Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 470 (2003) 1–7
www.elsevier.com/locate/ejphar

NF449, a novel picomolar potency antagonist at human $P2X_1$ receptors

Martin Hülsmann[a], Peter Nickel[b], Matthias Kassack[b], Günther Schmalzing[c], Günter Lambrecht[d], Fritz Markwardt[a,*]

[a] *Julius-Bernstein-Institute for Physiology, Martin-Luther-University Halle, Magdeburger Straße 6, D-06097 Halle/Saale, Germany*
[b] *Department of Pharmaceutical Chemistry, University of Bonn, An der Immenburg 4, D-53121 Bonn, Germany*
[c] *RWTH Aachen, Department for Molecular Pharmacology, Wendlingweg 2, D-52074 Aachen, Germany*
[d] *Department of Pharmacology, Biocentre Niederursel, University of Frankfurt, Marie-Curie-Straße 9, D-60439 Frankfurt/Main, Germany*

Received 6 January 2003; received in revised form 16 April 2003; accepted 23 April 2003

Abstract

The antagonistic effects of the novel suramin analogue 4,4′,4″,4‴-(carbonylbis(imino-5,1,3-benzenetriylbis(carbonylimino)))tetrakis-benzene-1,3-disulfonic acid (NF449) were analyzed at homomeric human $P2X_1$ and $P2X_7$ receptor subtypes ($hP2X_1$ and $hP2X_7$) heterologously expressed in *Xenopus* oocytes using the two-microelectrode voltage-clamp technique. At activating ATP concentrations of 1 μM ($hP2X_1$) and 100 μM ($hP2X_7$), IC_{50} values of 0.05 nM and 40 μM were found for $hP2X_1$ and $hP2X_7$ receptors, respectively. The Schild analysis revealed a pA_2 of 10.7 at $hP2X_1$. Wash-in and wash-out of 10 nM NF449 were nearly complete within 16 s and 4 min, respectively, at the $hP2X_1$ receptor. An increase in the activating ATP concentration to 100 μM shifted the NF449 concentration–inhibition curve rightwards for the $hP2X_1$ receptor. NF449 decelerated activation as well as desensitization of $hP2X_1$. It is concluded that NF449 acts as a reversible competitive antagonist at the $hP2X_1$ with much higher potency at $hP2X_1$ than at $hP2X_7$ receptors. NF449 may hence be excellently suited to discriminate between both receptors in native human tissues.
© 2003 Elsevier Science B.V. All rights reserved.

Keywords: ATP receptor; Oocyte; Antagonist; Voltage clamp; Whole cell; NF449 (4,4′,4″,4‴-(carbonylbis(imino-5,1,3-benzenetriylbis(carbonylimino))) tetrakis-benzene-1,3-disulfonic acid)

1. Introduction

Receptors for extracellular adenine and uracil nucleotides have been classified as P2 receptors (Ralevic and Burnstock, 1998). According to the signal transduction mechanisms, the P2X receptor subtypes constitute a class of ligand-gated cation channels, while the P2Y subtypes are G-protein-coupled metabotropic receptors. To date, seven P2X receptor subunits ($P2X_{1-7}$) have been identified, functionally characterized and their transcript distribution has been described (Khakh et al., 2001; North, 2002). Native cell types express multiple P2Y receptors and P2X subunits (Jabs et al., 2000; Adrian et al., 2000; Nörenberg and Illes, 2000; Lewis and Evans, 2001; Klapperstück et al., 2000b) and heterooligomerization of different P2X subunits may form P2X receptors with distinctive physiological and pharmacological profiles (Lewis et al., 1995; Torres et al., 1999; Liu et al., 2001; Brown et al., 2002; North, 2002). Therefore, selective agonists or antagonists of certain P2X receptor subtypes would be helpful to relate nucleotide-induced physiological responses in native cells and tissues to distinct P2X receptors (Jacobson et al., 2002; Lambrecht et al., 2002; North, 2002). Recently, it has been shown that the suramin analogue 8,8′-(carbonylbis(imino-3,1-phenylenecarbonylimino)bis(1,3,5-naphthalenetrisulfonic acid) (NF023) is able to discriminate between $P2X_1$, $P2X_2$, $P2X_3$ and $P2X_4$ subtypes (Soto et al., 1999). A further suramin analogue, 8,8′-(carbonylbis(imino-4,1-phenylene-carbonylimino-4.1-phenylenecarbonylimino))bis(1,3,5-naphthalenetrisulfonic acid) (NF279) has been shown to be a wholly selective $P2X_1$ receptor antagonist at native and recombinant P2 receptors being 13-fold more potent than NF023 at cloned $rP2X_1$ (Damer et al., 1998; Lambrecht, 2000; Klapperstück et al., 2000b; Rettinger et al., 2000). Very recently, we reported on the pharmacological profile of

* Corresponding author. Tel.: +49-345-55-71390; fax: +49-345-55-27899.
E-mail address: fritz.markwardt@medizin.uni-halle.de (F. Markwardt).

0014-2999/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0014-2999(03)01761-8

the new antagonist 4,4′,4″,4‴-(carbonylbis(imino-5,1,3-benzenetriylbis(carbonylimino)))tetrakis-benzene-1,3-disulfonic acid (NF449) at P2 receptors. This compound represents the backup derivative for its first-runners NF023 and NF279. NF449 was shown to possess a subnanomolar potency at recombinant rat $P2X_1$ receptors with the following rank order at recombinant (rat) and native (rat vas deferens, guinea-pig ileum) P2 receptors: P2X1>>P2X3>P2Y1>P2Y2 (Lambrecht et al., 2002; Braun et al., 2001).

The aim of the present study was to characterize the effect of NF449 on recombinant human $P2X_1$ and $P2X_7$ receptors expressed in *Xenopus* oocytes to get further information about the selectivity and antagonistic mechanism of the drug on human P2X receptor subtypes.

2. Materials and methods

2.1. Materials

Chemicals were obtained from Sigma (Deisenhofen, Germany) if not otherwise stated. The method for synthesis of NF449 as octasodium salt has been described previously (Hohenegger et al., 1998).

2.2. cDNA constructs and cRNA synthesis

cDNA constructs encoding the $hP2X_1$ and $hP2X_7^*$ subunits have been described previously (Klapperstück et al., 2000b). The $hP2X_7^*$ cDNA was isolated from an immortalized human B lymphocyte cell line and carries two amino acid exchanges ($Gly^{441}Arg$ and $Ala^{496}Glu$) as compared to published sequences (accession numbers Y09561 and Y12851-5). Gly^{441} and Ala^{496} were sequentially replaced by Arg and Glu, respectively, using the QuikChange site-directed mutagenesis kit (Stratagene, Heidelberg, Germany), to generate a $hP2X_7$ construct identical with the one described earlier by others (Rassendren et al., 1997). Capped cRNAs were synthesized from linearized templates with SP6 RNA polymerase (Pharmacia) and purified as described (Schmalzing et al., 1991). For oocyte injection, the cRNAs were dissolved in 5 mM Tris/HCl, pH 7.5 at 0.5 μg/μl, and quantified using optical density reading at 260 nm (OD 1.0 = 40 μg/μl).

2.3. Oocyte treatment and electrophysiology

The preparation of *Xenopus laevis* oocytes, injection of cRNA and the protocols for the measurement of ATP-induced $hP2X_1$- and $hP2X_7$-dependent whole-cell currents were performed as described before (Klapperstück et al., 2000a,b, 2001).

The experiments followed European Community guidelines for the use of experimental animals and have been approved by the local animal protection authority. Briefly, membrane currents were measured by the two-microelectrode voltage-clamp method. All experiments were carried out at room temperature (≈22 °C). Currents were measured 1 or 2 days after injection of 20 nl of cRNA (0.2 mg/ml). A 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 (Bretschneider and Markwardt, 1999). Measurements of P2X receptor-dependent currents were carried out in a bathing solution consisting of (mM): 100 NaCl, 2.5 KCl, 10 HEPES, pH 7.4, adjusted with NaOH. Ca^{2+} was omitted to avoid the activation of endogenous currents by a Ca^{2+} influx through the $P2X_7$ receptor channels. Mg^{2+} was omitted to prevent the Mg^{2+} complexation of ATP^{4-}, since otherwise the high ATP concentrations required for maximal activation of the $hP2X_7$ receptor would have been insoluble (Di Virgilio, 1995; Markwardt et al., 1997). This means that the concentrations of the total ATP and of the free ATP^{4-} are approximately equal in these solutions. The removal of divalent cations from the extracellular solution evoked a large conductance, which could be blocked by 0.1 mM flufenamic acid (Weber et al., 1995; Zhang et al., 1998). Currents were recorded at an holding potential of −40 mV and filtered at 100 Hz using an oocyte clamp amplifier (OC-725C, Warner Instrument, 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).

For the detailed analysis of the concentration-dependent effect of NF449 on the two subtypes of human P2X receptors, different concentrations of the antagonist were applied in random order. For the effects on $hP2X_1$ receptors, ATP was administered at the start of the experiment, three times for 6 s at an interval of 5 min. The third current response was taken as control current ($I_{P,cont}$). This procedure was chosen because pilot experiments showed that application of ATP at intervals of 5 min gave reproducible currents with amplitude variations <10%.

The $P2X_1$ receptor-dependent current I_P was measured as the peak current minus the holding current before agonist application. To account for the various degree of $hP2X_1$ receptor expression, the $P2X_1$-dependent current at different NF449 concentrations evoked by a certain concentration of ATP, I_P([ATP, NF449]), was normalized to the control $P2X_1$ receptor-dependent current $I_{P,cont}$ evoked by ATP in NF449-free solution (see Eq. (2)). To overcome the problem that application of ATP together with NF449 causes only a fraction of the total $P2X_1$ receptor pool to activate and subsequently to desensitize, a 6-s-lasting pulse of 1 or 100 μM ATP in NF449-free solution was always applied immediately before the 5-min-lasting recovery period to induce all $P2X_1$ receptors to enter the desensitized state. Accordingly, no other $P2X_1$ receptors than those which recovered from the desensitized state during the 4-min recovery period

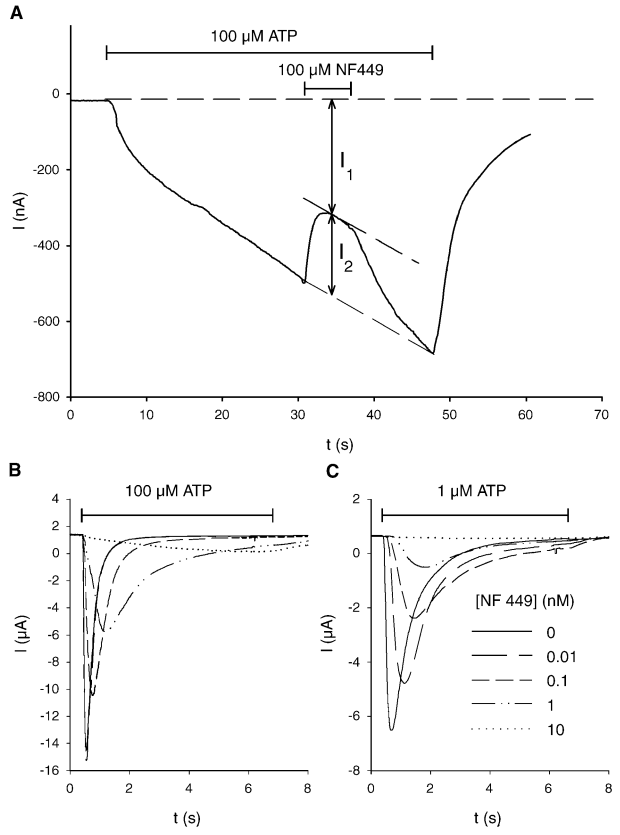

Fig. 1. Effect of NF449 on whole cell currents mediated by $hP2X_1$ and $hP2X_7$ receptors. (A) Principle and example of measurement of NF449 effects on $hP2X_7$-dependent currents. (B, C) Typical examples for the effect of NF449 on $hP2X_1$-dependent currents. Original current traces recorded from two different oocytes, which were first preincubated for 30 s with the indicated NF449 concentrations and then superfused with the same NF479 solution supplemented with 100 µM (A) or 1 µM ATP (B).

were available for activation by the next test pulse of ATP. The next control current (without NF449) and the following NF449-dependent current were then elicited at 5-min intervals.

Because of the low potency of NF449 at the $hP2X_7$ receptor, another protocol had to be chosen to avoid the consumption of large amounts of the drug. During sustained linearly increasing activation of $hP2X_7$ receptors (Klapperstück et al., 2000a), NF449 was washed in until the decrease of the inward current ceased and again a nearly linear current increase was reached. Some seconds later, NF449 was washed out for 15–30 s (see Fig. 1A).

Nonlinear approximations and presentation of data were performed using the program Sigmaplot (Jandel, Corte Madeira, USA). Averaged data are given as means ± S.E.M. if not otherwise stated. Statistical data were analyzed by one-way repeated measures analysis of variance. Statistical significance of differences between means was tested using an multiple comparison method versus control group procedure (Bonferroni) by the program Sigmastat (Jandel). Significance was taken at $P < 0.05$.

3. Results

3.1. Antagonism by NF449 on human $P2X_1$ and $P2X_7$ receptors

3.1.1. $hP2X_7$

Fig. 1A demonstrates an example of measuring the blocking effect of NF449 on $hP2X_7$ receptors. The $P2X_7$-dependent current at a distinct NF449 concentration (I_1) was normalized to the linearly extrapolated current without NF449 application at the same time ($I_1 + I_2$):

$$I_{\mathrm{rel}}([\mathrm{NF449}], \mathrm{P2X_7}) = \frac{I_1([\mathrm{NF449}])}{I_1 + I_2} \quad (1)$$

Within less than 5 s, the blocking effect of NF449 reached a steady state level, at which the $P2X_7$-dependent current again continued to increase linearly. At this time, the NF449-dependent current I_1 was measured. The wash-out of the drug seemed to be completed within about 10 s, when the current reached the level extrapolated for the unblocked current without NF449 application.

The concentration-dependent effect of NF449 on $hP2X_7$ was approximated according to a Hill equation (see Eq. (3) with d assumed to be 0). A $\log IC_{50}$ value of 4.4 ± 0.1 was determined corresponding to an IC_{50} value of 40 µM.

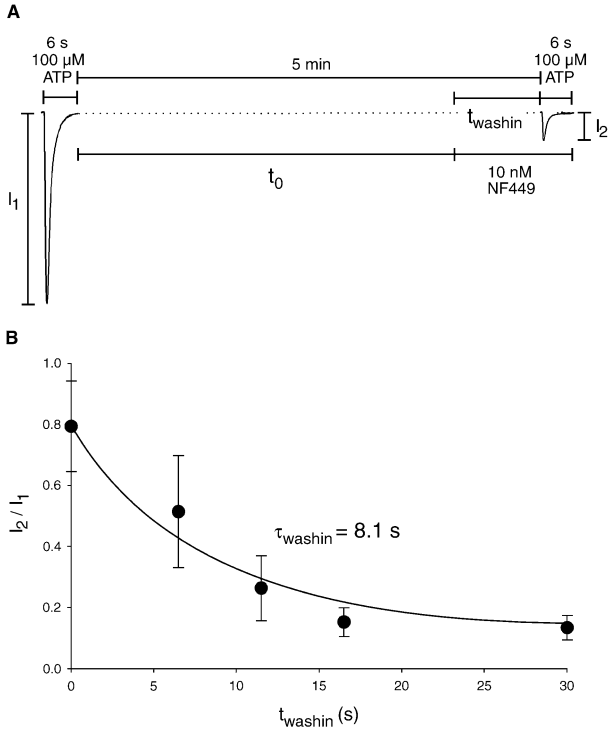

Fig. 2. Kinetics of wash-in of NF449. (A) Principle of measurement of the effect of different wash-in times ($t_{\mathrm{wash\text{-}in}}$) of 10 nM NF449 on $hP2X_1$-dependent currents. (B) Dependence of mean relative $hP2X_1$-dependent currents I_2/I_1 on $t_{\mathrm{wash\text{-}in}}$ ($N = 6$ oocytes). The time constant of the wash-in effect $\tau_{\mathrm{wash\text{-}in}}$ shown in the legend was determined by an exponential fit of the dependence of $I_2/I_1(t_{\mathrm{wash\text{-}in}})$.

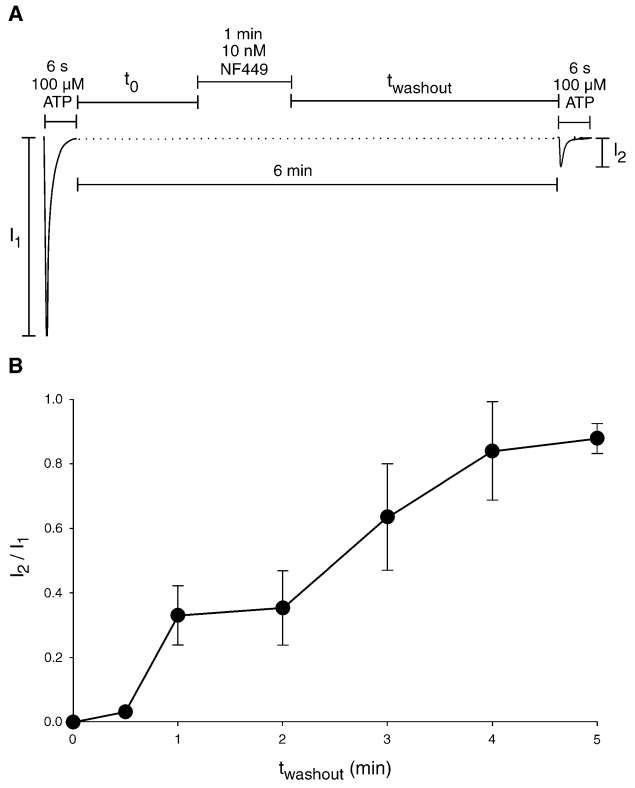

Fig. 3. Kinetics of wash-out of NF449. (A) Principle of measurement of the effect of different wash-out times ($t_{wash\text{-}out}$) of 10 nM NF449 on hP2X$_1$-dependent currents. (B) Dependence of mean relative hP2X$_1$-dependent currents I_2/I_1 on $t_{wash\text{-}out}$ ($N=6$ oocytes).

3.1.2. hP2X$_1$

The effect of NF449 on hP2X$_1$ was tested by preincubation of *Xenopus* oocytes expressing this receptor in divalent free extracellular solutions with different concentrations of the drug over a period of 1 min and subsequent activation of P2X$_1$-dependent currents by 1 or 100 µM ATP (see Materials and methods). As shown in Fig. 1B and C, the degree of block of the currents was dependent on the ATP concentration used to activate the receptor. To quantify this observation, the normalization of the currents was achieved by:

$$I_{rel}([NF449], P2X_1) = \frac{I_P([ATP, NF449])}{I_{P,cont}} \quad (2)$$

The time necessary to complete wash-in of NF449 was determined according to the protocol shown in Fig. 2A.

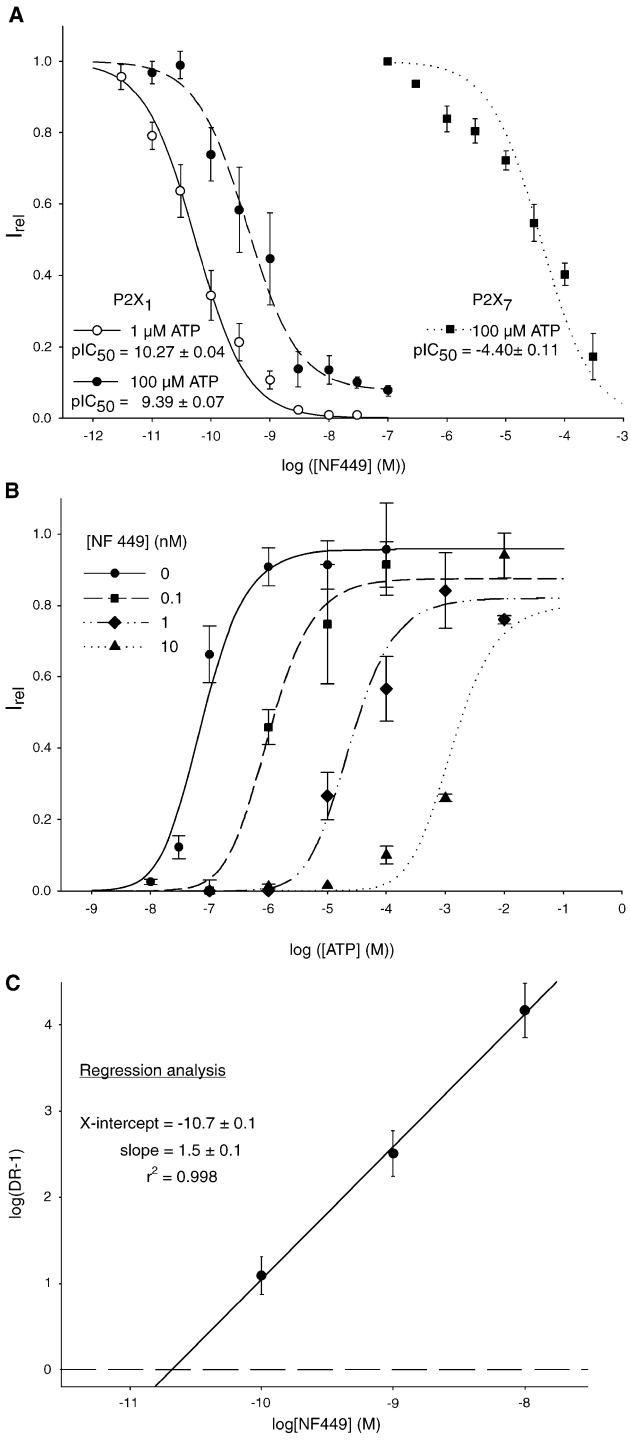

Fig. 4. Concentration dependent effect of NF449 on hP2X$_1$. (A) Concentration–inhibition curves for the blocking effect of NF449 on peak hP2X$_1$- and hP2X$_7$-dependent currents. The currents were evoked by application of ATP concentrations as indicated according to Fig. 1. The currents were normalized to the ATP-induced current without NF449 application (Eq. (2)). Means from seven (1 and 100 µM ATP, P2X$_1$) or six (P2X$_7$) oocytes are shown. The data for the relative ATP-induced currents I_{rel} were fitted according to Eq. (3). (B) Effect of NF449 on the concentration–response relation for ATP on hP2X$_1$-dependent currents. Peak currents were normalised to the currents evoked by 0.1 mM ATP 5 min before (Eq. (2)). The data were fitted according to Eq. (4):

$$I_{ATP,rel} = \frac{a}{\left(1 + \frac{10^{\log K_{ATP}}}{[ATP]}\right)^n} \quad (4)$$

where K_{ATP} is the apparent dissociation constant of ATP at hP2X$_1$ and a characterises the maximal relative current which was found to be not significantly dependent on [NF449]. The highest correlations were obtained using a uniform Hill coefficient of 2 for all four tested NF449 concentrations. (C) Schild plot of the data shown in B according to the approximated K_{ATP} values.

Without preincubation, the current elicited by simultaneous application of 10 nM NF449 and 100 μM ATP was reduced by only about 20% compared to the control current measured 5 min before. Within about 16 s, the effect of the wash-in reached a steady state (Fig. 2B). Therefore, the usual time of preincubation with NF449 was set to 1 min for measurement of concentration–effect curves.

The time course of the wash-out of the blocking effect of NF449 was measured according to the protocol illustrated in Fig. 3A. Like for all other measurements of $P2X_1$-dependent currents, the time interval between two ATP applications was set to 5 min to take the long-lasting desensitization into account. The wash-out reached a steady state value of 90% of the control value after about 4 min (Fig. 3B).

To determine the concentration-dependent effects of NF449 on $hP2X_1$, the relative peak currents I_{rel} (Eqs. (1) and (2), see also Materials and methods) were plotted against the concentration of the drug as shown in Fig. 4. The data were fitted according to Eq. (3):

$$I_{NF449,rel} = \frac{1-d}{1+\left(\frac{[NF449]}{10^{\log IC_{50}}}\right)^{n}} + d \qquad (3)$$

where IC_{50} is the NF449 concentration required for half maximal current block and d is the remaining ATP-induced current at infinite NF449 concentrations. By testing only for natural numbers of n, the best fits of $P2X_1$-dependent current amplitudes were achieved with a Hill coefficient of 1. For the $P2X_1$ receptor, the approximations for 1 and 100 μM ATP yielded $\log IC_{50}$ values (means ± S.E.M.) of -10.27 ± 0.04 and -9.39 ± 0.07, which are significantly different from each other. The approximated value of d was 0.01 ± 0.01 and 0.08 ± 0.03 for 1 and 100 μM ATP, respectively.

The shift of the NF449 concentration–response curve by ATP implicates a possible competitive effect of NF449. To test this further, ATP concentration–response curves were measured at different concentrations of NF449. As shown in Fig. 4B, the ATP concentration–response curves were significantly shifted rightwards by increasing NF449 concentrations. By the Schild analysis (Fig. 4C), a pA_2 value of 10.7 was approximated.

3.2. Effect of NF449 on receptor kinetics

As demonstrated in Fig. 1B and C, NF449 seemed to slow down the time course for activation and inactivation of $hP2X_1$. Therefore, for a formal description of the kinetics of $hP2X_1$, a Hodgkin–Huxley-like model of independent activation and desensitization was used:

$$I_{P2X_1}(t) = I_{act}\left(1 - e^{-\frac{t}{\tau_{act}}}\right)\left(ND + e^{-\frac{t}{\tau_{des}}}\right) + I_0 \qquad (5)$$

where I_{act} is the activating current, I_0 is the steady-state current without ATP application, ND is the non-desensitiz-

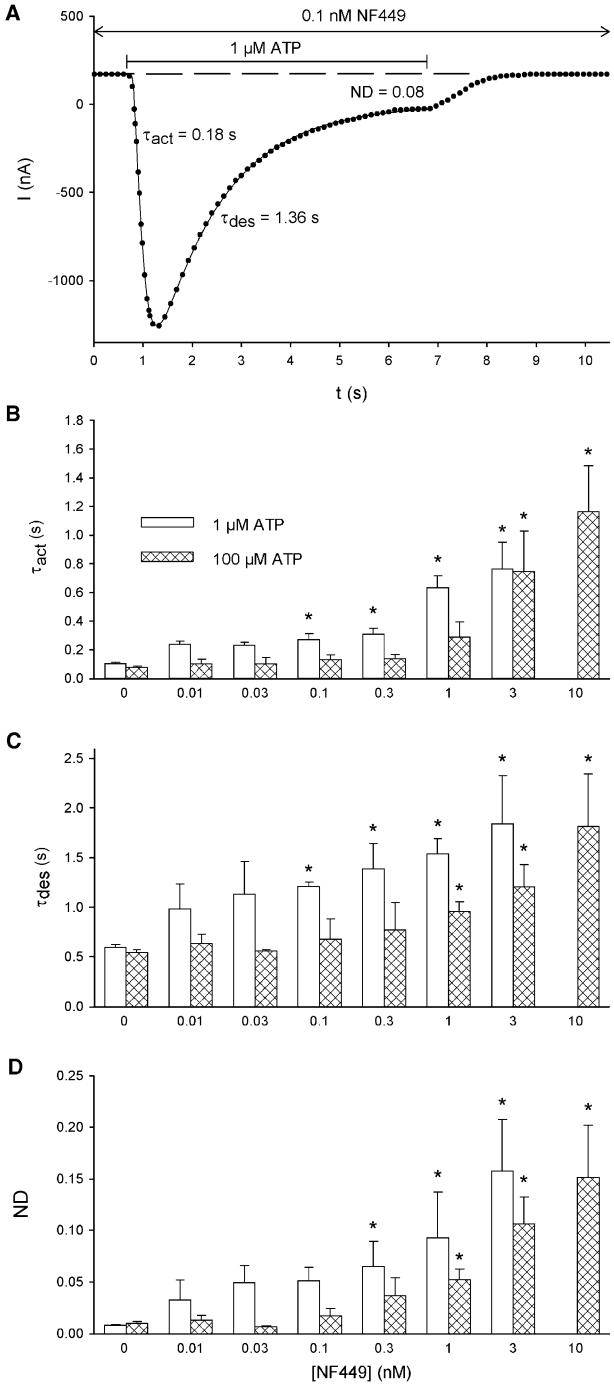

Fig. 5. Effect of NF449 on kinetics of $hP2X_1$-dependent currents. (A) Example of approximation of $hP2X_1$ receptor kinetics according to Eq. (3). NF449 (0.1 nM) was added to the bath 30 s prior to ATP application. The measured current is shown as circles and the approximation is drawn as line. Fitted parameters are shown as inset. (B–D) Statistical analysis of the dependence on the NF449 concentration of the activation time constant τ_{act} (B), the desensitization time constant τ_{des} (C), and the grade of non-desensitizing current ND (D) for currents induced by application of 1 μM (open bars) or 100 μM ATP (cross-hatched bars). Means that are significantly different from the control values are marked by asterisks. Means are data from four to seven different oocytes.

ing part of the activating current, and τ_{act} and τ_{des} are the time constants for activation and desensitization, respectively. Fig. 5B and C reveals a deceleration of both activation and desensitization of $hP2X_1$ by NF449. With the low ATP concentration of 1 μM ATP used for activation of $P2X_1$, the increase of the time constants for activation and desensitization occurred at lower NF449 concentrations than with 100 μM ATP. Similarly, the effect of NF449 to increase the non-desensitizing part of the $P2X_1$-dependent current was found with 1 μM ATP at lower NF449 concentrations compared to 100 μM activating ATP (Fig. 5D).

4. Discussion

The present study demonstrates a very high inhibitory potency of the novel suramin analogue NF449 towards the $hP2X_1$ receptor ($IC_{50} = 50$ pM for [ATP] = 1 μM). The recombinant $hP2X_1$ receptor seems therefore to be even more sensitive to NF449 than the rat homologue, for which an IC_{50} of 290 pM NF279 was found at activating ATP concentrations of 1 μM (Braun et al., 2001). The variance may be due to differences in the protein structure of the rat and human $P2X_1$ receptor. On the other hand, for another suramin analogue, NF023, similar IC_{50} values of 0.24 and 0.21 μM have been found for the rat and human $P2X_1$ homologue, respectively, heterologously expressed in *Xenopus* oocytes (Soto et al., 1999). NF279, another potent $P2X_1$ receptor antagonist, was even found to be more potent at $rP2X_1$ than at the $hP2X_1$ with IC_{50} values of 19 and 50 nM, respectively, for activating ATP concentrations of 1 μM (Klapperstück et al., 2000b; Rettinger et al., 2000).

The P2 receptor selectivity profile of NF449 is quantitatively and qualitatively unique and different from that of other known P2X receptor antagonists with nanomolar potency at recombinant $P2X_1$ receptors such as 2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP (TNP-ATP) and diinosine pentaphosphate (IP_5I). Although not yet investigated in detail, TNP-ATP and IP_5I seem to be of limited use in whole tissue experiments or under in vivo conditions due to their breakdown by ecto-nucleotidases whereas NF449 has a inhibitory potency at these enzymes (Lambrecht, 2000; Jacobson et al., 2002; Lambrecht et al., 2002).

We had to take into account that extracellular divalent cations reduce the concentration of free ATP^{4-}, the agonist at $P2X_7$ and $P2X_7$-like native P2X receptors, for example, in human B lymphocytes (Markwardt et al., 1997). Therefore, in our measurements, we omitted extracellular divalent cations like Mg^{2+} and Ca^{2+} to activate $hP2X_1$ and $hP2X_7$ receptors under identical conditions, i.e. to obtain comparable activating ATP concentrations on $hP2X_1$ and $hP2X_7$ receptors. The development of large ionic currents was prevented by adding flufenamic acid to the bath (see also Materials and methods). A significant influence of Mg^{2+} and flufenamic acid on $hP2X_1$- and $hP2X_7$-dependent current amplitude and kinetics and on the effect of NF449 on $hP2X_1$ seems unlikely because earlier investigations revealed no effect of Mg^{2+} on the block by NF279 of $hP2X_1$ receptors (Klapperstück et al., 2000b).

Three main findings point to a competition between NF449 and ATP at the agonist binding site at $P2X_1$ receptors. (i) Although the used method for solution exchange is probably too slow for an exact description of the kinetics of the $hP2X_1$ receptor, our data indicate that NF449 slows down the activation. (ii) Although the relationship between pIC_{50} and agonist concentration is not a simple linear one, for a competitive antagonist, the pIC_{50} should decrease as the agonist concentration is increased. Indeed, IC_{50} values for NF449 inhibition of $hP2X_1$ receptor-mediated currents varied eightfold whether currents were evoked by 1 ($pIC_{50} = 10.3$) or 100 μM ($pIC_{50} = 9.4$) ATP, respectively. (iii) The Schild analysis revealed a significant rightward shift of the ATP concentration–response curve by increasing concentrations of NF449. The same conclusion was already drawn for the action of NF279 and NF449 on smooth muscle preparations (Damer et al., 1998; Lambrecht, 2000; Braun et al., 2001) and recombinant $hP2X_1$ receptors (Klapperstück et al., 2000b) as well as for the effect of NF023 on rat and human $P2X_1$ (Soto et al., 1999).

NF449 also decelerates or even reduces (Fig. 5C and D, respectively) the desensitization of $hP2X_1$ receptors. This means that binding of NF449 not only blocks activation but also the desensitization of the $hP2X_1$ receptor. The effect of NF449 on both activation and desensitization kinetics could be antagonized by increasing the activating ATP concentration. Accordingly, currents which are activated by different concentrations of NF449 and ATP but with about the same amplitude have similar activation and desensitisation time constants (compare in Figs. 4 and 5 pairs of mean currents activated by 0.03 nM NF449/1 μM ATP and 0.3 NF/100 ATP, 0.1 NF/1 ATP and 1 NF/100 ATP, or 1 NF/1 ATP and 10 NF/100 ATP, respectively). Thus, changes in τ_{act} and τ_{des} are likely to simply reflect the differences in receptor occupancy in the absence and presence of NF449. This is reminiscent to the effects of NF279, which were interpreted as an action of NF279 on the ATP binding site only and the assumption that desensitization of $P2X_1$ is only possible after its activation (Klapperstück et al., 2000b).

Wash-in and wash-out experiments showed that NF449 is a reversible blocker at $hP2X_1$ receptors. Simultaneous application of NF449 and ATP resulted only in a small block of the channels. Therefore, a preincubation period for the cells under investigation of at least 30 s should be used. The wash-out reached a steady state value after about 4 min. After this time span, ATP-induced currents reached about 90% of the control value measured before. It remains unclear if the incomplete "wash-out" was due to a persisting drug effect, a long-lasting desensitization or a run-down of $P2X_1$ channel activity. Longer time intervals between ATP applications to $P2X_1$ would possibly have reduced part of these effects, but on the other hand would have resulted in very long time for the experiments.

Also, we show that NF449 is a poor blocker of $hP2X_7$ receptors. The potency at the $hP2X_7$ receptor is even lower than for NF279, another suramin analogue highly potent at $P2X_1$ receptors. This latter compound blocked the $hP2X_7$ receptor with an IC_{50} of 7 μM at the same activating ATP^{4-} concentration of 100 μM (Klapperstück et al., 2000b). Because of its low potency at $hP2X_7$, NF449 is not suitable as a $P2X_7$ receptor antagonist and, therefore, the mechanism of block was not further investigated in detail. Likewise, the deviation of the concentration–response curve from a simple Hill plot remains unexplained. Possibly, it originates from the existence of two functionally different binding sites for ATP at the $hP2X_7$ receptor (Klapperstück et al., 2001). Already, the effects of NF279 pointed to the fact that the activated $P2X_7$ receptor may exist in two different states and that the one of them which deactivates fast is preferentially blocked by this compound.

In conclusion, our results demonstrate that NF449 is an extremely potent and reversible antagonist at the $hP2X_1$ receptor. Considering additionally its low potency at $hP2X_7$, NF449 is a very useful tool for pharmacological discrimination between $P2X_1$- and $P2X_7$-mediated effects in native tissues, which express several P2X receptor subunits such as human B lymphocytes, in which messages for $P2X_1$, $P2X_4$ and $P2X_7$ were found (Klapperstück et al., 2000b).

Acknowledgements

This work was supported by grants of the Deutsche Forschungsgemeinschaft (La350/7-3, Schm536/2-3; Schm536-4; GRK 137/2 and 137/3) and the Fonds der Chemischen Industrie (Germany).

References

Adrian, K., Bernhard, M.K., Breitinger, H.G., Ogilvie, A., 2000. Expression of purinergic receptors (ionotropic P2X1-7 and metabotropic P2Y1-11) during myeloid differentiation of HL60 cells. Biochim. Biophys. Acta 1492, 127–138.

Braun, K., Rettinger, J., Ganso, M., Kassack, M., Hildebrandt, C., Ullmann, H., Nickel, P., Schmalzing, G., Lambrecht, G., 2001. NF449: a subnanomolar potency antagonist at recombinant rat $P2X_1$ receptors. Naunyn-Schmiedeberg's Arch. Pharmacol. 364, 285–290.

Bretschneider, F., Markwardt, F., 1999. Drug-dependent ion channel gating by application of concentration jumps using U-tube technique. Methods Enzymol. 294, 180–189.

Brown, S.G., Townsend-Nicholson, A., Jacobson, K.A., Burnstock, G., King, B.F., 2002. Heteromultimeric $P2X_{1/2}$ receptors show a novel sensitivity to extracellular pH. J. Pharmacol. Exp. Ther. 300, 673–680.

Damer, S., Niebel, B., Czeche, S., Nickel, P., Ardanuy, U., Schmalzing, G., Rettinger, J., Mutschler, E., Lambrecht, G., 1998. NF279: a novel potent and selective antagonist of P2X receptor-mediated responses. Eur. J. Pharmacol. 350, 5–6.

Di Virgilio, F., 1995. The P2Z purinoceptor: an intriguing role in immunity, inflammation and cell death. Immunol. Today 16, 524–528.

Hohenegger, M., Waldhoer, M., Beindl, W., Boing, B., Kreimeyer, A., Nickel, P., Nanoff, C., Freissmuth, M., 1998. Gs-selective G protein antagonists. Proc. Natl. Acad. Sci. U. S. A. 95, 346–351.

Jabs, R., Guenther, E., Marquordt, K., Wheeler-Schilling, T.H., 2000. Evidence for $P2X_3$, $P2X_4$, $P2X_5$ but not for $P2X_7$ containing purinergic receptors in Müller cells of the rat retina. Mol. Brain Res. 76, 205–210.

Jacobson, K.A., Jarvis, M.F., Williams, M., 2002. Purine and pyrimidine (P2) receptors as drug targets. J. Med. Chem. 45, 4057–4093.

Khakh, B.S., Burnstock, G., Kennedy, C., King, B.F., North, R.A., Seguela, P., Voigt, M., Humphrey, P.P.A., 2001. International union of pharmacology: XXIV. Current status of the nomenclature and properties of P2X receptors and their subunits. Pharmacol. Rev. 53, 107–118.

Klapperstück, M., Büttner, C., Böhm, T., Schmalzing, G., Markwardt, F., 2000a. Characteristics of $P2X_7$ receptors from human B lymphocytes expressed in *Xenopus* oocytes. Biochim. Biophys. Acta 1467, 444–456.

Klapperstück, M., Büttner, C., Nickel, P., Schmalzing, G., Lambrecht, G., Markwardt, F., 2000b. Antagonism by the suramin analogue NF279 on human $P2X_1$ and $P2X_7$ receptors. Eur. J. Pharmacol. 387, 245–252.

Klapperstück, M., Büttner, C., Schmalzing, G., Markwardt, F., 2001. Functional evidence of distinct ATP activation sites at the human $P2X_7$ receptor. J. Physiol. (Lond.) 534, 25–35.

Lambrecht, G., 2000. Agonists and antagonists acting at P2X receptors: selectivity profiles and functional implications. Naunyn-Schmiedeberg's Arch. Pharmacol. 362, 340–350.

Lambrecht, G., Braun, K., Damer, S., Ganso, M., Hildebrandt, C., Ullmann, H., Kassack, M.U., Nickel, P., 2002. Structure–activity relationships of suramin and pyridoxal-5′-phosphate derivatives as P2 receptor antagonists. Curr. Pharm. Des. 8, 2371–2399.

Lewis, C.J., Evans, R.J., 2001. P2X receptor immunoreactivity in different arteries from the femoral, pulmonary, cerebral, coronary and renal circulations. J. Vasc. Res. 38, 332–340.

Lewis, C., Neidhart, S., Holy, C., North, R.A., Buell, G., Surprenant, A., 1995. Coexpression of $P2X_2$ and $P2X_3$ receptor subunits can account for ATP-gated currents in sensory neurons. Nature 377, 432–435.

Liu, M., King, B.F., Dunn, P.M., Rong, W.F., Townsend-Nicholson, A., Burnstock, G., 2001. Coexpression of P2X3 and P2X2 receptor subunits in varying amounts generates heterogeneous populations of P2X receptors that evoke a spectrum of agonist responses comparable to that seen in sensory neurons. J. Pharmacol. Exp. Ther. 296, 1043–1050.

Markwardt, F., Löhn, M., Böhm, M., Klapperstück, M., 1997. Purinoceptor-operated cationic channels in human B lymphocytes. J. Physiol. (Lond.) 498, 143–151.

North, R.A., 2002. Molecular physiology of P2X receptors. Physiol. Rev. 82, 1013–1067.

Nörenberg, W., Illes, P., 2000. Neuronal P2X receptors: localisation and functional properties. Naunyn-Schmiedeberg's Arch. Pharmacol. 362, 324–339.

Ralevic, V., Burnstock, G., 1998. Receptors for purines and pyrimidines. Pharmacol. Rev. 50, 413–492.

Rassendren, F., Buell, G.N., Virginio, C., Collo, G., North, R.A., Surprenant, A., 1997. The permeabilizing ATP receptor, $P2X_7$—cloning and expression of a human cDNA. J. Biol. Chem. 272, 5482–5486.

Rettinger, J., Schmalzing, G., Damer, S., Müller, G., Nickel, P., Lambrecht, G., 2000. The suramin analogue NF279 is a novel and potent antagonist selective for the $P2X_1$ receptor. Neuropharmacology 39, 2044–2053.

Schmalzing, G., Gloor, S., Omay, H., Kroner, S., Appelhans, H., Schwarz, W., 1991. Up-regulation of sodium pump activity in *Xenopus laevis* oocytes by expression of heterologous beta 1 subunits of the sodium pump. Biochem. J. 279, 329–336.

Soto, F., Lambrecht, G., Nickel, P., Stühmer, W., Busch, A.E., 1999. Antagonistic properties of the suramin analogue NF023 at heterologously expressed P2X receptors. Neuropharmacology 38, 141–149.

Torres, G.E., Egan, T.M., Voigt, M.M., 1999. Hetero-oligomeric assembly of P2X receptor subunits—specificities exist with regard to possible partners. J. Biol. Chem. 274, 6653–6659.

Weber, W.M., Liebold, K.M., Reifarth, F.W., Uhr, U., Clauss, W., 1995. Influence of extracellular Ca^{2+} on endogenous Cl^- channels in *Xenopus* oocytes. Pflügers Arch. 429, 820–824.

Zhang, Y., McBride Jr., D.W., Hamill, O.P., 1998. The ion selectivity of a membrane conductance inactivated by extracellular calcium in *Xenopus* oocytes. J. Physiol. (Lond.) 508, 763–776.