Functional Expression of Three P2X$_2$ Receptor Splice Variants From Guinea Pig Cochlea

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Chen, Chu, Margaret S. Parker, Anthony P. Barnes, Prescott Deininger, and Richard P. Bobbin. Functional expression of three P2X$_2$ receptor splice variants from guinea pig cochlea. J. Neurophysiol. 83: 1502–1509, 2000. ATP has been suggested to act as a neurotransmitter or a neuromodulator in the cochlea. The responses to ATP in different cell types of the cochlea vary in terms of the rate of desensitization and magnitude, suggesting that there may be different subtypes of P2X receptors distributed in the cochlea. Recently three ionotropic P2X$_2$ receptor splice variants, P2X$_{2-1}$, P2X$_{2-2}$, and P2X$_{2-3}$, were isolated and sequenced from a guinea pig cochlear cDNA library. To test the hypothesis that these different splice variants could be expressed as functional homomeric receptors, the three P2X$_2$ receptor variants were individually and transiently expressed in human embryonic kidney cells (HEK293). The biophysical and pharmacological properties of these receptors were characterized using the whole cell patch-clamp technique. Extracellular application of ATP induced an inward current in HEK293 cells containing each of the three splice variants in a dose-dependent manner indicating the expression of homomeric receptors. Current-voltage (I-V) relationships for the ATP-gated current show that the three subtypes of the P2X$_2$ receptor had a similar reversal potential and an inward rectification index ($I_{SO} V/\Delta I_{SO}$ mV). However, the ATP-induced currents in cells expressing P2X$_{2-1}$ and P2X$_{2-2}$ variants were large and desensitized rapidly whereas the current in those cells expressing the P2X$_{2-3}$ variant was much smaller and desensitized slower. The order of potency to ATP agonists was 2-MeSATP > ATP > α,β-MeATP for all three expressed splice variants. The ATP receptor antagonists suramin and PPADS reduced the effects of ATP on all three variants. Results demonstrate that three P2X$_2$ splice variants from guinea pig cochlea, P2X$_{2-1}$, P2X$_{2-2}$, and P2X$_{2-3}$, can individually form nonselective cation receptor channels when these subunits are expressed in HEK293 cells. The distinct properties of these P2X$_2$ receptor splice variants may contribute to the differences in the response to ATP observed in native cochlear cells.

INTRODUCTION

Extracellular ATP has been suggested to serve as a neurotransmitter, neuromodulator, cytotoxin, and mitogen in various systems (see Bobbin 1996; Bobbin et al. 1998; Burnstock 1990; Housley 1997). Ionotropic (P2X) receptors for ATP are found on cells throughout the cochlea (Housley et al. 1999; Xiang et al. 1999). The response to ATP differs greatly among cell types in terms of the biophysics and pharmacology. For instance, the current response to ATP shows very little desensitization in outer hair cells (OHCs) (Chen et al. 1997; Housley et al. 1992; Kujawa et al. 1994; Nakagawa et al. 1990) whereas desensitization is relatively fast in Deiters’ cells, pillar cells, and inner hair cells (Ashmore and Ohmori 1990; Chen and Bobbin 1998; Chen et al. 1998; Skellett et al. 1997; Sugasawa et al. 1996a,b). In addition, the application of the ATP receptor antagonists also shows a difference in the blockade of ATP-gated current among the cell types (Chen et al. 1998; Skellett et al. 1997). Such variations in phenotype between cells may partly be caused by differences in the subunit composition of the receptors. Furthermore, alternate splicing of individual subunit mRNA may produce variants with different properties that contribute to the biophysical differences seen in vivo (e.g., Partin et al. 1996).

Seven subunits of the P2X receptors (i.e., P2X$_1$, P2X$_2$, P2X$_3$, P2X$_4$, P2X$_5$, P2X$_6$, and P2X$_7$) have been isolated (Barnard et al. 1997). To date P2X$_2$ is the only ATP receptor type that has been identified in the inner ear (Brandle et al. 1997; Housley et al. 1995; Parker et al. 1998; Troyanovskaya and Wackym 1998). In addition, several splice variants of P2X$_2$ have been identified in different tissues (Brandle et al. 1997; Housley et al. 1995; Koshimizu et al. 1998; Parker et al. 1999; Simon et al. 1997; Troyanovskaya and Wackym 1998). Heterologous expression of the wild-type P2X$_2$ (or P2X$_{2-1}$ or P2X$_{2-2}$) receptor isoform and the P2X$_{2-2}$ (or P2X$_{2-2}$) splice variant obtained from rats resulted in the formation of functional homomeric receptors with different characteristics (Brandle et al. 1997; Koshimizu et al. 1998; Simon et al. 1997). Parker et al. (1998) recently sequenced three splice variants of the P2X$_2$ receptor from a guinea pig organ of Corti cDNA library: P2X$_{2-1}$, P2X$_{2-2}$, and P2X$_{2-3}$ (or P2X$_{2-3}$). The sequences of the P2X$_{2-1}$ and P2X$_{2-2}$ variants are similar to those previously isolated from the rat. The P2X$_{2-3}$ has not been characterized functionally and is unusual in that a significantly long sequence of amino acids is included in the putative extracellular domain. Therefore the purpose of this study was to examine whether these three variants, especially the P2X$_{2-3}$, could be expressed as functional homomeric receptors. For this purpose the three splice variants were individually and transiently expressed in human embryonic kidney cells (HEK293).

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properties of the three P2X₂ receptor variants were characterized using the whole cell voltage-clamp technique.

METHODS

Construction of recombinant plasmids

The recombinant plasmids pEGFP-N1-P2X₂-1, pEGFP-N1-P2X₂-2, and pEGFP-N1-P2X₂-3, which carry the entire protein coding sequence of the guinea pig P2X2 splice variants, were constructed as follows. Previously described cDNA clones (Parker et al. 1998) isolated from a guinea pig organ of Corti library (Wilcox and Fex 1992) were digested with Bam HI and Hin dIII and subcloned into a eukaryotic expression vector (Enhanced Green Fluorescent Protein, pEGFP-N1, Clontech, Palo Alto, CA). The insertion of the cDNA eliminated the translation of the green fluorescent protein encoded downstream of the cloning site in pEGFP-N1 (data not shown). Transfection quality DNA was obtained by Qiagen Maxiprep column purification from One Shot competent cells (InvA/F, Invitrogen, Carlsbad, CA) bearing the respective expression plasmid. Verification of each variant inserted into the expression vector was confirmed by restriction mapping and by sequencing each variant at the 5' and 3' ends.

Functional expression in HEK293 cells

Cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) at 37°C in 5% CO₂. Approximately 10⁶ cells were seeded onto sterile coverslips within 35-mm culture dishes before transfection with the appropriate cDNA expression plasmid and the native pEGFP vector as a fluorescent reporter. Twenty-four hours after plating, cells were transfected using Lipofectin (Life Technologies, Gaithersburg, MD). For transfection, 1 μg of pEGFP-N1 and 1 μg of cDNA construct were mixed with 97 μL of OptiMEM (serum free; Life Technologies, Gaithersburg, MD) and incubated at room temperature for 30 min. Ten μL of Lipofectin and 90 μL of OptiMEM were also incubated at room temperature for 30 min. These reagents were combined and incubated for 10 min at room temperature before addition of 800 μL of OptiMEM to make the transfection medium. The culture medium was removed and replaced with MEM + 10% FCS. Twelve to twenty-four hours later, cells expressing green fluorescence were analyzed for electrophysiological properties.
Electrophysiological recordings

Single HEK293 cells demonstrating fluorescence were voltage-clamped using the whole cell variant of the patch-clamp technique as described before (Chen and Bobbin 1998). Membrane currents were filtered at 5 KHz (-3dB) using a four-pole low-pass Bessel filter digitized with a 12-bit A/D converter and stored for offline analysis using a computer. Voltage paradigms were generated from a 12-bit D/A converter using pClamp software. After the whole cell configuration had been established, series resistance and cell capacitance compensation were carried out before the recording, with 90% series resistance compensation normally applied. All experiments were conducted at room temperature (22–24°C).

Solutions and drugs

The modified Hank’s Balanced salt solution (HBS) utilized for transfected cells and perfusion bath contained (in mM) 140 NaCl, 5.4 KCl, 2.5 CaCl2, 0.5 MgCl2, 10 HEPES, and 10 glucose. The internal solution contained (in mM) 140 CsCl, 1.0 MgCl2, 5 HEPES, 11 EGTA, and 0.5 CaCl2. Internal and external solutions were adjusted to a pH of 7.4 and had osmolalities of 300 and 287 mOsm, respectively. The drugs tested had osmolalities of 300 and 287 mOsm, respectively. The drugs tested were freshly prepared from powder or from stocks at desired concentrations in the HBS external solution. All the chemicals and drugs including ATP were purchased from Sigma (St. Louis, MO) except for 2-methyl-ATP (2-MeSATP), ATP were purchased from Sigma (St. Louis, MO) except for 2-methyl-

RESULTS

Current-voltage relationship of the ATP-gated receptor channels

When HEK293 cells were individually and transiently transfected with the P2X2 splice variants, a homomeric ATP-gated ion channel was formed as evidenced by the inward currents that were evoked at negative holding potentials in response to the application of ATP to the HEK293 cells expressing the splice variants (Fig. 1, A–C). No ATP-induced current was elicited in nontransfected cells (data not shown). To determine the current-voltage (I-V) relationships of the ATP-gated current in cells expressing individual subunits, ATP (30 μM for the P2X2-1, 10 μM for the P2X2-2, and 1 mM for the P2X2-3) induced current responses were recorded at various holding potentials from -100 to +20 mV (Fig. 1D). The reversal potential of the ATP-gated currents was -1.2 ± 0.4 (SE) mV (n = 4) for the P2X2-1, -0.5 ± 0.3 mV (n = 6) for the P2X2-2, and -1.5 ± 0.6 mV (n = 5) for the P2X2-3, suggesting that the P2X receptors in HEK293 cells form nonselective cation channels. The I-V relationship (Fig. 1D) for the current exhibited inward rectification. This rectification was quantified by calculating a ratio of amplitudes of the ATP-gated current at 50 mV as described by Evans et al. (1996) and Chen and Bobbin (1998). The rectification indices for the three subunits were similar (P2X2-1: 0.13 ± 0.01, n = 5; P2X2-2: 0.11 ± 0.01, n = 4; P2X2-3: 0.14 ± 0.02, n = 5) and suggested a strong inward rectification of three P2X2 splice variants. This index was smaller than that of cloned P2X2 and P2X2 (Evans et al. 1996) but similar to the ATP-induced current recorded in Deiters’ cells (Chen and Bobbin 1998).

ATP-induced dose-response relationship

Figure 2 shows that ATP-gated current responses exhibited...
a dose-dependency in all three subunits, although there were differences in the magnitude of the current response. The currents were much larger in the cells expressing the P2X2–1 and P2X2–2 variants than in cells expressing the P2X2–3 variant. In addition, the concentration of ATP required to evoke a response was much higher in the cells expressing the P2X2–3 variant compared with the other two variants (Fig. 2). The EC50 for ATP in cells expressing the P2X2–1 and P2X2–2 variants was 10 and 8 μM, respectively, whereas it was 55 μM for the P2X2–3 variant. In most cells expressing the P2X2–2 variant, at a holding potential of −60 mV, 100 μM ATP induced a current amplitude that was larger than the maximum current amplitude that can be measured with the Axopatch 200A amplifier (i.e., 20 nA). Therefore the dose-response curve for ATP only extend up to 30 μM for the P2X2–2 variants (Fig. 2).

Rate of desensitization of the ATP-induced response

One of the distinguishing characteristics of different P2X receptors is the rate of desensitization. The currents evoked by ATP in the cells expressing the P2X2–1 and P2X2–2 variants desensitized rapidly in the presence of the purinoceptor agonists whereas the P2X2–3 variant desensitized very slowly (Fig. 3A). The desensitization was more pronounced during prolonged application of a high concentration of ATP (30 μM for the P2X2–1 and P2X2–2 variants; 1 mM for the P2X2–3 variant; Fig. 3A). Because the P2X2–3 variant exhibited virtually no desensitization, the current waveforms could not be fit with exponential functions and no time constant was obtained. Because desensitization rate is concentration and activity dependent, the slow desensitization of the P2X2–3 variant may be caused by the lack of agonist affinity. The current decay for the P2X2–1 and P2X2–2 variants was fitted well by a double exponential function. The time constant (τ) of the current decay was τ1 = 3.92 ± 0.6 s and τ2 = 15.77 ± 2.99 s (n = 10) for the P2X2–1 subunit and τ1 = 0.99 ± 0.2 s and τ2 = 7.56 ± 0.57 s (n = 10) for the P2X2–2 subunit (Fig. 3B). The differences in two time constants between the P2X2–1 and P2X2–2 variants were significant (P < 0.01). In addition, the difference in the percentage of current remaining at the end of ATP application to cells expressing the P2X2–1 subunit (4.5 ± 0.9%, n = 10) and the P2X2–2 subunit (1.7 ± 0.4%, n = 10) was significant (P < 0.05). Approximately 95% (P2X2–1) and 98% (P2X2–2)
of the current was desensitized during the 1-min period of agonist application.

**Effects of purinoceptor agonists**

Another distinguishing characteristic of different P2X receptors is the potency of various agonists such as 2-MeSATP and α,βMeATP (Burnstock 1990; Chen and Bobbin 1998; Fredholm et al. 1997; Kennedy and Leff 1995). Therefore these agonists were used to determine pharmacologic differences among the three P2X2 receptor splice variants. Figure 4 shows that the three variants exhibited a similar response to 2-MeSATP and α,β-MeATP except for differences in current amplitudes. Compared with ATP, 2-MeSATP exhibited strong agonist activity, whereas α,β-MeATP exhibited weak agonist activity (Fig. 4, A–C). The order of agonist potency is very similar to that observed in native Deiters’ cells (Chen and Bobbin 1998).

**Effect of purinergic antagonists**

During coapplication of ATP with the ATP receptor antagonist suramin (100 μM), the currents evoked by ATP were reversibly reduced in HEK293 cells expressing P2X2–1, P2X2–2, and P2X2–3 splice variants (Fig. 5, A–D). Suramin reduced the ATP-induced current to a greater degree in the cells expressing the P2X2–3 splice variants (Fig. 5, C and D) and the reduction in cells expressing the P2X2–1 was not significant (Fig. 5D). During coapplication of ATP with the ATP receptor antagonist PPADS (100 μM), the currents evoked by ATP were significantly reduced in HEK293 cells expressing P2X2–3 splice variants but not in cells expressing the P2X2–1 or P2X2–2 splice variants (Fig. 6, A–D). Immediately after washing out the PPADS the current evoked by ATP was significantly reduced in cells expressing P2X2–1, P2X2–2, and P2X2–3 splice variants (Fig. 6, A–D). A similar delayed block of ATP-induced currents by PPADS was obtained previously in cells from the organ of Corti (Chen et al. 1998).

**DISCUSSION**

Results demonstrate that when P2X2–1, P2X2–2, and P2X2–3 splice variants were individually and transiently transfected in HEK293 cells, each formed homomeric ATP-gated ion channels. The homomeric P2X2–3 receptor differed greatly from P2X2–1 and P2X2–2 receptors in that the former was less sensitive to various agonists and required a higher concentration of agonist to elicit a measurable response. Even at high agonist concentrations the P2X2–3 receptor induced less overall current than the P2X2–1 and P2X2–2 receptors. At the concentrations of agonists tested, the P2X2–3 receptor exhibit little
desensitization. However, because desensitization is a function of agonist concentration and induced response, the lack of desensitization may be the result of the low potency of agonists at this receptor.

The low potency of agonists suggests the P2X_{2–3} receptor splice variant, if expressed in vivo as a homomeric receptor, would probably have little sensitivity for endogenous ATP. However, the homomeric P2X_{2–3} receptor expressed in HEK293 may not reflect the same sensitivity as the endogenously expressed receptor. For instance, it may be that in vivo the P2X_{2–3} subunit is part of a heteromeric receptor with one or both of the other variants, in which case the P2X_{2–3} receptor would act as a modulator or modifier. This can be addressed in the future by studying stable transfected HEK293 cells containing all of the various combinations of the subunits. In addition, one cannot exclude the possibility that other variants or proteins not described to date are present which also have modulatory activity either alone or in combinations with the identified splice variants. Overall the obstacles for elucidating the structures that determine the response to ATP in vivo may be elusive as it has been for other agonists and their receptors. For example, striking differences have been described between natively and recombinantly expressed NMDA receptors (for review see Sucher et al. 1996).

Presumably, the P2X_{2–3} splice variant found in the guinea pig cannot be found in the rat. The molecular mechanism which produces this variant in the guinea pig is the inclusion of an intron which codes for an additional nine amino acids (i.e., intron VIII; Parker et al. 1998). In the rat this intron contains a stop codon. Therefore if this sequence were included in the rat mRNA, presumably it would result in a truncated protein lacking the second transmembrane spanning region of the receptor and would therefore be nonfunctional. This raises the question of why the guinea pig makes this splice variant and...
the rat does not and what effect this variant has on the function of the cochlea and other tissue where it may be expressed (Parker et al. 1999). To date there is only preliminary data on this question. Chen et al. (1997) failed to obtain responses to application of ATP in a majority of rat OHCs, yet obtained large responses in guinea pig OHCs. Because the guinea pig OHC response to ATP is a nondesensitizing response (Chen et al. 1997; Housley et al. 1992; Nakagawa et al. 1990), the P2X_2–3 splice variant failed to demonstrate desensitization and the P2X_2–3 splice variant is absent from the rat; this variant is a candidate for being the receptor present in guinea pig OHCs and the one being absent from rat OHCs.

It appears that the recombinant P2X_2–1 and P2X_2–2 receptors fairly accurately reflect the activity of the receptors expressed in Deiters’ cells and possibly other supporting cells such as pillar cells in both species (Chen et al. 1998). The nucleotide sequences of these guinea pig receptors are very similar to the P2X_2–1 and P2X_2–2 receptor sequences found in the rat (Parker et al. 1998) and so the responses should be similar in both species. The homomeric P2X_2–1 and P2X_2–2 receptors both desensitize rapidly and exhibit similar dose response curves to various agonists including ATP. Therefore they respond similarly to the receptors on Deiters’ cells in both the rat and the guinea pig (Chen and Bobbin 1998; Chen et al. 1997). Brandle et al. (1997) and Simon et al. (1997) found the rat P2X_2–2 receptor had a faster desensitization time constant than the P2X_2–1 receptor when the subunits were expressed in Xenopus oocytes. A similar relationship was observed between the guinea pig P2X_2–1 and P2X_2–2 receptors in the present study; however, the guinea pig time constants were approximately twice the rates of those found in the rat. This difference between rat and guinea pig may be because Brandle et al.

**FIG. 6.** Effects of the purinoceptor receptor antagonist, pyridoxal-phosphate-6-azophenyl-2’,4’-disulfonic acid (PPADS; 100 μM) on the ATP induced current in HEK293 cells expressing homomeric (A) P2X_2–1 (10 μM ATP), (B) P2X_2–2 (10 μM ATP), and (C) P2X_2–3 (300 μM ATP) guinea pig ATP receptor splice variants. Shown are the induced currents in response to the application of ATP alone (ATP), of an ATP with PPADS combination (PPADS) and of ATP alone after washing the bath (Wash). D: summary of the effect of PPADS on the ATP-induced current response demonstrating the greater reduction in the evoked current after the application and washout of the PPADS (Wash) than obtained with coapplication of ATP with PPADS (PPADS). First ATP alone application was used to derive the current reduction (%). Each bar represents the mean ± SE for the indicated number of cells. Asterisk indicates a significant difference from a pre-PPADS ATP-induced control current (***P < 0.01).
(1997) and Simon et al. (1997) made their measurement on receptors expressed in Xenopus oocytes which might have different posttranslational modification steps than the HEK293 cells used in this study. However, similar slower rate constants for rat P2X<sub>2</sub>-1 and P2X<sub>2</sub>-2 using Ca<sup>2+</sup> flux in HEK293 cells and neurons was reported by Koshimiizu et al. (1998).

In summary, P2X<sub>2</sub>-1, P2X<sub>2</sub>-2, and P2X<sub>2</sub>-3 splice variants formed homomeric ATP-gated ion channels when they were individually and transiently transfected into HEK293 cells. These ion channels exhibited differences in the rate of desensitization, magnitude, and pharmacology of the ATP-induced current responses among three splice variants. The distinct properties of these P2X<sub>2</sub> receptor splice variants may contribute to the differences in the response to ATP observed in native cochlear cells.

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