/C ratio of 0.70 was measured in oocytes expressing GluR3-flip alone, which is virtually identical to the value of 0.71 obtained in oocytes expressing GluR3-flip + GluR2-flop (1 : 1). As for oocytes expressing GluR3-flip alone, we previously obtained a lower P/C ratio (0.20) when 100 μM glutamate, 25 μM PEPA and 25 μM cyclothiazide were used (Sekiguchi *et al*., 1997). In the present study, the use of AMPA as an agonist and cyclothiazide at 100 μM may be responsible for the discrepancy of these values (0.70 versus 0.20). Namely, in oocytes expressing GluR3-flip alone, 100 μM cyclothiazide potentiated AMPA (10 μM) response only 3.59 fold (Table 1), whereas 25 μM cyclothiazide potentiated the glutamate (100 μM) response more efficaciously (13.4 fold) (Sekiguchi *et al*., 1997). In the homomeric GluR3-flip receptor, allosteric structure between the binding sites for AMPA and cyclothiazide may be different from that between the binding sites for glutamate and cyclothiazide. Indeed, it has been suggested that glutamate, AMPA, and kainate bind to different, overlapping substructures on recombinant AMPA receptors (Stein *et al*., 1992).

Although the cell-to-cell heterogeneity of AMPA receptors revealed by use of two allosteric potentiators was explained in the present study by the different sensitivity of subunits and splice-isoforms for these potantiators, there was a clear discrepancy between the data from cultured cells and oocytes. Namely, our PCR data (Figure 4) suggested that GluR1-flip and GluR2-flip are predominant in the hippocampal cultures used. Since the P/C ratio in oocytes co-expressing these subunits were 0.20 or 0.19 (Table 1), it is expected that there are many culture cells in which the P/C ratio is approximately 0.20. However, very few culture cells have a P/C ratio equal to or near 0.2 (Figure 7b). One plausible explanation for this discrepancy is as follows; in the culture cells expressing both GluR1-flip and GluR2-flip, the potentiation by PEPA may be too weak to prolong the depolarization mediated by the AMPA receptors. In such a condition, the PEPA potentiation is underestimated, which causes a lower P/C ratio than oocytes. Indeed, there are many cells in which P/C ratio is less than 0.15. As far as we could discern, there were no subunit- and splice variant-combinations in which the P/C ratio is less than 0.15. Therefore, it appears that the cells with a P/C ratio less than 0.15 are the cells expressing both GluR1-flip and GluR2-flip.

Furthermore, there are two possible factors which may cause discrepancies between the data from oocytes and cultured cells. One is that the kinetic properties of AMPA receptors are also modified by RNA editing at the R/G site (Mosbacher *et al*., 1994). Therefore, in order to obtain further details on the origin of the AMPA receptor heterogeneity detected in the present study, it may be important to examine the effects of R/G site editing upon the actions of allosteric potentiators. Another is the presence of calcium-permeable AMPA receptors. It is well known that GluR2-less AMPA receptors conduct calcium ions (Hollmann *et al*., 1991; Hume *et al*., 1991). Therefore, calcium-imaging should be used carefully on analysis of the heterogeneity in the cells of tissues where the expression of GluR2 is weak.

In conclusion, we investigated whether a recently developed allosteric potentiator for AMPA receptors, PEPA, can be utilized as an indicator of AMPA receptor heterogeneity. Our results suggest that the potentiation by PEPA alone reveals cell-to-cell heterogeneity of AMPA receptors, but comparing the actions of PEPA and cyclothiazide further facilitates the detection of AMPA receptor heterogeneity. The cell-to-cell difference in expression of AMPA receptor subunits and splice variants seems to be one of the major mechanisms underlying the detection of AMPA receptor heterogeneity by these potentiators.

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