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Characteristics of $P2X_7$ receptors from human B lymphocytes expressed in *Xenopus* oocytes

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Abstract

Human B lymphocytes express an ATP-gated ion channel (P2Z receptor), which shares similarities with the recently identified $P2X_7$ receptor. Using gene specific primers, we have now isolated $P2X_7$ cDNA from the total RNA of human B lymphocytes. This $hP2X_7$ receptor subtype was expressed in *Xenopus* oocytes and electrophysiologically characterized. The $hP2X_7$ receptor is similar to, but does not completely match, P2Z of human B cells. The $hP2X_7$ receptors resemble the P2Z receptors with regard to the ATP concentration of half maximal activation, reproducibility, permeation characteristics and lack of desensitization of the ATP-evoked currents. However, in contrast to the native lymphocytic P2Z receptor, the time course of activation of $hP2X_7$ displayed an additional linearly increasing current component. Furthermore, a second, small and slowly deactivating current component exists only in $hP2X_7$ expressed in oocytes. The activation and deactivation kinetics as well as permeation characteristics of $hP2X_7$ are different from rat $P2X_7$ recently expressed in oocytes. Unlike in mammalian cells, $hP2X_7$ expressed in *Xenopus* oocytes is not sufficient to induce large non-selective pores.

Keywords: B lymphocyte; Purinoceptor; P2X receptor; Permeation; *Xenopus* oocyte; Kinetics

1. Introduction

Cells of the immune and inflammatory system express a distinct subtype of receptors for extracellular ATP (P2X receptors), named P2Z. Like the other subtypes of the P2X receptor family, the P2Z receptor is regarded as a ligand-gated ion channel. It is opened by free uncomplexed ATP^{4-} in the 100 μM range. 3′-*O*-(4-Benzoyl)benzoyl-ATP (BzATP) which opens P2Z channels at about one tenth of the ATP concentrations needed to activate P2Z is considered to be the typical and selective agonist. Oxidized ATP (oATP) acts as an irreversible antagonist on P2Z [1]. In macrophages, fibroblasts and mast cells, ATP induces large pores in the cell membrane which allow the permeation of molecules as large as 900 Da [1].

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The precise in vivo function of P2 receptors in cells of hematopoetic origin is not yet known. The stimulation of proliferation, secretion of mediators, apoptosis or necrosis have been reported depending on cell type as well as on the duration and concentration of ATP application [2–4].

P2Z receptors in human B lymphocytes display the same pharmacological profile as in macrophages, fibroblasts and mast cells, but the ATP-induced pores allow permeation only for molecules <400 Da [5]. Electrophysiological studies demonstrated the gating by ATP^{4-} of a current which could be carried by small inorganic cations. The ATP^{4-} concentration for half maximal activation of the non-desensitizing currents was 200 μM. The activation and deactivation of this current were complete within less than 100 ms [6]. Single channel studies in Epstein-Barr virus transformed and tonsillar human B cells revealed ionic channels with a single channel conductance of 9 pS which also did not desensitize. The channels were opened by ATP and BzATP in a concentration range typical of P2Z. Therefore, these channels were assumed to carry at least one part of the current measured in the whole-cell configuration [7].

Recently, a subtype of the P2X receptor family, named $P2X_7$, was cloned with typical characteristics of pore-forming P2Z receptors [8,9]. After the short administration of ATP to mammalian cells expressing $P2X_7$, these receptors were reported to open ionic channels permeable to small cations only. However, extended applications of ATP or BzATP to $P2X_7$ receptors in solutions with low divalent cation concentration induced pores for large molecules like the propidium dye YO-PRO-1 (629 Da). For the rat phenotype, these pores closed very slowly (only after a few minutes) after the removal of the agonist [9].

Xenopus oocytes were used as an expression system for the electrophysiological characterization of the $hP2X_7$ receptor subtype found in human B lymphocytes. The aim of this study was to examine whether the previously characterized native P2Z of human B lymphocytes [6,7] exhibits exactly the same phenotype as the cloned $P2X_7$. To this end, we isolated the cDNA of the $P2X_7$ from human B lymphocytes and overexpressed the encoded receptor in *Xenopus* oocytes for electrophysiological characterization.

2. Methods

2.1. Chemicals

Chemicals were obtained from Sigma (Deisenhofen, Germany) if not otherwise stated.

2.2. Isolation of human $P2X_7$ cDNA from human B lymphocytes by RT-PCR

cDNA comprising the entire coding region of $P2X_7$ was amplified by reverse transcription-polymerase chain reaction (RT-PCR) from the total RNA of human B lymphocyte RNA using gene-specific primers (capitals) flanked by an *Xba*I-site (forward: 5′-aatctagaGCAGGGAGGGAGGCTGTCACCAT; reverse: 5′-aatctagaGCCTGGCTTCAGTAAGGACTCTTGAA). PCR primers were designed from the published human $P2X_7$ sequence ([8], accession number Y09561). PCR products were size-selected by agarose gel electrophoresis and cloned into an *Xba*I site of vector pNKS2 [10]. The insert was fully sequenced on both strands using the dideoxynucleotide method. The amino acid sequence deduced deviated in two positions (G441 and A496) from the published sequence [8].

2.3. cRNA synthesis

Capped cRNAs were synthesized from linearized templates with SP6 RNA polymerase (Pharmacia), purified by Sepharose chromatography and phenol–chloroform extraction [11] and dissolved in 5 mM Tris/HCl, pH 7.2, at 0.5 μg/μl, using the optical density reading at 260 nm for quantitative analysis ($OD_{1.0} = 40$ μg/μl).

2.4. Oocyte treatment

Xenopus laevis females were imported from the African *Xenopus* Facility (Knysna, Republic of South Africa). The animals were anaesthetized in an aqueous solution supplemented with tricaine (MS222, solution a, see Table 1). Parts of the ovary were removed through a small incision and treated overnight with collagenase (solution b) for defolliculation. After several washes in nominally Ca^{2+}-free salt solution (solution c), the oocytes were placed in

Table 1
Preparation solutions. Concentrations are in mM if not otherwise stated

	a	b	c	d	e
NaCl		100	100	100	100
KCl		1	1	1	1
$MgCl_2$		1	1	1	1
$CaCl_2$		1		1	1
HEPES	5	5	5	5	5
Penicillin (U/ml)					10 000
Streptomycin (mg/ml)					10
Collagenase (mg/ml)		1.5			
Tricaine (g/l)	2				

pH was adjusted to 7.4 with NaOH for Na^+-containing solutions or with choline-Cl for choline$^+$-containing solutions, respectively.

Petri dishes containing normal salt solution (solution d). Stage V or VI oocytes were injected with 20–50 nl of cRNA and then kept in normal salt solution supplemented with antibiotics (solution e) at 19°C until used 2–4 days later.

2.5. Electrophysiology

All experiments were carried out at room temperature (≈22°C). Fast and reproducible solution exchange was achieved using a small tube-like chamber (0.1 ml) combined with fast superfusion (≈75 μl/s). Switching between different bathing solutions was performed by a set of computer-controlled magnetic valves using a modified U-tube technique [12].

The measurement of membrane currents was performed by the two-microelectrode voltage clamp method. Microelectrodes were pulled from borosilicate glass and filled with 3 M KCl. Only electrodes with resistances of between 0.5 and 1 MΩ were used. Currents were recorded and filtered at 100 Hz using an oocyte clamp amplifier (OC-725C, Hamden, USA) and sampled at 85 Hz. Data were stored and analyzed on a personal computer using software programmed at our department (Superpatch 2000, SP-Analyzer by T. Böhm).

The holding potential was set to −40 mV, if not otherwise stated. For the characterization of the permeation behavior, the oocyte cell membrane was depolarized by voltage ramps going from −80 to +40 mV within 300 ms. The reversal potentials of the resulting ramp-like currents were determined by a linear fit of the current near the reversal potential which additionally yielded the slope conductance.

In oocytes injected with H_2O, 1 mM extracellular ATP induced no or very small inward currents (< 1 nA). In some cases, larger currents (< 4 nA) were evoked, but only by the first application of ATP.

Concentrations of agonists always refer to the free form not complexed by Ca^{2+}, Mg^{2+} or Ba^{2+}. That means, for bathing solutions containing 1 mM Ba^{2+} the absolute amounts of ATP had to be calculated to adjust the correct concentrations of the free nucleotide (ATP^{4-}). This was done by a computer program kindly provided by R. Schubert [13].

Non-linear approximations and presentation of data were performed using the program Sigmaplot (Jandel, Corte Madeira, USA). Averaged data are given as means ± S.D. if not otherwise stated. Statistical data were analyzed by one-way ANOVA. The statistical significance of differences between means was tested using the multiple *t*-test (Bonferroni) by the program Sigmastat (Jandel).

3. Results

3.1. Speed of solution exchange

The oocytes were held for 1–3 min at +40 mV to induce a Na^+-selective conductance [14]. Afterwards, the bathing solution was changed from a normal oocyte Ringer (solution A, see Table 2) to a low Na^+ high K^+ bathing solution (solution H, wash-in), thereby inducing a shift of the holding current, the time course of which reflected the time course of

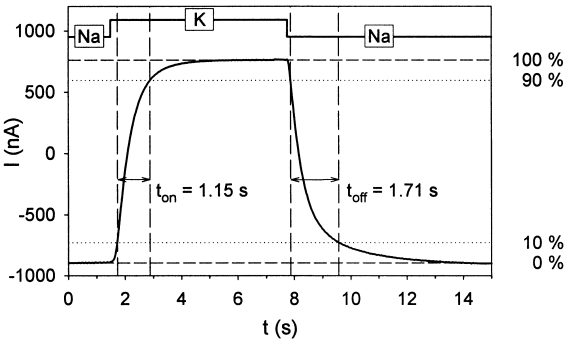

Fig. 1. Time course of solution exchange within the oocyte perfusion chamber. The oocyte was clamped at a holding potential of +40 mV. The bathing solution was changed by means of a set of computer-controlled magnetic valves from a high Na^+ low K^+ standard oocyte Ringer solution ('Na', solution A, see Table 1) to a low Na^+ high K^+ solution ('K', solution H) and back to the standard oocyte Ringer solution as indicated on the top of the figure.

wash-in. After 6 s, this solution was washed out by solution A. A typical example of the time course of shifting the holding current as a result of this solution exchange is shown in Fig. 1. The mean 10–90% exchange times were 1210 ± 70 ms for the wash-in and 1770 ± 170 ms for the wash-out.

3.2. Complex kinetics of $hP2X_7$-receptor currents in extracellular solutions containing Ba^{2+}

The impalement of the electrodes and measurement of the membrane potential of the oocytes was carried out in normal oocyte Ringer (solution A). To avoid the activation of endogenous currents activated by Ca^{2+} ions permeating the $hP2X_7$ receptor channels, the subsequent measurements of $hP2X_7$ receptor-dependent currents were carried out in bathing solutions with Ca^{2+} equimolar replaced by Ba^{2+}. This was reported to block Ca^{2+}-induced Cl^- currents [15]. Furthermore, Mg^{2+} ions were omitted to prevent complexation of ATP^{4-} by Mg^{2+}. The natural agonist of lymphocytic P2Z receptors is ATP^{4-} [1,7]. Free [ATP^{4-}] saturating the receptor could not be achieved in the presence of Mg^{2+}, Ca^{2+} or Ba^{2+} because of the complexation of ATP and the corresponding divalent ions. ATP and divalents are insoluble in the necessary high total concentrations.

During long term ATP applications in Ba^{2+}-containing solutions (solution C) complex kinetics of activation and deactivation were observed (Fig. 2A). Within the first 20–30 s the $hP2X_7$ displayed a fast exponential activation followed by a slow approximately linear current enlargement. Thereafter, the holding current as well as the conductance increased much faster, leading to very large currents. At the same time, the inward rectification of the ramp current changed to a slight outward rectification (Fig. 2B,C). The deactivation phase consisted mainly of two components: a first fast deactivation was followed by a very slow deactivation lasting more than 70 s. During the deactivation the outward rectification of the ramp currents became more prominent (Fig. 2D,E).

The observed slow deactivation seems to be similar to the deactivation kinetics of $P2X_7$ expressed in mammalian cells [8,9], in which a prolonged ATP application induces non-selective pores. On the other hand, the slow deactivating current component observed here could also be induced by the sustained influx of Ba^{2+} through the $hP2X_7$ channels. To dis-

Table 2
Bathing solutions

	A	B	C	D	E	F	G	H
NaCl	100	100	100	100	100			2.5
KCl	2.5	2.5	2.5	2.5	2.5	2.5	2.5	100
$MgCl_2$	1							1
$CaCl_2$	1							1
$BaCl_2$		1	1			1		
HEPES	5	5	5	5	5	5	5	5
Flufenamic acid			0.1	0.1	0.1	0.1	0.1	
EGTA					0.1			
CholineCl						100	100	

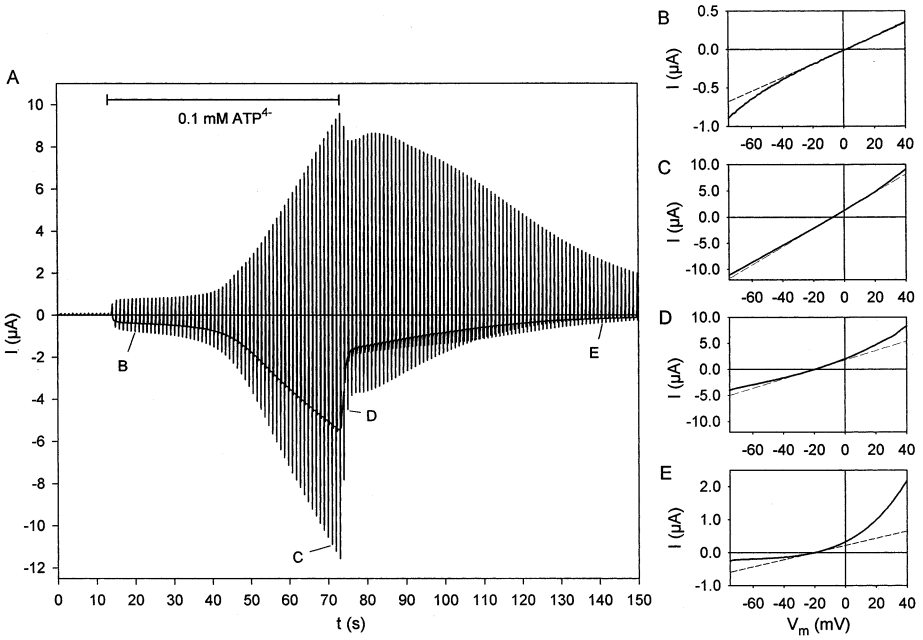

Fig. 2. Effect of long term applications of ATP to human $P2X_7$-receptors in Ba^{2+}-containing bathing solution. The concentration of free ATP (ATP^{4-}) was 0.1 mM. (A) Typical time course of $P2X_7$-receptor current. Bathing solution contained 1 mM Ba^{2+} as divalent cation (solution C, Table 2). (B–E) ATP-induced ramp currents (solid lines) as indicated in (A) but after subtraction of the mean ramp current before ATP application with linear fit of the current slope at the reversal potential (dashed lines). The fitted reversal potentials are +1 mV (B), −7 mV (C), −20 mV (D) and −21 mV (E).

criminate between these two possibilities the following experiments were carried out in extracellular solutions free from divalent cations. Under these circumstances any divalent-induced currents could be eliminated. Furthermore, the absence of divalent cations is reported to promote the induction of pores and a non-selective slowly deactivating conductance by $P2X_7$ in mammalian cells [8,9] That means, if pores are responsible for the slow deactivating component shown in Fig. 2A, it should be augmented under these experimental conditions.

3.3. Kinetics of hP2X7-receptor currents in divalent free extracellular solutions

The removal of divalent cations from the extracellular solution evoked a large conductance. It could be blocked by 0.1 mM flufenamic acid [16,17]. The effect of flufenamic acid on $hP2X_7$-dependent currents was measured in extracellular solution containing 1 mM Ba^{2+} to suppress the large conductance developing in the absence of flufenamic acid and divalent cations (Fig. 8A). Under these conditions,

Table 3
Kinetics of $hP2X_7$ expressed in oocytes activated by 1 mM ATP in solution D ($n=25$) or by 0.1 mM ATP^{4-} in solution B and C ($n=6$)

Parameter	1 ATP, 0 Ba, 0.1 fluf.	0.1 ATP, 1 Ba, 0 fluf.	0.1 ATP, 1 Ba, 0.1 fluf.	0.1 ATP, 1 Ba, 0.1 fluf./0 fluf.
τ_{act} (s)	0.9 ± 0.3	0.25 ± 0.1	0.37 ± 0.1	1.50 ± 0.32*
I_{act} (nA)	−244 ± 139	−101 ± 64	−92 ± 55	0.94 ± 0.08
c (nA/s)	−19 ± 8	−13 ± 7	−16 ± 8	1.25 ± 0.07*
$I_{deact,2}/I_{deact,1}$	0.20 ± 0.10	0.13 ± 0.07	0.10 ± 0.07	0.68 ± 0.32*
$\tau_{deact,1}$ (s)	0.9 ± 0.3	3.5 ± 0.6	3.3 ± 0.6	0.95 ± 0.08
$\tau_{deact,2}$ (s)	6.2 ± 2.0	14.8 ± 2.8	16.2 ± 4.8	1.20 ± 0.33

Abbreviations, see explanation of Eqs. 1 and 2. Means ± S.D., *$P < 0.05$.

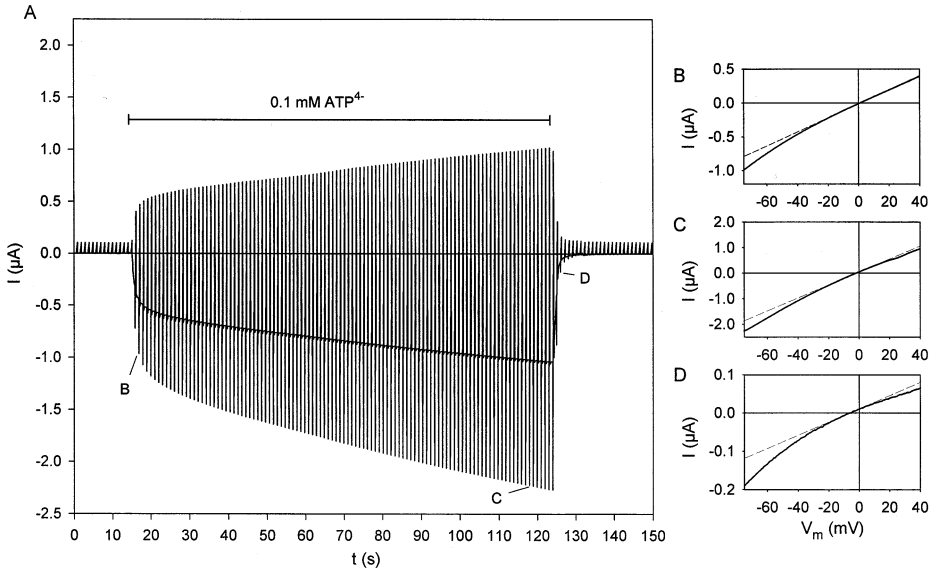

Fig. 3. Effect of extended applications of ATP to human $P2X_7$-receptors in divalent free bathing solution. (A) Typical time course of $P2X_7$-receptor current. Bathing solution without divalent cations (solution E, Table 2). The cell membrane was depolarized every 6 s by voltage ramps (see text) leading to ramp-like current deflections. Between these ramps the membrane was held at −40 mV. The current at −40 mV showed an exponential activation with a time constant of 0.9 s (fitted by Eq. 1) and a nearly linear slow current increase lasting until ATP washout. The removal of ATP led to a rapid deactivation with 90% of the current deactivating with a time constant $\tau_{deact,1} = 0.6$ s. The remaining current deactivated with a time constant $\tau_{deact,2} = 4.6$ s (fitted by Eq. 2). (B–D) ATP-induced ramp currents (solid lines) as indicated in (A). The fitted reversal potentials are +1 mV (A), −1 mV (B) and −6 mV (C). Same cell as in Fig. 2. For further explanations see Fig. 2.

only ATP^{4-}-concentrations up to 0.3 mM could be tested. At higher concentrations, ATP was not fully soluble. Flufenamic acid increased $hP2X_7$-dependent currents by about 16% at ATP^{4-}-concentrations of ≥0.1 mM (Fig. 8A) mainly due to an increase of the slope of the linearly rising component c (Table 3). The addition of flufenamic acid to the bath (change from solution B to C) never evoked inward currents >2 nA. Further effects of flufenamic acid are a deceleration of the activation and a decrease of the ratio of the two components of deactivation, $I_{deact,2}/I_{deact,1}$. A detailed pharmacological profile of the different components of the $hP2X_7$-dependent currents according to Eqs. 1 and 2 including the effects of flufenamic acid is currently under investigation. Nevertheless, the principal characteristics of $hP2X_7$-dependent currents (two components of activation and of deactivation as well as the activating concentration range for ATP^{4-}) seem not to be altered by flufenamic acid.

Fig. 3A demonstrates a current activated by ATP in divalent free extracellular solution. The current at −40 mV displayed after an initial phase of approximately exponential activation, a further current component that increased linearly with time. The activating part of the $hP2X_7$-receptor current ($I_{P2X7}(t)$) was fitted according to:

$$I_{P2X7,act}(t) = I_{act}\left(1 - e^{-\frac{t}{\tau_{act}}}\right) + c \cdot t + I_0 \quad (1)$$

where I_{act} is the activating current, I_0 is the steady-state current without ATP application, τ_{act} is the time constant for activation and c is the slope of the linearly rising current.

The best approximation of the deactivating current ($I_{P2X7,deact}$) during washout of ATP was achieved by use of:

$$I_{P2X7,deact}(t) = I_{deact}\left(I_{deact,1} \cdot e^{-\frac{t}{\tau_{deact,1}}} + I_{deact,2} \cdot e^{-\frac{t}{\tau_{deact,2}}}\right) + I_0 \quad (2)$$

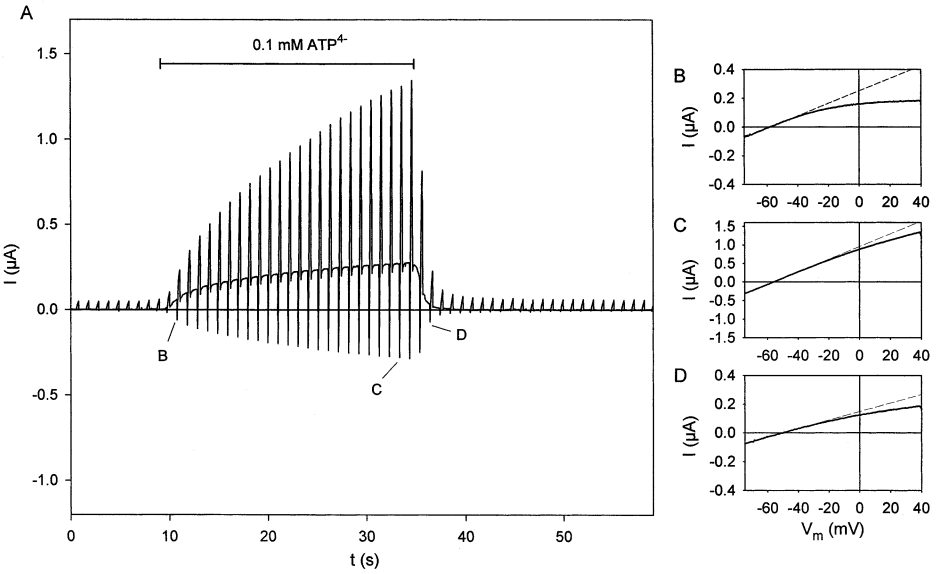

Fig. 4. Effect of long lasting applications of ATP to human $P2X_7$-receptors in Na^+-free bathing solution. (A) Typical time course of $P2X_7$-receptor current. Bathing solution contained choline$^+$ instead of Na^+ (solution G, Table 2). (B–D) ATP-induced ramp currents (solid lines) as indicated in (A). For further explanations see Fig. 3.

where I_0 has the same meaning as in Eq. 1 and I_{deact} is the absolute amplitude of the deactivating current. $I_{deact,1}$ and $I_{deact,2}$ are the relative amplitudes, and $\tau_{deact,1}$ and $\tau_{deact,2}$ are the time constants of the fast and slow deactivating component, respectively.

The activation and deactivation kinetics were not significantly altered by shortening the duration of ATP application to 6 s. The statistics of the kinetics of $hP2X_7$ are given in Table 3. The mean value of the slope of the linearly rising component c was significantly different from 0.

3.4. Permeation behavior during long term ATP applications

In Na^+-containing divalent free bathing solution, the reversal potential V_{rev} did not shift during 30 s of exposure to 0.1 mM ATP^{4-} (-1.8 ± 1.9 mV, not significantly different from 0). If Ba^{2+} was added to this solution, V_{rev} shifted significantly by -11.7 ± 5.0 mV.

In divalent free extracellular solutions with Na^+ substituted by choline$^+$ (Fig. 4) the current kinetics were similar to currents measured in divalent free oocyte Ringer. A fast exponential activation was followed by a linear rise of the holding current and conductance and the deactivation was complete within a few seconds (Fig. 4A). The very negative reversal potential was only slightly shifted to more positive values (Fig. 4B–D). On average the reversal potential was slightly but significantly shifted during 30 s of application of 0.1 mM ATP^{4-} by $+2.9 \pm 1.7$ mV.

After long term ATP applications (>20 s) in Na^+-free solutions containing Ba^{2+} (solution F, data not shown) the time of deactivation to the value of holding current before ATP^{4-}-application ± 20 nA lasted more than 1 min. Additionally, during 30 s of ATP^{4-}-application the inward rectification changed to an outward rectification and the reversal potential was significantly shifted by $+11.7 \pm 2.6$ mV.

3.5. Permeation behavior during short term ATP applications

The permeation was characterized by measuring the reversal potentials of the $hP2X_7$ receptor-dependent current in different extracellular solutions (Fig. 5).

To characterize the permeation of $hP2X_7$ by Ba^{2+} the reversal potential in Ba^{2+}-containing solutions was measured after short term ATP applications (<6 s). Under these circumstances the deactivation was not significantly different from the deactivation behavior in Ba^{2+}-free external solutions. This indi-

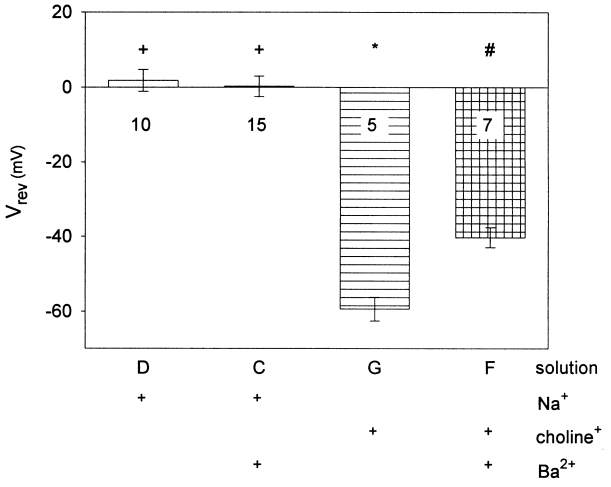

Fig. 5. Permeation behavior of human $P2X_7$-receptors. The reversal potentials of ramp currents measured according to Fig. 3B–D were determined 5 to 20 s after addition of 1 mM ATP in distinct extracellular solutions as indicated on the abscissa and on the bottom of the figure. Different symbols on the top indicate statistically different means. Numbers within the bars correspond to the number of investigated oocytes.

cated the absence of additional Ba^{2+}-induced current components.

The reversal potential in Na^+-containing Ringer solutions was about 0 mV independent of the Ba^{2+}-concentration. In bathing solution with Na^+ replaced by choline$^+$ the reversal potential was very negative indicating a high selectivity for Na^+ over the larger choline$^+$. The reversal potential in Na^+-free solutions was significantly shifted to more positive values by the addition of 1 mM Ba^{2+} to the bathing solution. This points to a substantial Ba^{2+} permeability of $hP2X_7$.

3.6. Reproducibility of activation of $hP2X_7$

As shown in Fig. 6A, the currents induced by ATP in divalent free bathing solution were highly reproducible even if ATP was applied several times in short intervals. The currents always returned within several seconds to the holding current before the first ATP application. The deactivation time course after each stimulation by ATP was obviously nearly identical. If the typical agonist of P2Z and $P2X_7$-receptors, BzATP, was used to activate the $hP2X_7$ receptors a potentiating effect on the current amplitude was observed which was pronounced in the first 2 min after the first BzATP application (Fig. 6B). The deactivation time course, on the other hand, was obviously not altered by repeated addition of either agonist to the bath.

3.7. ATP concentration–response relationship

For determination of the dependence of the amplitude of $hP2X_7$ receptor currents on the agonist concentration, different concentrations of ATP or BzATP, were applied in divalent free extracellular solution. The $hP2X_7$ receptor-dependent current I_P was measured as the current after 5 s of agonist application minus the current before agonist application. To account for the various extent of receptor expression the $hP2X_7$ receptor-dependent current evoked by a certain concentration of the agonists I_P([agonist]) was normalized to the control $hP2X_7$

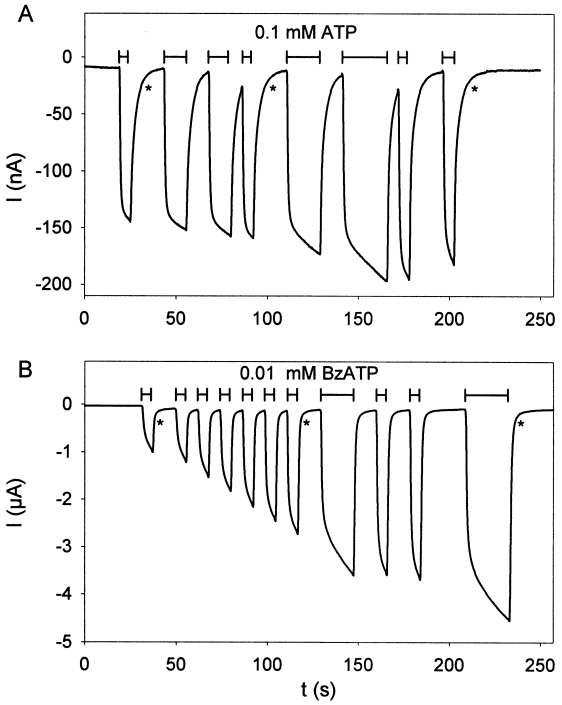

Fig. 6. Reproducibility of $P2X_7$-receptor currents gated by $P2X_7$-agonists. Solution E was used as extracellular solution. Typical examples of the effect of repeated application of ATP (A) or BzATP (B) on two different oocytes expressing $hP2X_7$. The duration of agonist application is marked by horizontal bars. The 90–10% deactivation times for the fraction of the current traces marked by an asterisk are 5.8, 6.1 and 5.9 s for ATP- (A) and 4.0, 1.8 and 2.1 s for BzATP-induced currents (B), respectively.

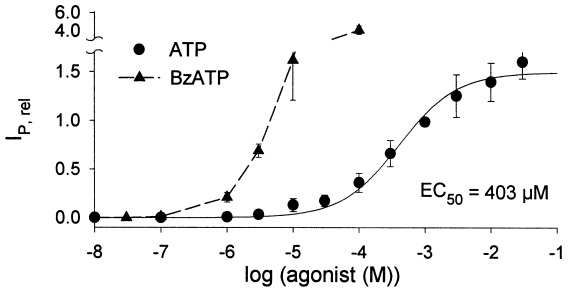

Fig. 7. Concentration–response curves for hP2X$_7$ receptor-dependent currents. hP2X$_7$ receptor-dependent currents evoked by application of different concentrations of agonist for 6 s were normalized to the current induced by at 1 mM ATP. Means from 12 oocytes are shown. Bathing solution (E) was used. The data for ATP-induced currents were fitted according to Eq. 3. For further explanations, see text.

receptor-dependent current $I_{P,cont}$ evoked by 1 mM ATP. The dose–response curve for ATP was fitted by the Hill equation with a Hill coefficient of 1:

$$I_{P,rel} = \frac{I_P([agonist])}{I_{P,cont}} = \frac{I_{P,rel,max}}{1 + \frac{10^{-\log EC_{50}}}{[agonist]}} \quad (3)$$

where $I_{P,rel,max}$ is the maximal relative hP2X$_7$ receptor current evoked by infinite concentrations of the agonist and EC_{50} is the concentration of half maximal current activation.

Models with different Hill coefficients did not describe the data significantly better [18]. The approximations yielded a $\log EC_{50}$ value of -3.40 ± 0.06 (see Fig. 7).

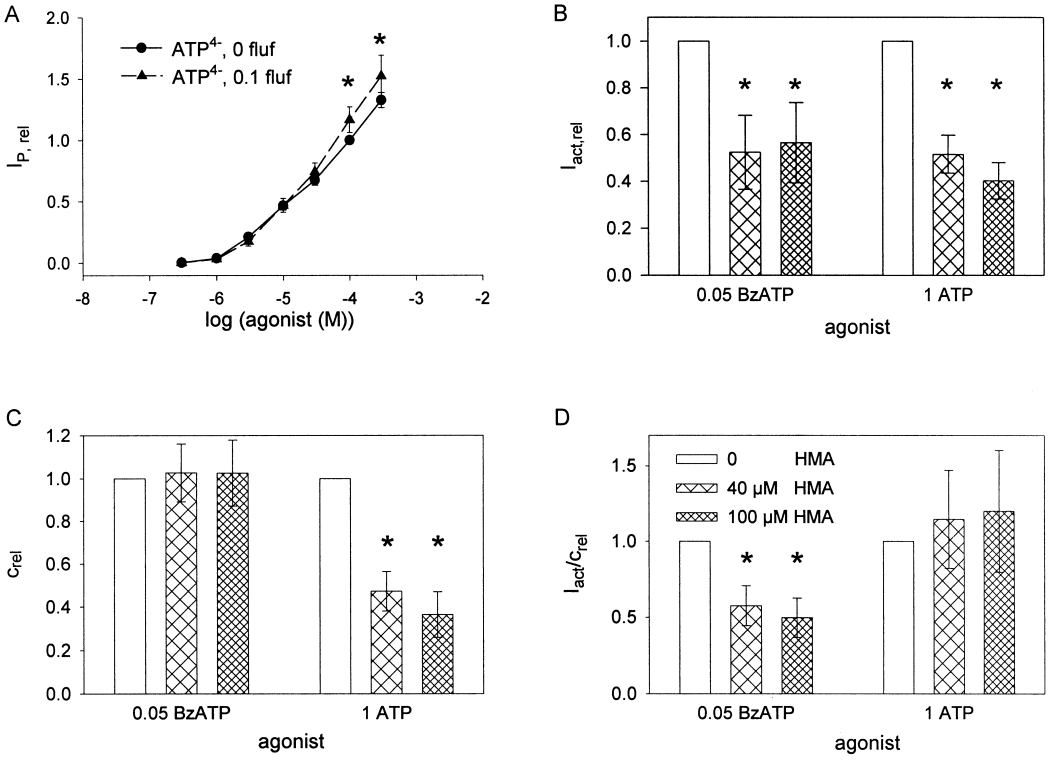

Fig. 8. Effect of flufenamic acid and HMA on hP2X$_7$ receptor-dependent currents. The agonist concentrations applied for 6 s to evoke hP2X$_7$-dependent inward currents are given at the abscissas. (A) Concentration–response curves for ATP^{4-} in bathing solutions containing 1 mM free Ba^{2+} and 0 or 0.1 mM flufenamic acid, respectively (bathing solution B versus C). At ATP^{4-}-concentrations of 0.1 and 0.3 mM, flufenamic acid induced a small but significant increase of the maximal hP2X$_7$-dependent current I_P (see Section 3.7 and Eq. 3). (B–D) Effect of HMA on the exponentially ($I_{act,rel}$) and the slope of the linearly-activating current (c_{rel}) as well as on the relation of these both components ($(I_{act}/c)_{rel}$). Bathing solution (D) was used supplemented with HMA as given in the legend to (D). Data are normalized to the controls measured in bathing solutions without HMA. Mean values significantly different from the controls are marked by asterisks.

3.8. Effect of hexamethylene amiloride (HMA) on hP2X$_7$-dependent currents

HMA is known as a blocker of large pores formed after the activation of purinergic receptors of macrophages [19]. As shown in Fig. 8B–D, in oocytes expressing hP2X$_7$, 100 μM HMA is not able to block BzATP- or ATP-evoked currents completely. This is in contrast to oocytes injected with macrophage mRNA. Interestingly, HMA blocks both the exponentially- as well as the linearly-activating current component of ATP-evoked currents to about 40–50% whereas it inhibits only the exponentially-activating component of currents elicited by the application of BzATP. This finding will be investigated further in future experiments.

4. Discussion

4.1. Relevance of hP2X$_7$ to the P2Z phenotype

The use of flufenamic acid to block currents activated by removal of divalent cations from the bathing solution [16,17] allowed the performance of experiments in extracellular solutions which were completely free from divalent cations. This simplified the investigated system because the influence of other ionic currents secondarily activated by an influx of divalent cations was avoided. The hP2X$_7$ receptor behaves similar to the native P2Z receptor found in human B lymphocytes with respect to the fast activation, ATP and BzATP concentration–response relationship, reproducibility, permeation characteristics and lack of desensitization of the ATP-evoked currents. However, two main differences exist: firstly, the hP2X$_7$-dependent current displays a slowly, linearly increasing current component during long ATP applications not detected in B lymphocytes [6]. The long term agonist addition to the bathing solution did not obviously change either rectification, reversal potential or the deactivation time course. Therefore, this additional current component seems not to be carried by other ionic channels, secondarily opened due to the gating of hP2X$_7$ or a slow enlargement of the hP2X$_7$ receptors channel pore.

Secondly, an additional, small and slowly deactivating current component exists only in hP2X$_7$ expressed in oocytes and was neither found in whole-cell [6] nor single channel measurements [7] of P2Z in human B lymphocytes. The presence of divalent ions in the experiments carried out in human B lymphocytes is probably not responsible for the different behavior of the lymphocytic P2Z receptor, because the linear-increasing component as well as the second slow deactivating component of hP2X$_7$-dependent currents was also measured in oocytes using Ba^{2+}-containing extracellular solutions (data not shown). The measurements of current kinetics may be somewhat biased by the time course of the solution exchange. Nevertheless, the finding that hP2X$_7$ displays slowly activating and deactivating components additional to the native lymphocytic P2Z receptor [6,7] is not in question because the time course of these components is obviously slower than the solution exchange.

4.2. Species-related comparison of the effects of agonists and the kinetics of P2X$_7$ expressed in different cell systems

Like in other described preparations, BzATP is the more potent and effective agonist at P2X$_7$ receptors in comparison to ATP. That means, similar to the lymphocytic P2Z receptor [20], ATP is only a partial agonist. However, the EC_{50} value of about 400 μM found for ATP^{4-} at hP2X$_7$ in oocytes is much higher than the 3 μM measured for the rat P2X$_7$ in the same expression system [21].

The concentration–response relationship for ATP^{4-} could be well fitted with a Hill coefficient of 1 similar to P2Z-mediated whole-cell currents in human B lymphocytes and HMA-insensitive BzATP-induced currents in oocytes injected with macrophage mRNA [19]. On the other hand, higher Hill coefficients were reported for the HMA-sensitive current in macrophage RNA-injected oocytes [19] and for P2Z-receptors mediated Ba^{2+}-influx in human leukemic B lymphocytes [4,5]. Therefore, it could be assumed that the currents measured here represent the rapid, HMA-insensitive component of the BzATP-inducible current carried by macrophage purinergic receptors and that the activation of this component requires the binding of a single molecule of BzATP or ATP to the receptor. Furthermore, it could be concluded that the Ba^{2+}-influx in human

leukemic B lymphocytes is not or not only carried by this component. We feel, however, that this assumption is highly speculative and that the number of agonist binding sites cannot be determined solely by concentration–response curves. Therefore, the approximation shown for the data of our experiments should mainly characterize the concentration range at which activation of $hP2X_7$ takes place. The concentration of half maximal activation of about 400 μM ATP^{4-} is not far from the 200 μM reported for human B lymphocytes. The difference may arise from the absence of a clearly defined plateau of the current amplitude at high concentrations of ATP^{4-} in lymphocytes. This is due to the fact that divalent cations have to be added to the extracellular solution to obtain stable tight seals for whole-cell voltage clamp recordings in lymphocytes. This leads to insoluble Ca-ATP complexes at high [ATP^{4-}] (see also Section 3.2).

The human $P2X_7$ expressed in oocytes (this study) conducted currents with nearly constant fast deactivation if agonists were repeatedly applied in divalent-free extracellular solution. This is in contrast to $hP2X_7$ expressed in HEK 293 cells [8,22] and to rat $P2X_7$ expressed in *Xenopus* oocytes [21] where progressively increasing deactivation time constants were measured under these conditions. It is a further indication of the disability of $hP2X_7$ to induce non-selective slowly deactivating pores in *Xenopus* oocytes. Additionally, in the oocyte expression system, the $hP2X_7$-dependent currents were potentiated by repeated application of BzATP, but the rat $P2X_7$ displayed a strong reduction of the current amplitude during consecutive BzATP applications [21]. Taken together, these findings point to profound species differences for $P2X_7$. Different expression systems seem also to be responsible for different properties of $P2X_7$ even of the same species.

Another interpretation of the different kinetics may be, that the deactivation measured in mammalian expression systems does not only reflect the closure of the $hP2X_7$-receptor channel pore but, beyond it, the deactivation of components which could have been additionally activated by the $P2X_7$-receptor. At least for the rat $P2X_7$, this putative component seems to be related to the opening of the large pores. It is still an unresolved issue if these pores arise from the enlargement of the $P2X_7$ ion conducting pathway itself or reflect the activation of other ion-conducting pathways. Indications for the former [23–25] as well as the latter [26–28] hypothesis have been published. It is also imaginable that both mechanisms may also act in parallel. It is tempting to speculate that these additional pore molecules once activated via the $P2X_7$-receptor are deactivated slowly after wash out of ATP causing a slow deactivation of the whole-cell current. If these pore molecules were only expressed in mammalian cells and not in *Xenopus* oocytes, the deactivating whole-cell currents measured in the oocyte expression system would reflect the closure of only the $hP2X_7$-pore. This would account for the faster deactivation of $hP2X_7$-dependent currents in oocyte system compared to mammalian cells. Single channel measurements of $hP2X_7$-dependent currents should help to clarify this issue.

4.3. Permeation characteristics

As reported for rat $P2X_7$ expressed in *Xenopus* oocytes [21], the reversal potential of ATP-induced currents of human $P2X_7$ did not change during extended ATP applications. Furthermore, HMA incompletely blocks both the exponentially and the linearly-activating component of ATP-evoked $hP2X_7$-dependent currents in oocytes to about the same extent. This is in contrast to oocytes injected with macrophage mRNA in which the formation of large pores is selectively inhibited. It argues against the induction of large pores by $P2X_7$ in oocytes which have been observed in mammalian cells expressing human, rat or mouse $P2X_2$, $P2X_4$ or $P2X_7$ receptors [8,9,23,24,29]. It has been reported [19] that at temperatures higher than room temperature the formation of large pores is promoted in cells expressing the P2Z phenotype. Unfortunately, it is not possible to perform experiments in oocytes at temperatures higher than room temperature which is known to promote pore formation. In HEK293 cells expressing the rat $P2X_7$ receptor, at room temperature Virginio et al. [25] observed a significant pore enlargement after the application of 3 μM BzATP within <20 s. Under their experimental conditions, 30 μM BzATP induced membrane blebbing and membrane disruption within 30 s. We can certainly not exclude that during very long (lasting several minutes) applications of ATP an enlargement of the $hP2X_7$ channel

pore also takes place in oocytes. But the application of 1 mM ATP for 2 h to unclamped oocytes expressing $hP2X_7$ never resulted in the cytolysis of the oocytes. Similarly, the survival rate of oocytes injected with $P2X_7$ cRNA was not obviously shorter than that of water-injected oocytes.

Therefore, like the rat $P2X_7$ [21], the human $P2X_7$ itself seems to be unable to generate large pores in *Xenopus* oocytes at least under our experimental conditions. It is possible that further components are involved in the induction of non-selective pores in mammalian cells.

However, if the extracellular solution contained 1 mM Ba^{2+}, we observed in oocytes extended deactivation components which were also described in mammalian cells expressing $P2X_7$. Additionally, we measured under these conditions a change from inward to outward rectification as well as a shift of the reversal potential to values in the range of −28 to −12 mV which is close to the reported reversal potential for Cl^--currents in *Xenopus* oocytes [30]. Because, like lymphocytic P2Z receptors [5,6], $hP2X_7$ channels are highly permeable to Ca^{2+} [31] and obviously also to Ba^{2+} (see Fig. 5) a Ba^{2+}-induced Cl^--conductance may be responsible for the additional current component. It is not clear if this conductance corresponds to ICl_{Ca} which could be transiently activated by Ca^{2+}-entry through voltage-dependent Ca^{2+}-channels. ICl_{Ca} could not be evoked by depolarization of the oocytes if Ca^{2+} was replaced by Ba^{2+} [15]. However, the depolarization-induced inward current is transient whereas the activation of $hP2X_7$ in oocytes might maintain a Ba^{2+}-influx for several seconds which could be sufficient to induce ICl_{Ca}.

The reversal potential of $hP2X_7$-dependent currents was found at about 0 mV in this study. This is in accordance with reports on $P2X_{1-4}$-subtypes [31] and the results point to an approximately equal permeability for Na^+ and K^+. Contrary to these findings, a reversal potential of –20 mV was found for rat $P2X_7$ expressed in oocytes [21]. This refers to different permeation characteristics of rat and human $P2X_7$-receptors. Substitution of extracellular Na^+ by $choline^+$ shifted the reversal potential by about −60 mV. This indicates that $hP2X_7$ receptors are scarcely permeable to this organic cation (molecular weight of 121 Da). This corresponds to other measurements on heterologously expressed $P2X_{1-4}$-subtypes [31] and to the shift of +53 mV observed in human B lymphocytes when intracellular Na^+ was replaced by $Tris^+$ (MW also 121 Da) [6]. Therefore, the permeation characteristics of P2Z in human B cells and $hP2X_7$ expressed in oocytes are similar to those of other P2X subtypes but differ from $hP2X_7$ heterologously expressed in mammalian cells and rat $P2X_7$ expressed in oocytes.

4.4. Prospects

The $hP2X_7$ receptor expressed in oocytes does not match the lymphocytic P2Z completely. One reason for these differences may be the non-mammalian expression system for $hP2X_7$ possibly differing in additional components necessary for the function of the lymphocytic P2Z receptor. Another reason could be inferred from the fact that P2X receptors are probably composed of three submits [32]. It is therefore conceivable that heteropolymerization of the $hP2X_7$ receptor with $hP2X_1$ and $hP2X_4$ subunits additionally found in human B lymphocytes (preliminary results) may produce ATP-gated currents that are different from currents carried by pure $hP2X_7$ subunits. Heteropolymerization of P2X subunits has already been observed in neurons ([33], for example). Further experiments like coexpression of different hP2X subunits together with single channel measurements are necessary for the detailed characterization of the structure and subunit assembly of the human lymphocytic P2Z receptor. The characterization of the investigated single $hP2X_7$ subtype may help in future experiments to reveal possible new characteristics of such putative ATP-gated channels.

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