



P2X receptors in cochlear Deiters' cells

Chu Chen¹ & Richard P. Bobbin

Kresge Hearing Research Laboratory of the South, Department of Otorhinolaryngology and Biocommunication, Louisiana State University Medical Center, 2020 Gravier Street, Suite A, New Orleans, LA 70112-2234, U.S.A.

- 1 The ionotropic purinoceptors in isolated Deiters' cells of guinea-pig cochlea were characterized by use of the whole-cell variant of the patch-clamp technique.
- 2 Extracellular application of adenosine 5'-triphosphate (ATP) induced a dose-dependent inward current when the cells were voltage-clamped at -80 mV. The ATP-induced current showed desensitization and had a reversal potential around -4 mV.
- 3 Increasing intracellular free Ca^{2+} by decreasing the concentration of EGTA in the pipette solution reduced the amplitude of the ATP-gated current.
- 4 The order of agonist potency was: 2-methylthioATP (2-meSATP) > ATP > benzoylbenzoyl-ATP (BzATP) > α,β -methyleneATP (α,β -meATP) > adenosine 5'-diphosphate (ADP) > uridine 5'-triphosphate (UTP) > adenosine 5'-monophosphate (AMP) = adenosine (Ad).
- 5 Pretreatment with forskolin ($10 \mu\text{M}$), 8-bromoadenosine-3',5'-cyclophosphate (8-Br-cyclic AMP, 1 mM), 3-isobutyl-1-methylxanthine (IBMX, 1 mM) or phorbol-12-myristate-13-acetate (PMA, $1 \mu\text{M}$) reversibly reduced the ATP-induced peak current.
- 6 The results are consistent with molecular biological data which indicate that P2X₂ purinoceptors are present in Deiters' cells. In addition, the reduction of the ATP-gated current by activators of protein kinase A and protein kinase C indicates that these P2X₂ purinoceptors can be functionally modulated by receptor phosphorylation.

Keywords: Deiters' cells; cochlea; ATP; purinoceptor; phosphorylation; protein kinase A (PKA); protein kinase C (PKC); voltage-clamp

Introduction

The effects of adenosine triphosphate (ATP) as a neurotransmitter or neuromodulator are now well characterized in various systems (see reviews: Bean, 1992; Dubyak & El-Moatassim, 1993; North & Barnard, 1997). At least 5 subtypes of the P2-purinoceptors have been identified based on physiological and pharmacological characteristics (Kennedy & Leff, 1995; Fredholm *et al.*, 1997). Both ligand-gated (P2X; ionotropic) and G protein coupled (P2Y; metabotropic) purinoceptors have been cloned (North & Barnard, 1997). Metabotropic purinoceptors activate intracellular second messenger-coupled signalling pathways which trigger release of intracellular stored Ca^{2+} (Dubyak & El-Moatassim, 1993). Ionotropic purinoceptors form non-selective cation channels which may mediate fast synaptic transmission (Burnstock, 1990; Bean, 1992; Dubyak & El-Moatassim, 1993; North & Barnard, 1997).

It appears that the majority of cells in the cochlea contain purinoceptors (Ashmore & Ohmori, 1990; Nakagawa *et al.*, 1990; Dulon *et al.*, 1993; Housley *et al.*, 1992; Chen *et al.*, 1995b,c; Dulon, 1995; Sugasawa *et al.*, 1996; 1997; Raybould & Housley, 1997; Skellet *et al.*, 1997; Chen *et al.*, 1997; 1998; Parker *et al.*, 1997; 1998). Increasing evidence shows that activation of P2X purinoceptors in the organ of Corti may play powerful roles in cochlear mechanics (Eybalin, 1993; Kujawa *et al.*, 1994a,b; Bobbin, 1996; Bobbin *et al.*, 1997; Skellet *et al.*, 1997; Chen *et al.*, 1998). Supporting cells in the organ of Corti are coupled to each other by gap junctions

(Iurato *et al.*, 1976) and function to maintain the homeostasis of the organ and appear to provide an undefined electrical and micromechanical influence on the auditory receptors, the inner hair cells (IHCs) and outer hair cells (OHCs; e.g., Frank & Kossel, 1996; Bobbin, 1996). Deiters' cells, supporting cells which form a structure that holds the OHCs at their base and apex, may play an important role in modifying cochlear mechanics by influencing the force produced by sound induced motion of the OHCs (Bobbin, 1996; Bobbin *et al.*, 1997).

Extracellular application of ATP to Deiters' cells has been shown to induce an inward cation current and to increase intracellular Ca^{2+} by apparent activation of P2X receptors (Dulon *et al.*, 1993; Skellet *et al.*, 1997; Chen *et al.*, 1997; 1998). Ligand-gated ion channels such as P2X receptors mediate rapid excitatory and inhibitory synaptic transmission in the nervous system (Bean, 1992; Dubyak & El-Moatassim, 1993; North & Barnard, 1997). No other ionotropic receptors (e.g., acetylcholine, glutamate) have been functionally observed in Deiters' cells (unpublished data). Thus, ATP-gated receptor channels may be of unique importance to Deiters' cells for induction of a rapid membrane depolarization and Ca^{2+} influx, possibly to trigger a cascade of intracellular reactions. These events may alter the stiffness of the Deiters' cell, which in turn may influence the motility of the OHCs and thereby affect cochlear mechanics (Skellett *et al.*, 1997; Chen *et al.*, 1997; 1998). However, the pharmacological properties and the functional subtype of the P2X receptors in Deiters' cells have not been described. Therefore, the purpose of the present study was to characterize the biophysical and pharmacological properties of the P2X receptors in Deiters' cells isolated from the organ of Corti of guinea-pig cochlea. Preliminary results have been presented to the Annual Meeting of the Society for Neuroscience (Chen & Bobbin, 1997).

¹ Author for correspondence at: Kresge Hearing Research Laboratory, LSU Medical Center, 2020 Gravier Street, Suite A, New Orleans, Louisiana 70112, U.S.A.

Methods

Isolation of Deiters' cells

Deiters' cells from pigmented guinea-pigs were isolated as described previously (Skellett *et al.*, 1997; Chen *et al.*, 1997; 1998). Briefly, animals were anaesthetized with pentobarbitone (35 mg kg^{-1} , i.p.), decapitated, and the bulla separated and placed in a modified Hank's balanced solution (HBS). The bone surrounding the cochlea was removed, and the organ of Corti was placed in $200 \mu\text{l}$ of the HBS containing collagenase (1 mg ml^{-1} , Type IV, Sigma) for 5 min. The cells were then isolated and transferred into the dishes containing a $150 \mu\text{l}$ drop of the HBS by use of a microsyringe and stored at room temperature until recordings.

Whole-cell voltage clamp

Single dispersed guinea-pig Deiters' cells were voltage-clamped by use of the whole-cell variant of the patch-clamp technique (Hamill *et al.*, 1981) with an Axopatch 200A patch-clamp amplifier (Axon Instruments). Patch electrodes were fabricated from borosilicated capillary tubing (Longreach Scientific Resources) with a micropipette puller (Sutter Instrument Co.), and fire polished on a microforge (Narashige Scientific Instrument Lab.) before use. Membrane currents were filtered

at 5 kHz (-3 dB) with a four-pole low-pass Bessel filter digitized with a 12-bit A/D converter (DMA Interface, Axon Instruments), and stored for off-line analysis by a micro-computer. Voltage paradigms were generated from a 12-bit D/A converter (DMA Interface, Axon Instruments) using pClamp software (Axon Instruments). After the whole-cell configuration had been established, series resistance and cell capacitance compensation were carried out before the recording, with 80% series resistance compensation normally applied. Whole-cell capacitance of the Deiters' cells was $33.6 \pm 1.0 \text{ pF}$ ($n=42$) and series resistance was $4.3 \pm 0.2 \text{ M}\Omega$ ($n=42$). The input resistance was $765 \pm 88 \text{ M}\Omega$ ($n=16$) and the zero current potential was $-26 \pm 1.1 \text{ mV}$ ($n=13$). During the recording the change of series resistance was negligible.

Solutions and drugs

The HBS utilized for isolating cells and perfusing the bath contained (in mM): NaCl 145, KCl 5.4, CaCl_2 2.5, MgCl_2 0.5, HEPES 10 and glucose 10. The HBS solution was adjusted to a pH of 7.40 with NaOH and to $300 \text{ mOsm kg}^{-1} \text{ H}_2\text{O}$ with sucrose. The K^+ internal solution contained (in mM): KCl 140, MgCl_2 0.5, HEPES 5, EGTA 11, CaCl_2 0.1, Na_2ATP 2 and Na_2GTP 0.1. As noted in some experiments the concentration of the EGTA in the internal solution was decreased from 11 mM to 0.1 mM. Free

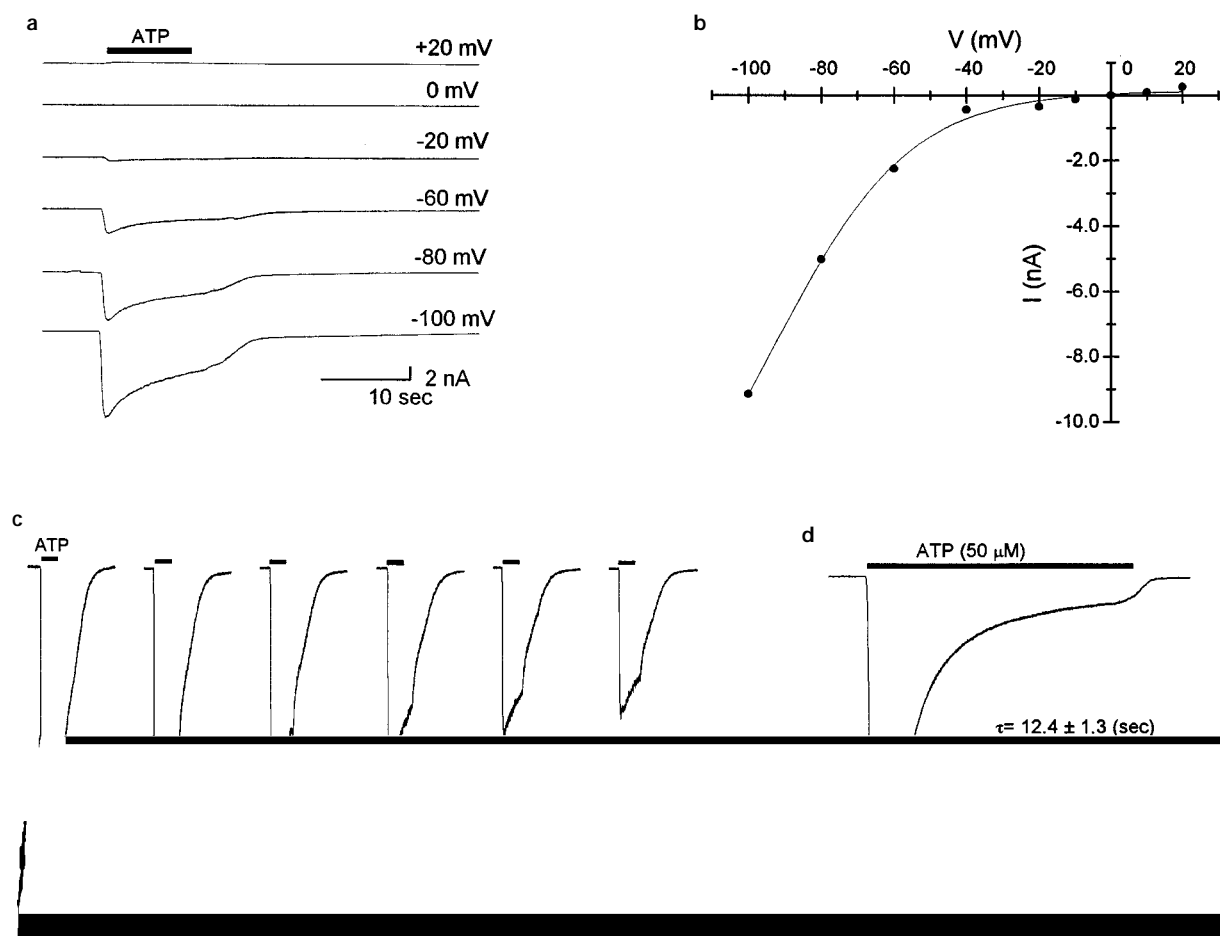


Figure 1 (a) Current traces of ATP ($10 \mu\text{M}$)-induced response at various indicated voltages recorded from a Deiters' cell. (b) Current-voltage (I - V) relationship for the ATP-induced current response measured from the peak current. The ATP-gated current showed an inward rectification (rectification index: 0.11 ± 0.04 , $n=7$) and reversal potential was around -4 mV . (c) Repeated applications of $10 \mu\text{M}$ ATP for six times with a one minute interval between the applications. (d) Pronounced desensitization of P2X current with a prolonged (1 min) application of $50 \mu\text{M}$ ATP. The time constant (τ) of desensitization was $12.4 \pm 1.3 \text{ s}$ ($n=14$).

Ca^{2+} concentration in the pipette solution was 1.2 nM for 11 mM EGTA and 1.1 μM for 0.1 mM EGTA (calculated by Chelator). The internal solution was adjusted to a pH of 7.35 with HCl and had an osmolality of 284 mOsm kg^{-1} H_2O adjusted with sucrose. The drugs tested were prepared freshly from powder or from stocks at desired concentrations in the HBS external solution. All the chemicals and drugs including ATP, adenosine 5'-diphosphate (ADP), uridine 5'-triphosphate (UTP), adenosine 5'-monophosphate (AMP), adenosine (Ad) and benzoylbenzoyl-ATP (BzATP) were purchased from Sigma Chemical Company (St. Louis, U.S.A.), except 2-methylthioATP (2-meSATP), α,β -methyleneATP (α,β -meATP), forskolin, 8-bromoadenosine-3',5'-cyclophosphate (8-Br-cyclic AMP), 3-isobutyl-1-methylxanthine (IBMX), phorbol-12-myristate-13-acetate (PMA) which were obtained from Research Biochemicals International (Natick, U.S.A.). All the drug solutions were delivered from a U-tubing system that applied drug solution around the cell for a select period of time while the bath was continuously exchanged with a perfusion system separate from the U-tubing system as described previously (Chen *et al.*, 1997). The solution surrounding a cell can be exchanged within 20 to 30 ms (Murase *et al.*, 1989). All experiments were conducted at room temperature (22–24°C).

Results

Current-voltage (*I-V*) relationship of the ATP-gated receptor channels

The data presented are based on observations made from 122 Deiters' cells, acutely isolated from 26 guinea-pigs. As described previously (Skellett *et al.*, 1997; Chen *et al.*, 1997; 1998) ATP evoked inward currents at a holding potential of -80 mV in Deiters' cells. To determine the *I-V* relationship of the ATP-gated current in Deiters' cells, ATP (10 μM)-induced current responses were recorded at various holding potentials from -100 to $+20$ mV. As indicated in Figure 1a, ATP induced an inward current when the membrane potential was held at negative potentials and an outward current at positive potentials. The reversal potential of the ATP-gated current was around -4 ± 1 mV ($n=4$), suggesting that the P2X receptors in Deiters' cells form non-selective cation channels. The *I-V* relationship (Figure 1b) for the current exhibited an inward rectification. This rectification was quantified by calculating a ratio of amplitudes of the ATP-gated current at 50 mV and -50 mV ($I_{50\text{ mV}}/I_{-50\text{ mV}}$) as described by Evans *et al.* (1996) and the rectification index was calculated as 0.11 ± 0.04 ($n=7$). This index was smaller than that of cloned P2X₁ and P2X₂

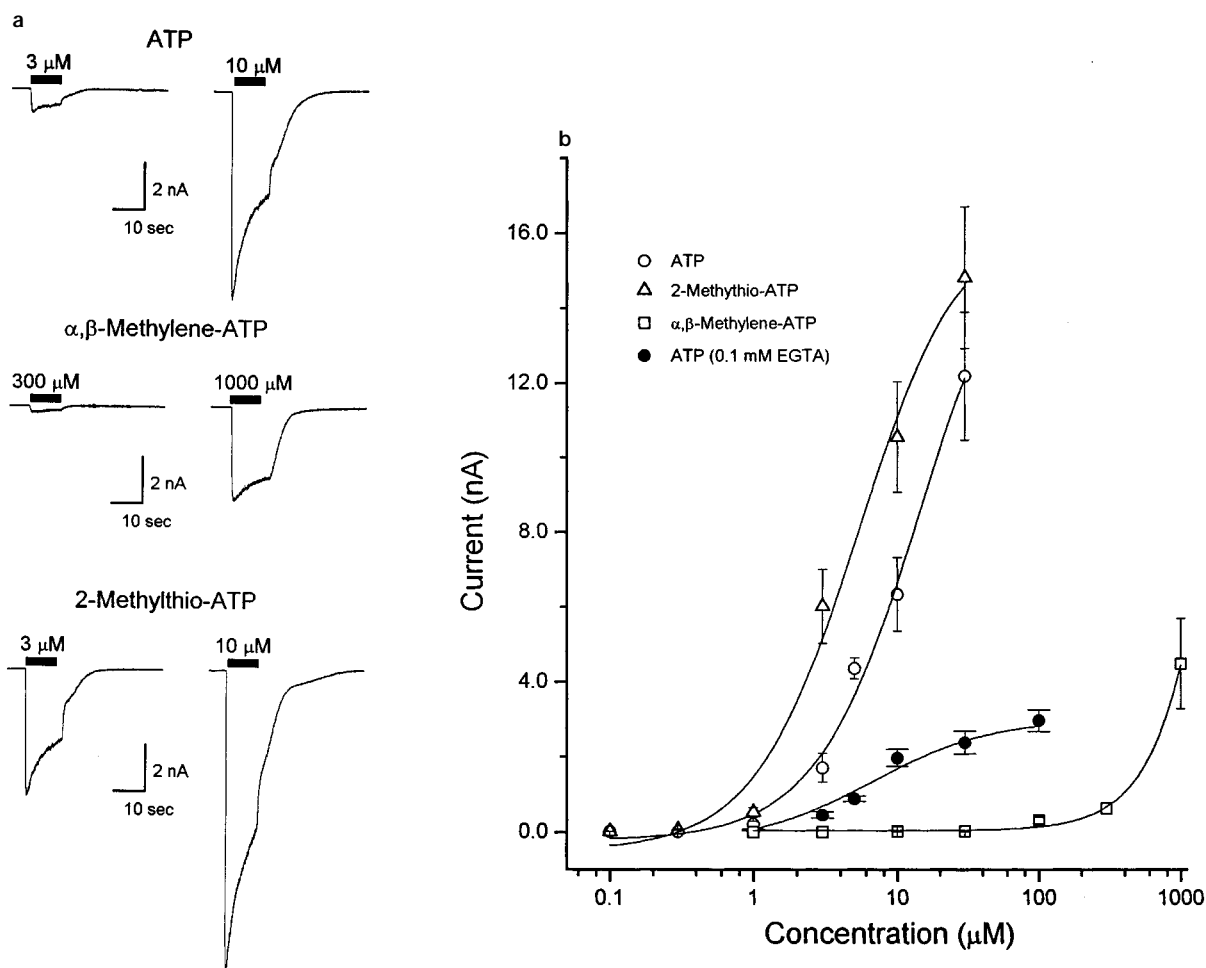


Figure 2 Dose-response curves for the purinoceptor agonist induced current responses. (a) Current traces of ATP (3 and 10 μM), α,β -methylene-ATP (300 and 1000 μM) and 2-methylthio-ATP (3 and 10 μM)-induced responses recorded from different Deiters' cells. (b) Dose-response curves for ATP, α,β -methylene-ATP, 2-methylthio-ATP-induced current responses. In one set of experiments the concentration of the EGTA in the internal solution was decreased from 11 mM to 0.1 mM (solid circles; 1.2 nM free Ca^{2+} for 11 mM EGTA and 1.1 μM free Ca^{2+} for 0.1 mM EGTA). Each symbol represents mean from 5 to 21 cells; vertical lines show s.e.mean. Solid lines are nonlinear least-square fits to a logistic equation.

receptors observed by Evans *et al.* (1996), suggesting a strong rectification of the P2X current in Deiters' cells. In addition, the ATP-induced current response exhibited desensitization in the presence of ATP (Figure 1c). The desensitization was more pronounced during prolonged application of a high concentration (50 μM) of ATP (Figure 1d). The fraction of current remaining at the end of the ATP application was 0.11 ± 0.01 ($n=14$), i.e., about 90% of the current being desensitized during the 1 min period of agonist application. The time constant (τ) of the current decay was 12.4 ± 1.3 s ($n=14$). This time constant was almost the same as the time constant for the P2X₂₋₂ splice variant observed by Brändle *et al.* (1997). Repetitive applications of ATP (10 μM) reduced the peak current, indicating a slow recovery from the desensitization (Figure 1c).

Dose-response curves for purinoceptor agonists

Since the functional classification for the subtypes of P2 receptors is usually based on the potency of the agonists such as 2-meSATP and α,β -meATP (Burnstock, 1990; Kennedy & Leff, 1995; Fredholm *et al.*, 1997), these agonists were used to define the subtype of P2X receptors in Deiters' cells pharmacologically. Dose-response curves for the purinoceptor agonist-induced current responses were constructed. Figure 2 shows that 2-meSATP was the strongest agonist for the P2X receptors tested, whereas α,β -meATP exhibited weak agonist activity. In most Deiters' cells, 100 μM ATP- and 2-meSATP-induced current amplitudes were larger than the maximum current amplitude that can be measured with the Axopatch 200A amplifier (i.e., 20 nA). Therefore, the dose-response curves for ATP and 2-meSATP only extend up to 30 μM (Figure 2b).

One of the important properties of ATP-gated channels is a permeability to Ca^{2+} . This has been confirmed in many different types of cells including Deiters' cells (Dulon *et al.*, 1993). In addition, inhibition of the response to ATP by high external Ca^{2+} concentrations has been described (Evans *et al.*, 1996). However, intracellular Ca^{2+} may also regulate receptor function (Nakazawa & Hess, 1993). To explore whether changes in intracellular free Ca^{2+} influence the ATP-gated current, the concentration of EGTA in the pipette solution was altered. As shown in Figure 2b, the ATP-gated current was greatly reduced when the concentration of EGTA in the pipette solution was reduced from 11 mM (free Ca^{2+} concentration: 1.2 nM) to 0.1 mM (free Ca^{2+} concentration: 1.1 μM).

Other related purinoceptor agonist-induced current responses

To identify further the subtype of P2X receptors in Deiters' cells, several related purinoceptor agonists, including adenosine (Ad), AMP, ADP, UTP and BzATP, were used. As shown in Figure 3, Ad and AMP did not elicit a detectable current when the cells were held at -80 mV. At a concentration of 1 mM UTP induced a very small inward current. A relatively large inward current was elicited by ADP (1 mM) and an even larger current was induced by BzATP at a concentration of 100 μM .

Effects of activators of protein kinases

Increasing evidence suggests that ionotropic receptor channels can be modulated by receptor phosphorylation (Huganir & Greengard, 1990; Swope *et al.*, 1992). For instance, the

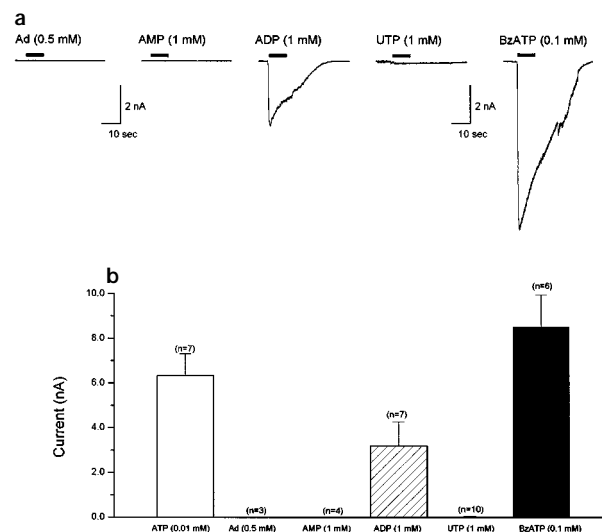


Figure 3 Effects of purinoceptor receptor-related agonists on Deiters' cells. (a) Current traces of adenosine (Ad), adenosine 5'-monophosphate (AMP), adenosine 5'-diphosphate (ADP), uridine 5'-triphosphate (UTP) and 2'- & 3'-O-(4-benzoylbenzoyl-adenosine 5'-triphosphate (BzATP)-induced responses in Deiters' cells. (b) Mean values of the purinoceptor agonist-induced current responses. Each column represents the mean \pm s.e. for the indicated number (n) of cells.

function of glutamate receptor channels can be modulated by adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase (Wang *et al.*, 1991; 1993; Raymond *et al.*, 1993). Because there are several putative sites for phosphorylation by protein kinase A (PKA) and protein kinase C (PKC) in the P2X receptor sequence (Séguéla *et al.*, 1996; Simon *et al.*, 1997; Brändle *et al.*, 1997; Parker *et al.*, 1997; 1998), it is likely that the P2X receptor can be modulated by protein phosphorylation. To determine whether the P2X receptors in Deiters' cells can be modulated by phosphorylation several activators of PKA and PKC were used. As shown in Figure 4a, pretreatment with forskolin (10 μM), which activates adenylate cyclase and so increases the level of intracellular cyclic AMP, an activator of PKA, reversibly reduced the ATP (5 μM)-induced current. In particular, the fast decaying phase disappeared. Application of 8-Br-cyclic AMP, a membrane permeable analogue of cyclic AMP, induced an effect on the ATP-gated current that was similar to that of forskolin (Figure 4b). As illustrated in Figure 4c, pretreatment with IBMX (1 mM), a phosphodiesterase inhibitor that prevents the breakdown of cyclic AMP, for 2 to 3 min also decreased the ATP-induced peak current, indicating that an increase in intracellular cyclic AMP reduces the ATP-gated current. To examine whether phosphorylation by PKC exerts an effect on the ATP-gated current, PMA, an activator of PKC was employed. The cells were pretreated with PMA (1 μM) for 2 to 3 min. As indicated in Figure 4d, the ATP (5 μM)-induced current response was reduced by PMA. The percentage decreases in the ATP (5 μM)-induced peak current by activators of PKA and PKC are shown in Figure 4e.

Discussion

The results show that the P2X receptors in cochlear Deiters' cells gate a non-selective cation channel, as observed in other systems, and that intracellular Ca^{2+} may play an important

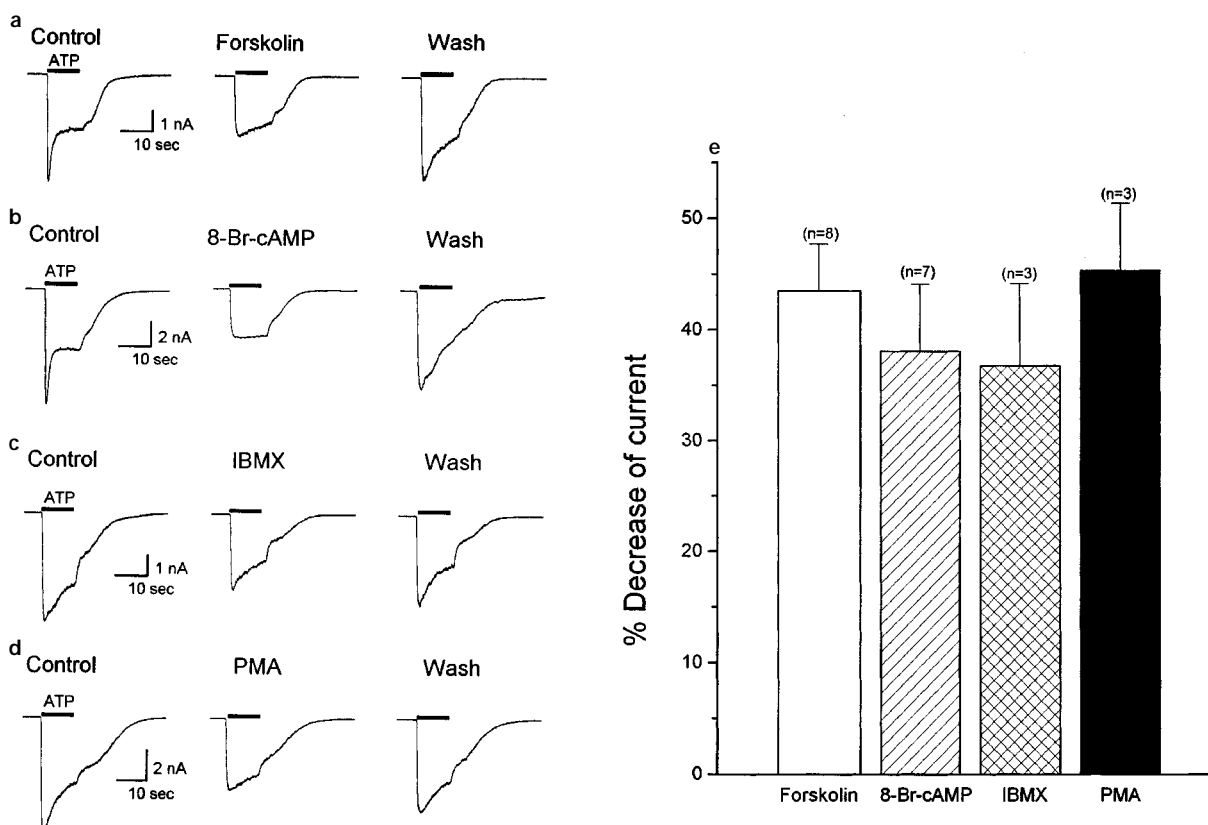


Figure 4 Phosphorylation of the P2X receptors. (a) ATP ($5 \mu\text{M}$)-induced current response at a holding potential of -80 mV before (control), after treatment with forskolin ($10 \mu\text{M}$) for 2 to 3 min and washout. (b) ATP ($5 \mu\text{M}$)-induced current response before, after treatment with 8-bromoadenosine-3',5'-cyclophosphate (8-Br-cyclic AMP, 1 mM) for 2–3 min and washout. (c) ATP ($5 \mu\text{M}$)-induced current response before, after treatment with 3-isobutyl-1-methylxanthine (IBMX, 1 mM) for 2 to 3 min and washout. (d) ATP ($5 \mu\text{M}$)-induced current response before, after treatment with phorbol-12-myristate-13-acetate (PMA, $1 \mu\text{M}$) for 2 to 3 min and washout. (e) The percentage decrease in the ATP ($5 \mu\text{M}$)-induced peak current by activators of PKA and PKC. Data represent the mean \pm s.e. for the indicated number of cells.

role in regulating the ATP-gated channel. In addition, the results show that P2X receptors can be functionally modulated by phosphorylation with PKA and PKC.

Functional subtype of the P2X receptor in Deiters' cells

To date seven P2X receptor subunits (P2X₁–P2X₇) have been cloned (Brake *et al.*, 1994; Valera *et al.*, 1994; Chen *et al.*, 1995a; Lewis *et al.*, 1995; Collo *et al.*, 1996; Surprenant *et al.*, 1996; Buell *et al.*, 1996). The amino acid sequences of these P2X receptor subunits share a predicted structure with intracellular C and N domains, two transmembrane spanning regions and a large extracellular loop. When these subunits are expressed, each of them can form non-selective cation channels. Each can be characterized and distinguished by differences in their sensitivities to purinoceptor agonists and antagonists as well as their desensitization in the presence of agonists (Surprenant *et al.*, 1995; Collo *et al.*, 1996; Buell *et al.*, 1996; Werner *et al.*, 1996).

Sensitivity to α,β -meATP has proved to be one of the useful criteria for distinguishing between P2X receptors. α,β -meATP is a full agonist at the P2X₁ and P2X₃ subtypes, whereas it is a weak or ineffective agonist at the P2X₂ and P2X_{4–7} purinoceptors (Surprenant *et al.*, 1995; 1996; Collo *et al.*, 1996; Robertson *et al.*, 1996; Ugur *et al.*, 1997). In Deiters' cells, α,β -meATP is a relative weak agonist compared to ATP and 2-meSATP, suggesting that P2X₁ and P2X₃ purinoceptors may not be functionally expressed in these cells.

It has been shown that P2X₄ and P2X₆ receptors are insensitive to suramin and PPADS (Collo *et al.*, 1996). In Deiters' cells the ATP-induced current response was inhibited by suramin, pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) and cibacron blue (Skellott *et al.*, 1997; Chen *et al.*, 1998). Thus, it is unlikely that the P2X₄ and P2X₆ subtypes are functionally expressed in Deiters' cells. In summary, the possible functional expressions of P2X₁, P2X₃, P2X₄ and P2X₆ receptors in Deiters' cells are excluded based on these criteria (Surprenant *et al.*, 1995; Collo *et al.*, 1996; Fredholm *et al.*, 1997).

Having excluded P2X₁, P2X₃, P2X₄, P2X₆, then the remaining possibilities are P2X₂, P2X₅ and P2X₇. A distinguishing property of P2X receptors is the rate of desensitization. In Deiters' cells, the purinoceptor agonist-induced responses (e.g., ATP) exhibited relatively fast desensitization, and the desensitization was more pronounced with prolonged (1 min) application of a relative high concentration of ATP ($50 \mu\text{M}$). It is unlikely that the P2X receptor that is functionally expressed in Deiters' cells is the P2X₇, because the P2X₇ receptor exhibits no desensitization even though we observed that BzATP, a P2X₇ receptor agonist, elicited an inward current in Deiters' cells (Ugur *et al.*, 1997). Both P2X₂ and P2X₅ receptors were originally identified as purinoceptors with slow rates of desensitization (Brake *et al.*, 1994; Surprenant *et al.*, 1995; Collo *et al.*, 1996; Brändle *et al.*, 1997). Thus, our data would suggest that neither P2X₂ or P2X₅ receptors are present in Deiters' cells.

However, Parker *et al.* (1997, 1998) recently described the sequencing of cDNA of three P2X₂ splice variants from a guinea-pig organ of Corti library and the localization of the mRNA to cells in the organ of Corti, including Deiters' cells by *in situ* hybridization, confirming and extending the results of others (Glowatzki *et al.*, 1995; Housley *et al.*, 1995; Brändle *et al.*, 1997). In addition, recent evidence shows that splice variants of the P2X₂ receptor exhibit different desensitization rates (Brändle *et al.*, 1997; Simon *et al.*, 1997). One splice variant (P2X₂₋₂, Brändle *et al.*, 1997; P2X_{2(b)}, Simon *et al.*, 1997) is desensitized relatively fast, insensitive to α,β -meATP and blocked by suramin and PPADS. The characteristics of the P2X receptor in Deiters' cells are consistent with these observations, suggesting that there may be a functional expression of a similar P2X₂ splice variant in Deiters' cells. However, we cannot exclude the possibility that Deiters' cells contain multiple individual P2X subunits, a novel subunit that has not so far been cloned, or novel P2X heteropolymers.

Effects of purinoceptor antagonists

Three different purinoceptor antagonists, suramin, cibacron blue and PPADS have been shown to block the ATP-gated current in Deiters' cells, and the effects of these antagonists exhibit distinct patterns (Skellett *et al.*, 1997; Chen *et al.*, 1998). Co-application of suramin and ATP reduced the current, but, shortly after the co-application, the ATP-induced current was potentiated (Skellett *et al.*, 1997). Cibacron blue is more potent than suramin in blocking the ATP-induced current and PPADS is the most potent (Skellett *et al.*, 1997; Chen *et al.*, 1998). Interestingly, the co-application of PPADS (100 μ M) and ATP (5 μ M) did not reduce the ATP-induced peak current, though the current did exhibit a rapid decay during the application. Shortly after the co-application, the current elicited by ATP (5 μ M) is completely blocked (Chen *et al.*, 1998). It has been shown that the kinetics of the block by PPADS at P2X₁, P2X₂ and P2X₅ receptors are slow in onset (Buell *et al.*, 1996). This may be the reason why PPADS was not able to block the P2X receptors immediately. These differences in the effects of suramin, cibacron and PPADS on the ATP-gated current response may reflect an unusual property of the P2X receptor in Deiters' cells.

Modulation of the P2X receptors by intracellular Ca²⁺

It has been shown that the ATP-induced current response is reduced when the extracellular Ca²⁺ concentration is raised while the current is increased when the extracellular Ca²⁺ concentration is decreased in PC 12 pheochromocytoma cells (Nakazawa *et al.*, 1990; Nakazawa & Hess, 1993), rat parasympathetic cardiac ganglion neurones (Fieber & Adams, 1991), guinea-pig IHCs (Sugasawa *et al.*, 1996) and OHCs (Raybould & Housley, 1997), and in P2X₁ and P2X₂ expressed in Chinese hamster ovary cells (Evans *et al.*, 1996). In the present study, the ATP-gated current was greatly reduced when intracellular Ca²⁺ was raised by decreasing the EGTA concentration from 11 mM to 0.1 mM. A voltage-dependent inhibition by internal Ca²⁺ of the ATP-gated currents has been observed in the inside-out patches of PC 12 pheochromocytoma

cells (Nakazawa & Hess, 1993). These findings suggest that the P2X receptors can be modulated by extracellular Ca²⁺ and by intracellular Ca²⁺. However, the mechanisms by which intracellular Ca²⁺ modulate the P2X receptors in Deiters' cells are still not clear and require further investigation.

Phosphorylation of the P2X purinoceptor

Protein phosphorylation is widely recognized as one of the primary modes of regulation of cellular functions. It has been demonstrated that ionotropic acetylcholine and glutamate receptors can be phosphorylated by protein kinase A and C (see reviews: Haganir & Greengard, 1990; Swope *et al.*, 1992). However, to date, there are no data showing that ATP-gated purinoceptors are regulated by protein phosphorylation. Sequences of cDNAs for the P2X₂ receptor splice variants from guinea-pig organ of Corti reveal that there are several potential phosphorylation sites for PKC and/or PKA (Parker *et al.*, 1997; 1998) as there are in other P2X receptor sequences (e.g. Séguéla *et al.*, 1996; Simon *et al.*, 1997; Brändle *et al.*, 1997), indicating that the P2X receptors may be functionally modulated by protein phosphorylation. In the present study, it was found that activation of PKA by forskolin, 8-Br-cyclic AMP and IBMX reduced the ATP-gated current amplitude. The current was also decreased by pretreatment with PMA, an activator of PKC. The reduction of the ATP-gated current is probably due to the facilitation of desensitization of the P2X receptors. This is the first evidence in the literature that the functions of the P2X receptors are regulated by PKA and PKC phosphorylation.

Functional significance of the P2X purinoceptors in Deiters' cells

Increasing evidence suggests that ATP may serve as a neurotransmitter or modulator in the mammalian organ of Corti (Bobbin, 1996; Skellett *et al.*, 1997; Bobbin *et al.*, 1997; Chen *et al.*, 1998). Both ATP and the ATP antagonist, suramin, have large effects on distortion product otoacoustic emissions (DPOAEs; Kujawa *et al.*, 1994a,b; Skellett *et al.*, 1997). Since DPOAEs are generated by the active and passive mechanics of the cochlea, it appears that the P2X₂ receptors may play an important role in cochlear mechanics. The source of the ATP in the cochlea is unknown. Recently, Wangemann (1996) demonstrated release of ATP from cells of the isolated organ of Corti, though no particular cell was determined as the release site. Burgess *et al.* (1997) demonstrated nerve fibre innervation of the supporting cells, including Deiters' cells and Hensen's cells. Whether these nerve fibres are the source(s) of the ATP acting on ATP receptors on these cells in the organ of Corti has not been determined.

Thanks to Julie Campbell, B.S. for technical assistance. This work was supported in part by research grant numbers R01 DC 00722 and PO1 DC00379 from the National Institute on Deafness and Other Communication Disorders, National Institutes of Health, and DAMD 17-93-V-3013, Kam's fund for Hearing Research, and the Louisiana Lions Eye Foundation.

References

- ASHMORE, J.F. & OHMORI, H. (1990). Control of intracellular calcium by ATP in isolated outer hair cells of the guinea-pig cochlea. *J. Physiol.*, **428**, 109–131.
- BEAN, P.B. (1992). Pharmacology and electrophysiology of ATP-activated ion channels. *Trends Pharmacol. Sci.*, **13**, 87–90.

- BOBBIN, R.P. (1996). Chemical receptors on outer hair cells and their molecular mechanisms. In *Hair Cells and Hearing Aids*, ed. Berlin, C.I. pp. 29–55. San Diego: Singular Publ.
- BOBBIN, R.P., CHEN, C., NENOV, A.P. & SKELLETT, R.A. (1997). Transmitters in the cochlea: The quadratic distortion product and its time varying response may reflect the function of ATP in the cochlea. In *Otoacoustic Emissions*, ed. Berlin, C.I. San Diego: Singular Publ. (in press).
- BRAKE, A.J., WAGENBACH, M.J. & JULIUS, D. (1994). New structural motif for ligand-gated ion channels defined by an ionotropic ATP receptor. *Nature*, **371**, 519–523.
- BRÄNDLE, U., SPIELMANN, P., OSTEROTH, R., SIM, J., SURPRENANT, A., BUELL, G., RUPPERSBERG, J.P., PLINKERT, P.K., ZENNER, H.P. & GLOWATZKI, E. (1997). Desensitization of the P2X₂ receptor controlled by alternative splicing. *FEBS Lett.*, **404**, 294–298.
- BUELL, G.N., LEWIS, C., COLLO, G., NORTH, R.A. & SURPRENANT, A. (1996). An antagonist-insensitive P2x receptor expressed in epithelia and brain. *EMBO J.*, **15**, 55–62.
- BURGESS, B.J., ADAMS, J.C. & NADOL, J.B. JR. (1997). Morphologic evidence for innervation of Deiters' and Hensen's cells in the guinea pig. *Hear. Res.*, **108**, 74–82.
- BURNSTOCK, G. (1990). Purinergic mechanism. *Ann. N.Y. Acad. Sci.*, **603**, 1–18.
- CHEN, C., AKOPIAN, A.N., SIVILOTTI, L., COLQUHOUN, D., BURNSTOCK, G. & WOOD, J.N. (1995a). A P2x receptor expressed by a subset of sensory neurons. *Nature*, **377**, 428–430.
- CHEN, C. & BOBBIN, R.P. (1997). Differences in effects of purinergic antagonists on P2x receptor in the cochlea. *Soc. Neurosci. Abstr.*, **23**, 1782.
- CHEN, C., LEBLANC, C. & BOBBIN, R.P. (1997). Differences in the distribution of responses to ATP and acetylcholine between outer hair cells of rat and guinea pig. *Hear. Res.*, **110**, 87–94.
- CHEN, C., NENOV, A.P., NORRIS, C. & BOBBIN, R.P. (1995b). ATP modulation of L-type Ca²⁺ channel currents in guinea pig outer hair cells. *Hear. Res.*, **86**, 25–33.
- CHEN, C., NENOV, A.P. & BOBBIN, R.P. (1995c). Noise exposure alters the response of outer hair cells to ATP. *Hear. Res.*, **88**, 215–221.
- CHEN, C., SKELLETT, R.A., FALLON, M. & BOBBIN, R.P. (1998). Additional pharmacological evidence that endogenous ATP modulates cochlear mechanics. *Hear. Res.*, (in press).
- COLLO, G., NORTH, R.A., KAWASHIMA, E., MERLO-PICH, E., NEIDHART, S., SURPRENANT, A. & BUELL, G. (1996). Cloning of P2X₅ and P2X₆ receptors and the distribution and properties of an extended family of ATP-gated ion channels. *J. Neurosci.*, **16**, 2495–2507.
- DUBYAK, G.R. & EL-MOATASSIM, C. (1993). Signal transduction via P₂-purinergic receptors for extracellular ATP and other nucleotides. *Am. J. Physiol.*, **265**, C577–C606.
- DULON, D. (1995). Ca²⁺ signaling in Deiters cells of the guinea-pig cochlea active process in supporting cells? In *Active Hearing*, ed. Flock, A., Ottoson, D. & Ulfendahl, M. pp. 195–207. Great Britain: Elsevier Science Ltd.
- DULON, D., MOATAZ, R. & MOLLARD, P. (1993). Characterization of Ca²⁺ signals generated by extracellular nucleotides in supporting cells of the organ of Corti. *Cell Calcium*, **14**, 245–254.
- EVANS, R.J., LEWIS, C., VIRGINIO, C., LUNDSTROM, K., BUELL, G., SURPRENANT, A. & NORTH, R.A. (1996). Ionic permeability of, and divalent cation effects on, two ATP-gated cation channels (P2X receptors) expressed in mammalian cells. *J. Physiol.*, **497**, 413–422.
- EYBALIN, M. (1993). Neurotransmitters and neuromodulators of the mammalian cochlea. *Physiol. Rev.*, **73**, 309–373.
- FIEBER, L.A. & ADAMS, D.J. (1991). Adenosine triphosphate-evoked currents in cultured neurons dissociated from rat parasympathetic cardiac ganglia. *J. Physiol.*, **434**, 239–256.
- FRANK, G. & KOSSL, M. (1996). The acoustic two-tone distortions 2f1-f2 and f2-f1 and their possible relation to changes in the operating point of the cochlear amplifier. *Hear. Res.*, **98**, 104–115.
- FREDHOLM, B.B., ABBRACCHIO, M.P., BURNSTOCK, G., DUBYAK, G.R., HARDEN, T.K., JACOBSON, K.A., SCHWABE, U. & WILLIAMS, M. (1997). Towards a revised nomenclature for P1 and P2 receptors. *Trends Pharmacol. Sci.*, **18**, 79–82.
- GLOWATZKI, E., WILD, K., BRÄNDLE, U., FAKLER, G., FAKLER, B., ZENNER, H.P. & RUPPERSBERG, J.P. (1995). Cell-specific expression of the $\alpha 9$ n-ACh receptor subunit in auditory hair cells revealed by single-cell RT-PCR. *Proc. R. Soc. Lond. B*, **262**, 141–147.
- HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.*, **391**, 85–100.
- HOUSLEY, G.D., GREENWOOD, D., BENNETT, T. & RYAN, A.F. (1995). Identification of a short form of the P2xR1-purinoceptor subunit produced by alternative splicing in the pituitary and cochlea. *Biochem. Biophys. Res. Commun.*, **212**, 501–508.
- HOUSLEY, G.D., GREENWOOD, D. & ASHMORE, J.F. (1992). Localization of cholinergic and purinergic receptors on outer hair cells isolated from the guinea-pig cochlea. *Proc. R. Soc. Lond. B.*, **249**, 265–273.
- HUGANIR, R.L. & GREENGARD, P. (1990). Regulation of neurotransmitter receptor desensitization by protein phosphorylation. *Neuron*, **5**, 555–567.
- IURATO, S., FRANKE, K., LUCIANO, L., WERMBTER, G., PANNESE, E. & REALE, E. (1976). Intercellular junctions in the organ of Corti as revealed by freeze fracturing. *Acta Otolaryngol.*, **82**, 57–69.
- KENNEDY, C. & LEFF, P. (1995). How should P2x purinoceptors be classified pharmacologically? *Trends Pharmacol. Sci.*, **16**, 168–174.
- KUJAWA, S.G., EROSTEGUI, C., FALLON, M., CRIST, J. & BOBBIN, R.P. (1994a). Effects of adenosine 5'-triphosphate and related agonists on cochlear function. *Hear. Res.*, **76**, 87–100.
- KUJAWA, S.G., FALLON, M. & BOBBIN, R.P. (1994b). ATP antagonists cibacron blue, basilen blue and suramin alter sound-evoked responses of the cochlea and auditory nerve. *Hear. Res.*, **78**, 181–188.
- LEWIS, C., NEIDHART, S., HOLY, C., NORTH, R.A., BUELL, G. & SURPRENANT, A. (1995). Heteropolymerization of P2x receptor subunits can account for ATP-gated currents in sensory neurons. *Nature*, **377**, 432–435.
- MURASE, K., RYU, P.D. & RANDIC, M. (1989). Excitatory and inhibitory amino acids and peptide-induced responses in acutely isolated rat spinal dorsal horn neurons. *Neurosci. Lett.*, **103**, 56–63.
- NAKAGAWA, T., AKAIKE, N., KIMITSUKI, T., KOMUNE, S. & ARIMA, T. (1990). ATP-induced current in isolated outer hair cells of guinea pig cochlea. *J. Neurophysiol.*, **63**, 1068–1074.
- NAKAZAWA, K., FUJIMORI, K., TAKANAKA, A. & INOUE, K. (1990). An ATP-activated conductance in pheochromocytoma cells and its suppression by extracellular calcium. *J. Physiol.*, **428**, 257–272.
- NAKAZAWA, K. & HESS, P. (1993). Block by calcium of ATP-activated channels in pheochromocytoma cells. *J. Gen. Physiol.*, **101**, 377–392.
- NORTH, R.A. & BARNARD, E.A. (1997). Nucleotide receptors. *Curr. Opin. Neurobiol.*, **7**, 346–357.
- PARKER, M.S., BOBBIN, R.P. & DEININGER, P.L. (1997). Guinea pig organ of Corti contains mRNA for ATP receptor type P2X₂. *ARO Abstr.*, **20**, 217.
- PARKER, M.S., LARROQUE, M.L., CAMPBELL, J.M., BOBBIN, R.P. & DEININGER, P.L. (1998). Sequence and distribution of P2X₂ ATP receptor variants in the guinea pig organ of Corti. *ARO Abstr.*, **21**, 84.
- RAYBOULD, N.P. & HOUSLEY, G.D. (1997). Variation in expression of the outer hair cell P2x receptor conductance along the guinea pig cochlea. *J. Physiol.*, **498**, 717–727.
- RAYMOND, L.A., BLACKSTONE, C.D. & HUGANIR, R.L. (1993). Phosphorylation and modulation of recombinant GluR6 glutamate receptors by cAMP-dependent protein kinase. *Nature*, **361**, 637–641.
- ROBERTSON, S.J., RAE, M.G., ROWAN, E.G. & KENNEDY, C. (1996). Characterization of a P2X-purinoceptor in cultured neurones of the rat dorsal root ganglia. *Br. J. Pharmacol.*, **118**, 951–956.
- SIMON, J., KIDD, E.J., SMITH, F.M., CHESSELL, I.P., MURRELL-LAGNADO, R., HUMPHREY, P.P.A. & BARNARD, E.A. (1997). Localization and functional expression of splice variants of the P2x₂ receptor. *Mol. Pharmacol.*, **52**, 237–248.
- SÉGUÉLA, P., HAGHIGHI, A., SOGHOMONIAN, J.-J. & COOPER, E. (1996). A novel neuronal P2x ATP receptor ion channel with widespread distribution in the brain. *J. Neurosci.*, **16**, 448–455.
- SKELLETT, R.A., CHEN, C., FALLON, M., NENOV, A.P. & BOBBIN, R.P. (1997). Pharmacological evidence that endogenous ATP modulates cochlear mechanics. *Hear. Res.*, **111**, 42–54.
- SUGASAWA, M., EROSTEGUI, C., BLANCHET, C. & DULON, D. (1996). ATP activates non-selective cation channels and calcium release in inner hair cells of the guinea-pig cochlea. *J. Physiol.*, **491**, 707–718.

- SUGASAWA, M., EROSTEGUI, C., BLANCHET, C. & DULON, D. (1997). ATP activates a cation conductance and a Ca^{2+} -dependent chloride conductance in Hensen cells of the guinea-pig cochlea. *Am. J. Physiol.*, **271**, C1817–C1827.
- SURPRENANT, A., BUELL, G. & NORTH, R.A. (1995). P2x receptors bring new structure to ligand-gated ion channels. *Trends Neurosci.*, **18**, 224–229.
- SURPRENANT, A., RASSENDREN, F., KAWASHIMA, E., NORTH, R.A. & BUELL, G. (1996). The cytolytic P2z receptor for extracellular ATP identified as a P2x receptor (P2x₇). *Science*, **272**, 735–738.
- SWOPE, S.J., MOSS, S.J., BLACKSTONE, C.D. & HUGANIR, R.L. (1992). Phosphorylation of ligand-gated ion channels: a possible mode of synaptic plasticity. *FASEB J.*, **6**, 2512–2523.
- UGUR, M., DRUMMOND, R.M., ZHOU, H., SHENG, P., SINGER, J.J. & WALSH, J.V. JR. (1997). An ATP-gated cation channel with some P2z-like characteristics in gastric smooth muscle cells of toad. *J. Physiol.*, **498**, 427–442.
- VALERA, S., HUSSY, N., EVANS, R.J., ADAML, N., NORTH, R.A., SURPRENANT, A. & BUELL, G. (1994). A new class of ligand-gated ion channel defined by P2x receptor for extracellular ATP. *Nature*, **371**, 516–519.
- WANG, L.-Y., SALTER, M.W. & MACDONALD, J.F. (1991). Regulation of kainate receptors by cAMP-dependent protein kinase and phosphorylations. *Science*, **253**, 1132–1135.
- WANG, L.-Y., TAVERNA, F.A., HUANG, X.-P., MACDONALD, J.F. & HAMPSON, D.R. (1993). Phosphorylation and modulation of a kainate receptor (GluR6) by cAMP-dependent protein kinase. *Science*, **259**, 1173–1175.
- WANGEMANN, P. (1996). Ca^{2+} -dependent release of ATP from the organ of Corti measured with a luciferin-luciferase bioluminescence assay. *Aud. Neurosci.*, **2**, 187–192.
- WERNER, P., SEWARD, E.P., BUELL, G.N. & NORTH, R.A. (1996). Domains of P2x receptors involved in desensitization. *Proc. Natl. Acad. Sci. U.S.A.*, **93**, 15485–15490.

(Received November 27, 1997)

Revised January 6, 1998

Accepted February 24, 1998)