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Characterization of Ca^{2+} influx through recombinant P2X receptor in C6BU-1 cells

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1 The effects of exogenous adenosine 5′-triphosphate (ATP) and α,β-methylene ATP (α,βmeATP) on C6BU-1 cells transfected with $P2X_2$ and $P2X_3$ subtypes, separately or together ($P2X_{2+3}$), were investigated using fura-2 fluorescence recording and whole-cell patch clamp recording methods.

2 Untransfected C6BU-1 cells showed no intracellular Ca^{2+} ($[Ca^{2+}]_i$) increase in response to depolarizing stimulation with high K^+ or stimulation with ATP. There was no current induced by ATP under voltage clamp conditions in untransfected C6BU-1 cells. ATP caused Ca^{2+} influx only from extracellular sources in C6BU-1 cells transfected with the P2X subtypes, suggesting that the C6BU-1 cell line is suitable for the characterization of Ca^{2+} influx through the P2X subtypes.

3 In C6BU-1 cells transfected with the $P2X_2$ subtype, ATP (more than 10 μM) but not α,βmeATP (up to 100 μM) evoked a rise in $[Ca^{2+}]_i$.

4 In the cells transfected with the $P2X_3$ subtype, current responses under voltage clamp conditions were observed at ATP concentrations higher than 0.1 μM of α,βmeATP were required. This discrepancy in the concentration dependence of the agonist responses with respect to the $[Ca^{2+}]_i$ rise and the current response was seen only with the $P2X_3$ subtype. In addition, the agonist-induced rise in $[Ca^{2+}]_i$ was observed only after the first application because of desensitization of this subtype.

5 In C6BU-1 cells co-transfected with $P2X_2$ and $P2X_3$, ATP at 1 μM evoked a $[Ca^{2+}]_i$ rise. This responsiveness was higher than that of the other subtype combinations tested. The efficiency of expression was improved by co-transfection with $P2X_2$ and $P2X_3$, when compared to transfection with the $P2X_3$ subtype alone. The desensitization of the $P2X_{2+3}$ was apparently slower than that of the $P2X_3$ subtype alone. Therefore, this combination could respond to the repeated application of agonists each time with a $[Ca^{2+}]_i$ rise.

6 These results suggest that the $P2X_2$ and $P2X_3$ subtypes assemble a heteromultimer and that this heterogeneous expression acquires more effective Ca^{2+} dynamics than that by homogenously expressed $P2X_2$ or $P2X_3$

Keywords: ATP; P2X; C6BU-1; fura-2 and patch clamp

Introduction

ATP is released from nerve terminals and acts as a neurotransmitter in the nervous system. Extracellular ATP causes a depolarization of the postsynaptic membrane by opening cationic P2X receptor-channels (reviewed in Burnstock, 1995; Surprenant *et al*., 1995). These ATP-activated channels have been cloned recently as P2X receptors and classified into seven subtypes ($P2X_1$; Valera *et al*., 1994; $P2X_2$; Brake *et al*., 1994; $P2X_3$; Chen *et al*., 1995; Lewis *et al*., 1995; $P2X_4$; Bo *et al*., 1995; Buell *et al*., 1996a; Seguela *et al*., 1996; Soto *et al*., 1996; Wang *et al*., 1996; $P2X_5$ and $P2X_6$; Collo *et al*., 1996; $P2X_7$; Surprenant *et al*., 1996). In the nervous system, P2X receptors are present in certain areas important for nociception (Collo *et al*., 1996). The $P2X_3$ subtype is associated exclusively with sensory neurons of peripheral ganglia, such as dorsal root ganglia (DRG). Further, the $P2X_3$ subtype was expressed selectively in a subpopulation of nociceptive DRG neurons (Chen *et al*., 1995; Lewis *et al*., 1995). Other P2X subtypes were detected not only in DRG neurons but also in spinal neurons of the dorsal horn where nociception is relayed from DRG neurons.

A relatively high permeability of ATP-activated cation channels to Ca^{2+} was found in rat pheochromocytoma cells (PC12) (Nakazawa *et al*., 1990), nucleus tractus solitarii neurons (Ueno *et al*., 1992) and human embryonic kidney (HEK 293) cells with exogenously transfected P2X receptors under the voltage-clamp condition (Evans *et al*., 1996). Fura-2 fluorescence recording also showed that ATP causes a rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$) associated with catecholamine release in PC12 cells (Nakazawa & Inoue, 1992) and $[Ca^{2+}]_i$ influx into rat cultured DRG neurons through a P2X receptor channel complex (Bouvier *et al*., 1991). There is increasing evidence that Ca^{2+} influx through the P2X receptor plays an important role in the modulation of this receptor type (Burnstock & Wood, 1996; Khiroug *et al*., 1997). In addition, pain-related neurotransmitters are known to be Ca^{2+}-dependent and the Ca^{2+} signal in sensory neurons is known to be involved in nociception and pathological states such as neuropathy. In DRG neurons, the $P2X_2$ and $P2X_3$ subtypes are mainly expressed (Collo *et al*., 1996) and an electrophysiological analysis suggested that the $P2X_2$ and $P2X_3$ subtypes might form a heteromultimeric complex (Lewis *et al*., 1995). However, there has been no precise characterization of the heterogeneous expression of recombinant $P2X_2$ and $P2X_3$ subtypes in terms of $[Ca^{2+}]_i$ influx under more physiological conditions.

In the present study, we focused on the calcium dynamics of $P2X_2$, $P2X_3$ and $P2X_{2+3}$, using the C6BU-1 cell line. We found that a heteromultimer complex of $P2X_2$ and $P2X_3$ acquired a more efficient expression level and greater Ca^{2+} influx ability than the homomers of $P2X_2$ or $P2X_3$.

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Methods

Expression of P2X receptors

cDNA constructs for P2X receptor subtypes were provided by T. Brake, University of California San Francisco (rat pheochromocytoma cell line; (PC12; $P2X_2$), G. Buell, Glaxo Institute Geneva, Switzerland (rat $P2X_3$). P2X receptor subtype cDNAs were transferred to the eukaryotic expression vector pcDNA3 (InVitrogen, San Diego, CA, U.S.A.) for expression in HEK 293 and C6BU-1 cells. A cDNA construct for CD8 in the eukaryotic expression vector piH3 was provided by B. Seed (Massachusetts General Hospital, Boston, MA, U.S.A.).

cDNA constructs were introduced into HEK 293 cells (Japanese Collection of Research Bioresources, Tokyo, Japan) or C6BU-1 cells (provided by Dr. Higashida, Kanazawa University, Kanazawa, Japan) using polycationic liposomes. Both cell lines were maintained in a Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Gibco, Gaithersburg, MD, U.S.A.), 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2. Cells were passaged twice a week and were used for 8–16 passages. For transfection, cells were plated at 10^5 cells/35-mm culture dish the day before transfection, and cDNAs (0.5 μg of each subunit plus 0.1 μg CD8 cDNA) and Lifpofectamine (Gibco) dissolved in Opti-Mem (Gibco) were placed in the dish. Cells were rinsed after 5 h and maintained in normal culture medium at 37°C. Recordings were made 24–72 h after transfection.

Cells that expressed a high level of protein from exogenous cDNA were identified using the bead-labeling technique (Jurman *et al*., 1994). cDNA coding for CD8 was coexpressed with P2X receptor subtypes, and cells expressing high levels of CD8 were identified with beads that had a covalently coupled antibody to CD8 (Dynabeads, Dynal, Lake Success, NY, U.S.A.).

$[Ca^{2+}]_i$ measurements

The increase in $[Ca^{2+}]_i$ in single cells was measured by the fura-2 technique (Grynkiewicz *et al*., 1985) with minor modifications (Koizumi *et al*., 1994). All procedures including incubation, washing and drug application, were made with a balanced salt solution (BSS) of the following composition (in mM): NaCl 150, KCl 5.0, $CaCl_2$ 1.2, $MgCl_2$ 1.2, NaH_2PO_4 1.2, NaH_2PO_4 1.2, D-glucose 10, ethylenediamine-N,N,N′,N′-tetraacetic acid (EDTA) 0.1 and 2-[4-(2-hydroxyethyl)-1-piperazinyl]ehtanesulphonic acid (HEPES) 25, pH adjusted to 7.4 with NaOH. For Ca^{2+}-free experiments, we used a medium in which Ca^{2+} was removed and 1 mM O,O′-bis(2-aminoethyl)ethyleneglycol-N,N,N′N′-teraacetic acid (EGTA) was added (Ca^{2+}-free BSS). The cells were washed with BSS and incubated with 5 μM 1-[6-amino-2(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2-amino-5-methylphenoxy)-ethane-N,N,N′,N′-tetraacetic acid, pentaacetoxymethyl ester (fura-2 AM) (Dojindo, Kumamoto, Japan) in BSS at 37°C. After 30 min incubation, the cells were washed with BSS. The coverslips were mounted on an inverted epifluorescence microscope (TMD-300 Nikon, Tokyo, Japan) equipped with a 75W xenon-lamp and bandpass filters of 340 nm wavelength (F340) for measurement of the Ca^{2+}-dependent signal, and 360 nm wavelength (F360) for measurement of the Ca^{2+} independent signal. The emission fluorescence was measured at 510 nm. Measurements were carried out at room temperature. Image data, recorded by a high-sensitivity silicon intensifier target camera (C-2741-08, Hamamatsu Photonics, Co., Hamamatsu, Japan) were processed by a Ca^{2+}-analyzing system (Furusawa Laboratory appliance Co., Kawagoe, Japan). The sampling rate was 3 Hz. The absolute $[Ca^{2+}]_i$ was estimated from the ratio of emitted fluorescence (F340/F360) according to a calibration curve obtained by using Ca^{2+}-buffers (Molecular Probes Inc., C-3712 with 1 mM $MgCl_2$).

Electrical recording

Recordings were made using the conventional whole cell patch-clamp method (Hamill *et al*., 1981). All experiments were performed at room temperature (21–23°C). The pipette solution contained (in mM): CsCl 140, $MgCl_2$ 2, EGTA 5, HEPES 10, pH adjusted to 7.2 with CsOH. The pipette resistances were 3–5 MOhm. Series resistance (2–8 MOhm) and cell capacitance (12–40 pF) were compensated up to 80%. In all cases, data were obtained from isolated single cells. Currents were filtered at 300 Hz with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA, U.S.A.) and measured with an Axopatch 200A amplifier (Axon Instruments, Foster City CA, U.S.A.). Data were then sampled at 1 kHz and stored on-line with a 486 PC using the pClamp software (Axon Instruments). Concentration-effect data were fitted using the Hill equation with the SigmaPlot software (Jandel Scientific Software, SanRafael, CA). Drugs were dissolved in an external solution of the following composition (in mM): NaCl 150, KCl 5.0 $CaCl_2$ 2, $MgCl_2$ 1, D-glucose 10, HEPES 10, pH adjusted to 7.4 with NaOH. The solutions of drugs were applied with a polyethylene Y-tube (equilibration time $<$20 ms) (Ueno *et al*., 1997). The bath was continuously perfused with normal external solution from a separate perfusion line, and the solution was removed from the bath with a Leiden aspirator (Medical Systems, Greenvale, NY, U.S.A.).

Drugs

Drugs used were ATP (Sigma, St. Louis, MO, U.S.A.), α,βmeATP (Sigma). The pH of solutions containing ATP or α,βmeATP was readjusted to 7.4 with NaOH.

Statistics

The reported probabilities for significance of differences were obtained using the Mann–Whitney rank sum test.

Results

Agonist-induced currents in C6BU-1 cells transfected with P2X receptors

In Figure 1, the representative ATP- or α,βmeATP-activated currents for each P2X subtype combination are shown. The $P2X_2$ subtype did not respond to α,βmeATP, while both the $P2X_3$ and the $P2X_{2+3}$ were sensitive to α,βmeATP. The peak amplitudes in response to ATP decreased in the order $P2X_{2+3} > P2X_2 > P2X_3$ (Table 1). In Figure 2, the concentration-response curves for each subtype are shown. With the $P2X_2$ subtype, the ATP-activated currents at a holding potential of -50 mV were observed usually at ATP concentrations higher than 10 μM, and the current was activated relatively slowly and showed small desensitization

in the presence of ATP. At the $P2X_2$ subtype ATP had an EC_{50} value of 48 μM and a Hill coefficient of 1.5. In contast, with the $P2X_3$ subtype, ATP-activated currents were evoked at concentrations higher than 0.1 μM and showed a markedly rapid activation and desensitization. ATP had the lowest EC_{50} value of 1.6 μM and a Hill coefficient of 1.0 at the $P2X_3$ subtype. When $P2X_2$ and $P2X_3$ were coexpressed in C6BU-1 cells, the ATP-activated current kinetics possessed characteristics intermediate between those at $P2X_2$ and $P2X_3$. In this case, the activation and desensitization of currents were faster than those at the $P2X_2$ subtype but slower than those at the $P2X_3$ subtype. For the $P2X_2$ and $P2X_3$ coexpression, the current threshold was around 1 μM, the EC50 value was 22 μM, and the Hill coefficient 1.0. We also examined the concentration-response relationship for the α,βmeATP activation of the $P2X_3$ and the $P2X_{2+3}$ subtypes. The EC_{50} value and the Hill coefficient with the $P2X_3$ subtype were 6 μM and 0.9 ($n=3$), and those with the $P2X_{2+3}$ subtypes were 30 μM and 0.9 ($n=3$), respectively. Hence, C6BU-1 cells expressed transfected P2X receptors, and agonist-activated currents recorded under the voltage clamp condition had characteristics similar to those recorded in the HEK293 expression system (Lewis *et al*., 1995), in terms of sensitivity to α,βmeATP and current kinetics.

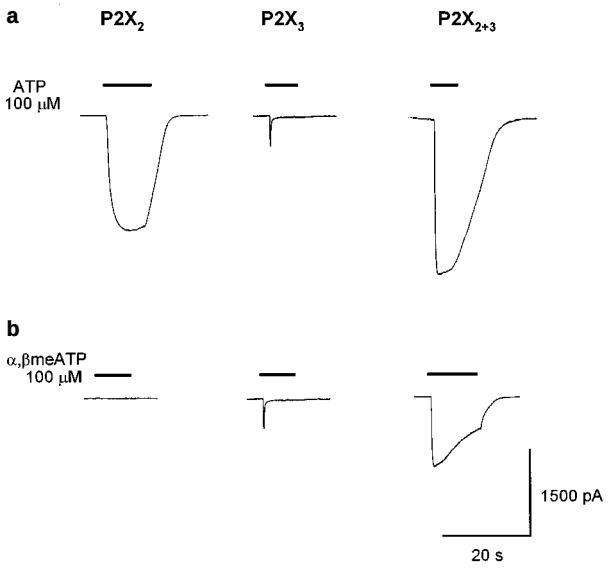

Figure 1 Current responses to ATP (a) or α,βmeATP (b) in C6BU-1 cells transfected with P2X receptor subtypes. The currents induced by ATP (100 μM) are shown for each subtype combination. The subtypes are indicated. Cells were held at -50 mV.

Comparison of responses to ATP application between untransfected C6BU-1 cells and C6BU-1 cells transfected with P2X receptors

The C6BU-1 cell line was established from the C6-glioma cell. Depolarizing stimulation with high K^+ (80 mM) solution did not affect $[Ca^{2+}]_i$ in the untransfected C6BU-1 cells tested ($n=48$) using fura-2 fluorescence recording. This result suggested that there were no functional voltage-dependent Ca^{2+} channels in the C6BU-1 cell line. ATP-activated currents were not observed under whole-cell recording conditions at a holding potential of -50 mV ($n=32$). In the absence and presence of extracellular Ca^{2+}, 100 μM of ATP did not alter $[Ca^{2+}]_i$ in more than 99% (758 of 763) of the untransfected

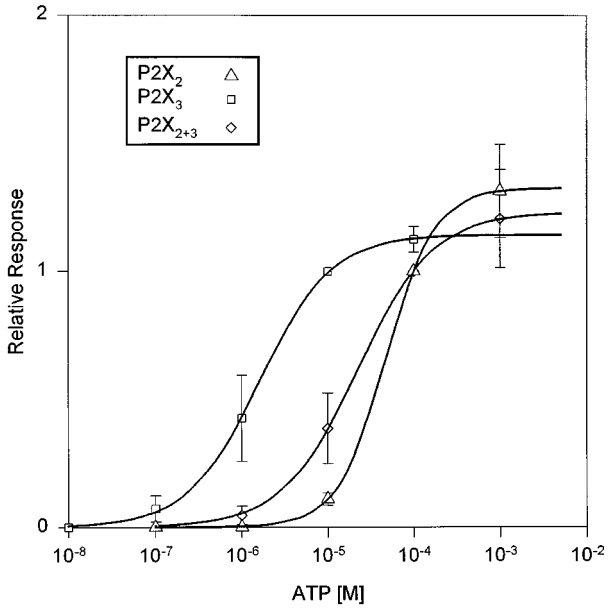

Figure 2 ATP-induced currents in cells transfected with recombinant P2X receptors. Concentration-response curves for the ATP-elicited currents are plotted. The peak amplitude of inward current elicited by ATP application was measured and this response was normalized to the response of the same cell to 100 μM ATP ($P2X_2$, $P2X_{2+3}$) or to 10 μM ATP ($P2X_3$). Results obtained from C6BU-1 cells transfected with $P2X_2$ (Δ, mean $\pm$ s.e.m. for data from four to six cells at various concentrations), $P2X_3$ ($\square$, for data from four to five cells) alone or $P2X_2$ and $P2X_3$ ($\diamond$, for data from four to six cells). The smooth lines show the values predicted by the Hill equation (1):

$$I(X) = I_{max}(X/EC_{50})^n/(1+(X/EC_{50})^n) \quad (1)$$

where I is the current elicited by the ATP concentration X, I_{max} is the fitted maximal current, EC_{50} is the concentration producing a half maximal response, and n is the Hill coefficient.

Table 1 Responses to 100 μM ATP OF C6-BU1 cells

C6-BU1 cells	*Current response (average $\pm$ s.e.) pS/pF*	*Fraction of current responding (responding/tested)*	*$[Ca^{2+}]_i$ response (average $\pm$ s.e.) Δratio (F340/F360)*	*Fraction of ($[Ca^{2+}]_i$) responding (responding/tested)*
$P2X_2$	3007 $\pm$ 811	1.0 (6/6)	0.61 $\pm$ 0.04	0.97 (30/31)
$P2X_3$	298 $\pm$ 99	0.67 (10/15)	0.30 $\pm$ 0.08	0.33 (6/18)
$P2X_{2+3}$	5292 $\pm$ 1188	1.0 (6/6)	0.72 $\pm$ 0.08	1.0 (22/22)

ATP (100 μM) was used as a test dose because it gave maximal or close-to-maximal responses for the subtype combinations tested. Cells were identified using the cotransfected marker CD8. The fraction of responsive cells was estimated based on the observed fraction of responding cells, which was the current of $[Ca^{2+}]_i$ rise response to ATP. The current responses are given in units of conductance divided by membrane capacitance (pS/pF) to account for the effects of the holding potential and cell size. For the $P2X_3$, the current response is significantly smaller than those for $P2X_2$ or $P2X_{2+3}$ ($P<0.01$). The $[Ca^{2+}]_i$ rise responses are compared using the relative increase of ratio (F340/F360) from the basal level before the applications. $[Ca^{2+}]_i$ responses for $p2X_3$ are also significantly smaller than those for $P2X_2$ or $P2X_{2+3}$ ($P<0.05$).

C6BU-1 cells tested (Figure 3a). These data indicate that the C6BU-1 cell line fails to express functional ionotropic or G protein coupled ATP receptors.

Only C6Bu-1 cells transfected with cDNAs of P2X receptors responded to the application of ATP, whereas these transfected cells were still insensitive to high K^+ application. In the whole-cell mode, ATP activated an inward current at a holding potential of -50 mV in transfected cells. In the presence but not absence of extracellular Ca^{2+}, ATP induced a transient $[Ca^{2+}]_i$ increase at the $P2X_2$ subtype (Figure 3b). Transfection with the $P2X_3$ subtype or a combination of subtypes resulted in a similar response. The C6BU-1 cell line enabled us to measure the Ca^{2+} influx via recombinant P2X receptors under physiological condition.

Concentration-dependence of the agonist-elicited rise in $[Ca^{2+}]_i$

The concentration-dependence of the ATP-elicited $[Ca^{2+}]_i$ rise for each subtype combination is shown in Figure 4. Figure 4a shows the actual $[Ca^{2+}]_i$ changes obtained from single cells at 1, 10 and 100 μM of ATP. In C6BU-1 cells transfected with $P2X_2$ alone, ATP could evoke a $[Ca^{2+}]_i$ increase in a concentration-dependent manner from 1 μM to 100 μM. To identify transfected cells, we used a surface marker of CD8. Marker expression on the surface was detected by the binding of small beads coated with antibody to the CD8 (see Methods). Most bead-positive cells (23 of 29) did not respond to the application of ATP (1 μM). At 10 μM of ATP, 26 of 29 cells were responsive. Finally, at 100 μM of ATP, all cells were responsive ($n=29$). Most C6BU-1 cells transfected with $P2X_3$ alone did not respond to ATP at 1 and 10 μM concentrations. ATP (1 μM) did not evoke a $[Ca^{2+}]_i$ increase in any of the bead labeled cells tested ($n=31$). One hundred μM of ATP evoked a $[Ca^{2+}]_i$ increase in some, but not in all bead-labeled cells (Table 1). Thus, we plotted only data obtained in $P2X_3$-transfected cells responsive to 100 μM ATP. In C6BU-1 cells cotransfected with $P2X_2$ and $P2X_3$, 18 of 22 cells were responsive to ATP at the 1 μM level. ATP at 10 μM or higher concentrations evoked a $[Ca^{2+}]_i$ rise in all cells tested ($n=22$). The amplitude of the responses of $P2X_{2+3}$ at 1 μM was larger than that of $P2X_2$ alone. These data indicated that the order of sensitivity to ATP was $P2X_{2+3} > P2X_2 > P2X_3$.

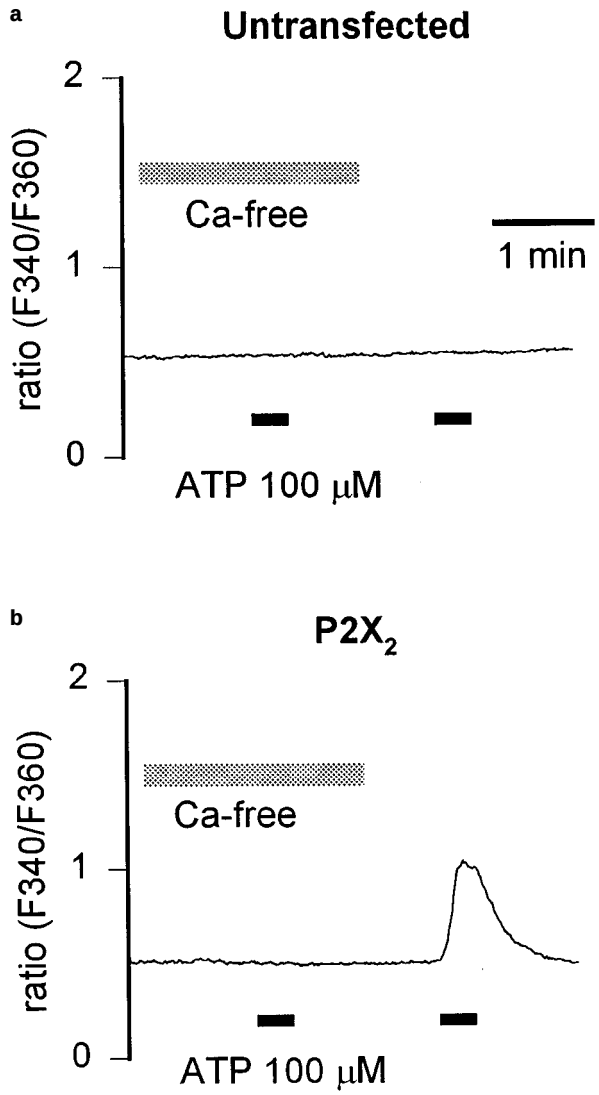

Figure 3 Effect of ATP on $[Ca^{2+}]_i$ in C6BU-1 cells untransfected (a) and transfected (b) with the $P2X_2$ receptor. Horizontal solid bars show the application of 100 μM ATP. The period when external Ca^{2+} was removed is indicated by hatched bars. Normal external solution included 1.2 mM Ca^{2+}. The time scale bar is shown on the right of the top panel.

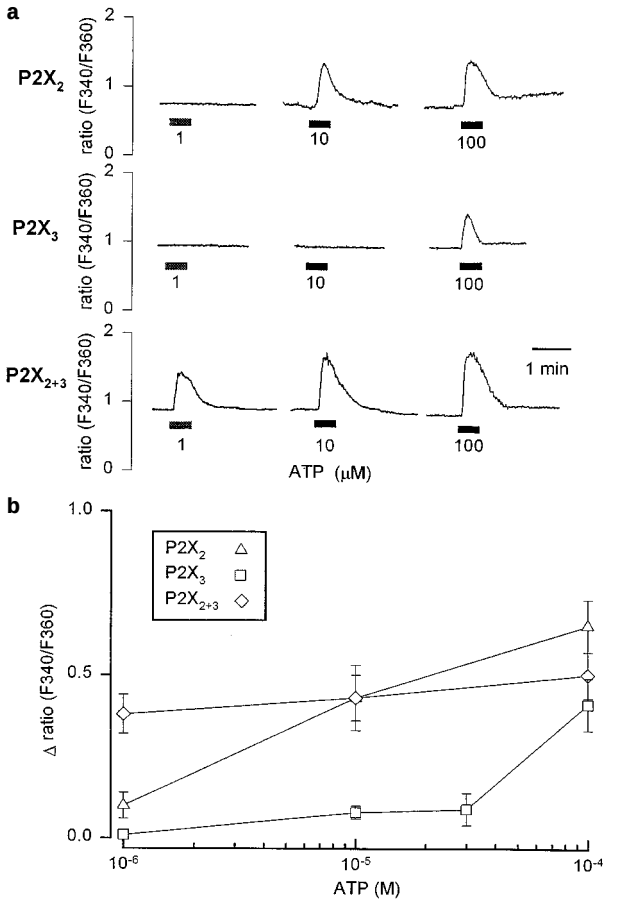

Figure 4 ATP-elicited $[Ca^{2+}]_i$ changes in C6BU-1 cells transfected with P2X subtypes. (a) Representative $[Ca^{2+}]_i$ changes in response to concentrations of ATP from 1 μM to 100 μM are shown for transfected cells. ATP (1, 10 and 100 μM) was applied every 10 min for periods indicated by the horizontal bars. Data were obtained from a single cell for each subtype. (b) Concentration-dependence of the ATP-evoked peak rise in $[Ca^{2+}]_i$ in C6BU-1 cells transfected with the P2X receptor. Results were obtained from cells responsive to ATP (100 μM). Values show the maximal $[Ca^{2+}]_i$ rise above basal. Data are mean $\pm$ s.e. of 19–31 cells. $[Ca^{2+}]_i$ response for the $P2X_{2+3}$ at 1 μM is significantly larger than those for $P2X_2$ or $P2X_3$ ($^{**}P<0.01$).

Effect of α,βmeATP on C6BU-1 cells transfected with P2X receptor subtypes

In $P2X_2$-transfected cells, ATP concentrations higher than 10 μM evoked a transient rise of $[Ca^{2+}]_i$, but α,βmeATP (up to 100 μM) failed to increase $[Ca^{2+}]_i$. This result is consistent with the electrophysiological data obtained in C6BU-1 cells expressing P2X receptors. In cells transfected with the $P2X_3$ subtype, 100 μM of α,βmeATP was necessary to induce a significant $[Ca^{2+}]_i$ rise (Figure 5). When α,βmeATP (100 μM) was applied twice with an interval of 10 min, the $P2X_3$ and $P2X_{2+3}$ subtypes reacted in a different manner. C6BU-1 cells transfected with the $P2X_3$ subtype responded to the first but not to the second application of α,βmeATP (100 μM) (Figure 6). The second response of the $[Ca^{2+}]_i$ rise was less than one-tenth of the initial one. In contrast, the $P2X_{2+3}$ subtype retained its responsiveness to repeated applications of α,βmeATP (100 μM). This concentration caused a submaximal current under patch-clamp conditions.

Discussion

As a P2X receptor expression system, HEK 293 cells and oocytes have been used mainly to characterize the current responses of P2X receptor subtypes (Evans *et al*., 1996). The HEK 293 cell line has been extensively utilized as an expression system since this cell line can express exogenous receptor subtypes efficiently and is suitable for electrophysiological studies. We initially tried to investigate the intracellular Ca^{2+} dynamics of recombinant P2X receptor subtypes using HEK 293 cells. Although ATP application did not elicit currents in HEK 293 cells under patch-clamp recording, more than 30% of untransfected HEK cells tested showed an ATP-evoked $[Ca^{2+}]_i$ rise in the absence of external Ca^{2+}, presumably through G protein coupled wild-type ATP receptors. The $[Ca^{2+}]_i$ rise triggered by these endogenous receptors could have interfered with the $[Ca^{2+}]_i$ rise induced by exogenous P2X receptor. In contrast, ATP (100 μM) caused no current response and no rise in $[Ca^{2+}]_i$ in the untransfected C6BU-1 cells. In this cell line, it has been reported that the endogenous P2Y receptor does not initiate a rise in $[Ca^{2+}]_i$ *via* activation of the phospholipase C pathway (Nicholas *et al*., 1996). These results indicated that the C6BU-1 cell line is suitable as an expression system to characterize Ca^{2+} influx through recombinant P2X receptor subtypes.

The kinetics of the ATP-activated current under voltage-clamp conditions greatly differed between each subtype, while the $[Ca^{2+}]_i$ rise evoked by ATP did not. This may be due to a number of factors. When $[Ca^{2+}]_i$ was measured by fura-2 microfluorometry, the cells were not voltage-clamped. Furthermore, the decay phase of the $[Ca^{2+}]_i$ response depended on the uptake of Ca^{2+} into intracellular stores and on the efflux of Ca^{2+} into the extracellular space neither of which was *via* P2X receptors. Thus, the kinetics of the $[Ca^{2+}]_i$ responses in our experiments did not correspond to the kinetics of the inward currents recorded under voltage-clamp conditions.

In the C6BU-1 cells transfected with the $P2X_2$ alone or with the $P2X_{2+3}$, the estimated efficacies of bead selection obtained

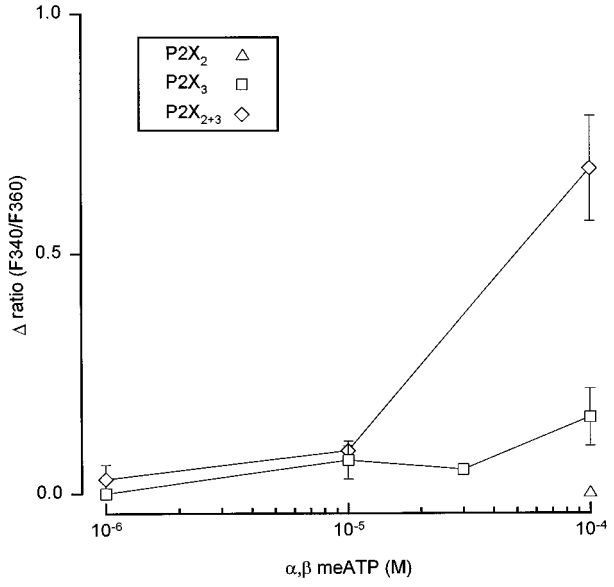

Figure 5 Concentration-dependence of α,βmeATP-evoked peak rise in $[Ca^{2+}]_i$ in C6BU-1 cells transfected with the P2X receptor. Values show the maximal $[Ca^{2+}]_i$ rise above basal. Data are mean ± s.e. of 20–30 cells tested.

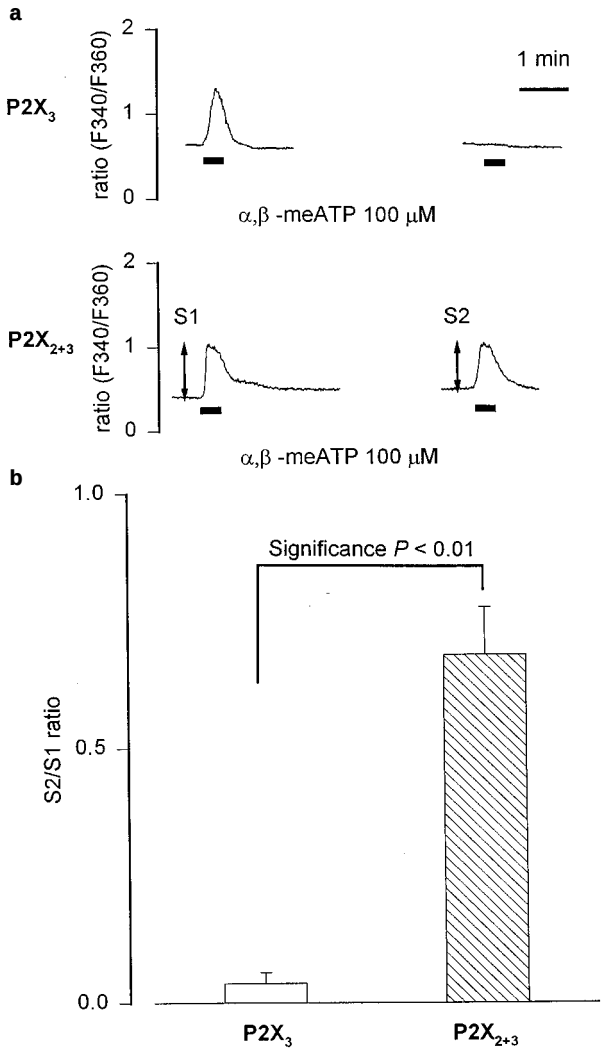

Figure 6 Effects of repeated applications of α,βmeATP. (a) Typical $[Ca^{2+}]_i$ responses to αβmeATP (100 μM) in C6BU-1 cells transfected with the $P2X_3$ or the $P2X_{2+3}$ subtypes at every 10 min. Horizontal solid bars show the application of 100 μM α,βmeATP. (b), Summary of the ratio of the second response to the first response (S2/S1), as obtained in (a). Data are mean s.e. of 7–10 cells tested.

from the fura-2 recording data were almost equal to those from the electrophysiological data. The concentration-dependence of ATP for both peak currents and peak $[Ca^{2+}]_i$ rise also showed the same tendency. In contrast, for the $P2X_3$, the fraction of cells showing an ATP-evoked $[Ca^{2+}]_i$ rise in the bead-labeled cells was lower than that of cells with ATP-evoked current responses. The low efficacy of bead labeling for $P2X_3$ was presumably due to the low efficiency of $P2X_3$ expression and the desensitization of the receptor. Indeed, a recent study revealed that the expression level of $P2X_3$ was lower than those of $P2X_2$ or $P2X_{2+3}$ after transfection (Radford *et al*., 1997) and the response *via* the $P2X_3$ subtype could not appear without pretreatment of apyrase in several preparations (Buell *et al*., 1996b). In our preparation, apyrase pretreatment improved the efficacy of bead-labeling for the $P2X_3$ expression in terms of both current and $[Ca^{2+}]_i$ rise. However, the fraction of cells responding with a $[Ca^{2+}]_i$ rise was still lower than that responding with currents. The fact that the currents but not the Ca^{2+} influx were detectable suggests that low concentrations of ATP could not induce significant Ca^{2+} entry via the $P2X_3$ subtype. This notion was supported by the following observation. Application of low concentrations of ATP (up to 3 μM) elicited reproducible currents via the $P2X_3$ subtype, while repeated applications of ATP at concentrations higher than 10 μM caused rapidly decreasing responses. With respect to the relation between $[Ca^{2+}]_i$ and the receptor activity, a simultaneous recording of current and $[Ca^{2+}]_i$ under voltage-clamp conditions demonstrated that the recovery of $[Ca^{2+}]_i$ to the control level was a prerequisite to obtain reproducible current responses in PC12 cells (Khiroug *et al*., 1997). Further, desensitization of $P2X_3$ was thought to be due to the activation of calcineurin by the $[Ca^{2+}]_i$ rise through this channel (Burnstock & Wood, 1996). In conclusion, although the $P2X_3$ subtype demonstrated high permeability for Ca^{2+} under voltage-clamp conditions (Evans *et al*., 1996), the $P2X_3$ receptor requires a relatively high concentration of ATP. The significant entry of Ca^{2+} may result in a loss of sensitivity to repeated agonist applications.

Cotransfection with $P2X_2$ and $P2X_3$ subtypes resulted in new properties, namely efficient Ca^{2+} influx at 1 μM ATP, sensitivity to α,βmeATP and relatively constant responses to repeated applications of ATP. These properties were different from those of $P2X_2$ alone or $P2X_3$ alone. These results strongly suggest that the $P2X_2$ and $P2X_3$ subunits assemble a heteromultimeric channel. This possibility was also supported by an electrophysiological study (Lewis *et al*., 1995) and a recent Western blot study showing direct evidence for heteromeric assembly of $P2X_2$ and $P2X_3$ receptors (Radford *et al*., 1997). When the characteristics of Ca^{2+} dynamics *via* the $P2X_3$ and the $P2X_{2+3}$ are compared, the newly acquired properties of the P2X receptor heteromultimer appear to be beneficial for synaptic transmission followed by Ca^{2+} signal transduction.

In the present voltage-clamp experiments, the sensitivity of the $P2X_{2+3}$ subtypes was lower than in previous studies (Lewis *et al*., 1995). However, when cotransfected with the $P2X_2$ and $P2X_3$ subtypes, the cells might expresss not only the heteromeric complex but also the homomeric $P2X_2$ or $P2X_3$ subtypes. If so, the ATP concentration-response curve for $P2X_{2+3}$ would be affected by the homomeric channels of $P2X_2$ and $P2X_3$. To exclude the effects of these homomeric channels, we investigated the concentration dependence of α,βmeATP for the $P2X_{2+3}$. Interestingly, α,βmeATP was also less potent than previously reported (Lewis *et al*., 1995). Considering that the stoichiometry of $P2X_{2+3}$ is unknown, in our preparation the heteromeric channel of the $P2X_{2+3}$ might exhibit a ratio of $P2X_2$ and $P2X_3$ different from that observed by others (Lewis *et al*., 1995).

There are already several reports that ATP, ATP-analogues and P2 receptor antagonists modulate nociception at the spinal level (Doi *et al*., 1987; Salter & Henry, 1985). The $P2X_3$ subtype expressed in sensory neurons is thought to be involved in nociception (Chen *et al*., 1995; Lewis *et al*., 1995) and thereby might play an important role in initial pain transmission (Burnstock, 1996). Although the $P2X_3$ subtype co-exists with other P2X receptor subtypes in sensory neurons, the assembly of the channel remains unclear. Our results indicate that a heteromultimeric channel containing a $P2X_3$ subunit constantly promotes Ca^{2+} influx from extracellular sources, suggesting that this Ca^{2+} signaling may play an important role for chronic pain.

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