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Antagonist effects on human $P2X_7$ receptor-mediated cellular accumulation of YO-PRO-1

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1 We have examined the interaction of P2 antagonists with the human $P2X_7$ receptor by studying their effect on 2′ and 3′-O-benzoyl-benzoyl-ATP (DbATP) stimulated cellular accumulation of the fluorescent, DNA binding dye, YO-PRO-1 (MW = 375Da).

2 In suspensions of HEK293 cells expressing human recombinant $P2X_7$ receptors, DbATP produced time and concentration-dependent increases in YO-PRO-1 fluorescence. This response presumably reflects YO-PRO-1 entry through $P2X_7$ receptor channels and binding to nucleic acids. When studies were performed in a NaCl-free, sucrose-containing buffer, full concentration-effect curves to DbATP could be constructed.

3 The P2 antagonists, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) and periodate oxidized ATP (oATP), reduced the potency of DbATP and decreased its maximum response. 1-[N,O-bis(1,5-isoquinolinesulphonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN62) and its analogue, KN04, reduced the potency of DbATP. Schild slopes for KN62 and KN04 were shallow and exhibited a plateau at concentrations of compound greater than 1 μM, indicating that these compounds were not competitive antagonists.

4 Calmidazolium and a monoclonal antibody to human $P2X_7$ receptors attenuated DbATP-stimulated YO-PRO-1 accumulation but they were not competitive antagonists and only produced 2–3 fold decreases in the potency of DbATP.

5 The effects of PPADS and KN62 were partially reversible whereas those of oATP were not. PPADS protected cells against the irreversible antagonist effects of oATP suggesting a common site of action. In contrast KN62 was not effective suggesting that it may bind at a different site to oATP and PPADS.

6 This study has demonstrated that $P2X_7$ receptor function can be quantified by measuring DbATP stimulated YO-PRO-1 accumulation and has provided additional information about the interaction of P2 receptor antagonists with the human $P2X_7$ receptor.
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Keywords: $P2X_7$ receptor; KN62; YO-PRO-1; PPADS; oxidized ATP

Abbreviations: CEC, concentration-effect curve; DbATP, 2′ and 3′-O-benzoyl-benzoyl-ATP; DMSO, dimethyl sulphoxide; G418, geneticin sulphate; KN04, N-[1-[N-methyl-p-(5-isoquinolinesulphonyl)benzyl]-2-(4-phenylpiperazine)ethyl]-5-isoquinoline-sulfonamide; KN62, 1-[N,O-bis(1,5-isoquinolinesulphonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine; oATP, periodate oxidized ATP; P5P, pyridoxal 5-phosphate; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid; YO-PRO-1, quinolinium,4-[(3-methyl-2(3H)-benzoxazolylidene)methyl]-1-[3-(triemthylammonio) propyl]-diiodide

Introduction

The $P2X_7$ receptor is the most recently identified member of the P2X family of extracellular ATP-gated ion channels and is thought to represent the previously described P2Z receptor (Surprenant *et al*., 1996). Similar to other members of the P2X family, the $P2X_7$ receptor can function as a non-selective cation channel. However, with repeated or prolonged activation, the channel can dilate and become permeable to molecules with MW of 400–800 D (Surprenant *et al*., 1996), this later feature being most extensively studied for the P2Z receptor (Gomperts, 1983; Steinberg *et al*., 1987). The mechanisms underlying the process of channel dilation from a non-selective cation channel permeable to Na^+, K^+ and Ca^{2+} ions ('channel-form') to a channel permeable to molecules with molecular weights up to 800 ('large pore-form') are not understood and were originally thought to be conferred by the C-terminus region of the $P2X_7$ receptor (Surprenant *et al*., 1996). However, the process of channel dilation has recently been identified in other members of the family lacking the long C-terminus and so may be a general feature of P2X receptors (Khakh *et al*., 1999; Virginio *et al*., 1999).

Although the biophysical properties and agonist sensitivity of $P2X_7$ receptors have been extensively studied there have been few studies on the antagonist sensitivity of the recombinant $P2X_7$ receptor. Furthermore, in those studies where antagonists have been examined their effects have only been evaluated against single concentrations of agonist (Surprenant *et al*., 1996; Rassendren *et al*., 1997; Virginio *et al*., 1997; although see Chessell *et al*., 1998). We have recently identified conditions for studying the pharmacological properties of the $P2X_7$ receptor by measuring the cellular accumulation of YO-PRO-1 (quinolinium,4-[(3-methyl-2(3H)-benzoxazolylidene) methyl]-1-[3-(triemthylammonio) propyl]-, diiodide), a fluorescent, DNA binding dye with a MW of 375 Da (Michel *et al*., 1999b). The method is based upon the presumed ability of YO-PRO-1 to enter through the dilated or 'large pore-form' of the $P2X_7$ receptor and to bind to

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intracellular DNA/RNA whereupon it increases its fluorescence intensity many fold. While this general method has been used to study $P2X_7$ receptor function in suspensions of cells in fluorimeter cuvettes, (Gomperts, 1983; Steinberg *et al*., 1987; Tatham *et al*., 1988, Hickman *et al*., 1994) we have found that it is possible to perform this assay using cells in a 96 well plate, thus providing a relatively simple and robust method for performing quantitative studies on the $P2X_7$ receptor. The aim of this study was therefore to examine the interaction of antagonists with the $P2X_7$ receptor using this methodology and in particular to provide additional quantitative information about the recently described $P2X_7$ receptor antagonists, KN62 (1-[N,O -bis(1,5 - isoquinolinesulphonyl) - N-methyl-L-tyrosyl]-4-phenylpiperazine) and KN04 (N-[1-[N-methyl-p-(5-isoquinolinesulphonyl)benzyl] - 2 - (4- phenylpiperazine)ethyl]-5-isoquinoline-sulphonamide) (Blanchard *et al*., 1995, Gargett & Wiley, 1997). A preliminary account of these data has been presented to the British Pharmacological Society (Michel *et al*., 1999a).

Methods

The methods for studying YO-PRO-1 accumulation have been described previously (Michel *et al*., 1999b). Briefly, HEK293 cells, stably expressing the human recombinant $P2X_7$ receptor, were grown as monolayer cultures at 37°C in a humidified atmosphere (95% air; 5% CO_2) in a 1 : 1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 nutrient mix (Gibco) which was supplemented with 10% foetal bovine serum and 0.5 mg ml^{-1} geneticin sulphate (G418). Cells were harvested in Trypsin/EDTA solution (Gibco), collected by centrifugation ($200 \times g$ for 5 min), and washed once in ice cold sucrose assay buffer before re-suspending in sucrose assay buffer at 37°C. The sucrose assay buffer comprised (in mM):- sucrose 280, KCl 5, $CaCl_2$ 0.5, glucose 10, HEPES 10, N-methyl-D-glucamine 10 with the pH adjusted to 7.4. This sucrose-containing buffer was used because the potency of DbATP for the human $P2X_7$ receptor is approximately 20 fold higher in this buffer than in NaCl-containing buffer (Michel *et al*., 1999b). As a consequence of this higher potency, it is possible to construct full concentration-effect curves (CEC) to DbATP even in the presence of antagonists that produce right-ward shifts in the CEC.

Cell suspensions were added to 96 well Costar 1/2 well plates (approximately 40,000 cells $well^{-1}$) containing the $P2X_7$ receptor agonist, 2′ and 3′-O-benzoyl-benzoyl-ATP (DbATP), and YO-PRO-1 (1 μM). Plates were incubated at 37°C and changes in YO-PRO-1 fluorescence were monitored in a Canberra Packard Fluorocount 96 well plate fluorimeter. Fluorescence signals were measured using an excitation wavelength of 485 nm and emission wavelength of 530 nm. In the majority of studies a 20 min agonist incubation period was used. When the effects of antagonists were studied, the cells were pre-incubated (see Results for pre-incubation times) with antagonist prior to DbATP addition and the antagonist was also present after DbATP addition.

In some studies, to determine the effect of delayed addition of YO-PRO-1, the cell suspension was added to varying concentrations of DbATP and preincubated for 30 or 60 min before addition of YO-PRO-1. In studies to examine the reversibility of antagonists, cells were pre-incubated with antagonist or vehicle for 30 min in a volume of 3 ml and washed by diluting 10 fold with assay buffer at 37°C, followed by centrifugation at $200 \times g$ for 5 min. The cell pellet obtained was re-suspended in 30 ml of assay buffer at 37°C and centrifuged at $200 \times g$ for 5 min. Finally, the cell pellet was re-suspended in 3 ml of assay buffer at 37°C, cells were added to plates containing DbATP and YO-PRO-1 and fluorescence was determined as described above. Using this procedure results in at least a 10,000 fold dilution of antagonist.

In studies to examine potential interactions between antagonists and periodate oxidized ATP (oATP), cells were pre-incubated for 15 min with assay buffer, KN62, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), suramin or pyridoxal 5-phosphate (P5P) before addition of either 100 μM oATP or assay buffer. Following a further 30 min incubation the cells were diluted 10 fold with assay buffer at 37°C and washed as described above before constructing a CEC to DbATP as described above.

Materials

All tissue culture reagents were from Gibco. YO-PRO-1 was obtained from Molecular Probes (Oregon). Suramin was from Bayer. PPADS was from RBI/Sigma. KN62 and KN04 were from Seikagaku (MD, U.S.A.). ATP, calmidazolium, DbATP, oATP, P5P and all other chemicals were obtained from Sigma. KN62, KN04 and calmidazolium were dissolved at 1 – 10 mM in dimethyl sulphoxide (DMSO) before dilution into assay buffer. DMSO had no effect on the CEC to DbATP at concentrations of up to 1%. A 30 mM stock solution of P5P was prepared in 5 mM Tris base and the pH adjusted to 7.4 with HCl before dilution into assay buffer. The monoclonal antibody to the human $P2X_7$ receptor has been described previously (Buell *et al*., 1998).

Data analysis

For graphical purposes the data are either presented in relative fluorescence units or as a percentage of the maximal response obtained in one of the treatment groups. In some of the studies the basal YO-PRO-1 fluorescence was substracted to obtain the specific DbATP-stimulated YO-PRO-1 fluorescence.

All statistical comparisons, determinations of initial rates and calculations of agonist EC_{50} values were performed on the raw, non-transformed data. The data are the mean $\pm$ s.e.mean of 3 – 6 experiments with each experiment being performed in duplicate. Concentration-effect curves were fitted to a four parameter logistic function using either GraphPad Prism or SigmaPlot to determine the maximum and minimum responses and to calculate the EC_{50} and Hill slope parameters. The kinetic data were fitted to a single exponential using GraphPad Prism to determine the maximal level of YO-PRO-1 accumulation as well as the rate constant and initial rate of YO-PRO-1 accumulation at each concentration of DbATP. Linear regression analysis was performed using GraphPad Prism. In experiments to determine the reversibility of antagonist effects the maximum and pEC_{50} values for the DbATP CEC were determined both in the continued presence of antagonist and in cells that had been exposed to antagonist and washed. The significance of any differences in maximum response or pEC_{50} between the groups were determined using a one way ANOVA followed by Tukey's test using GraphPad Prism.

Results

Quantitative analysis of DbATP-stimulated YO-PRO-1 accumulation

Addition of DbATP to cell suspensions of HEK293 cells expressing the human $P2X_7$ receptor caused a time- and

concentration-dependent increase in YO-PRO-1 fluorescence (Figure 1). For simplicity this will be subsequently referred to as DbATP-stimulated YO-PRO-1 accumulation. This response was not observed in non-transfected HEK293 cells even when incubations were extended to 2 h and the concentration of DbATP increased to 128 μM (data not shown). In order to provide a quantitative analysis of DbATP-stimulated YO-PRO-1 accumulation in the cells expressing the human P2X$_7$ receptor, the kinetics of the response were examined in detail. At concentrations of greater than 0.25 μM, DbATP-stimulated YO-PRO-1 accumulation occurred without detectable delay and the response exhibited an exponential rise with a maximum rate achieved at a DbATP concentration of 2 μM ($t_{1/2}$ 17$\pm$1.2 min; $n=5$). At DbATP concentrations of 0.125 and 0.25 μM, YO-PRO-1 accumulation appeared to increase after a slight delay, although the data could still be described by an exponential function. These data were quantified by estimating initial rates of YO-PRO-1 accumulation as described in the methods. The pEC$_{50}$ value determined using this approach was 6.27$\pm$0.06 and the Hill slope 3.24$\pm$0.6 (Figure 1B).

pEC$_{50}$ values were also estimated from data obtained at individual time points selected from the linear phase of the time course for DbATP stimulated YO-PRO-1 accumulation.

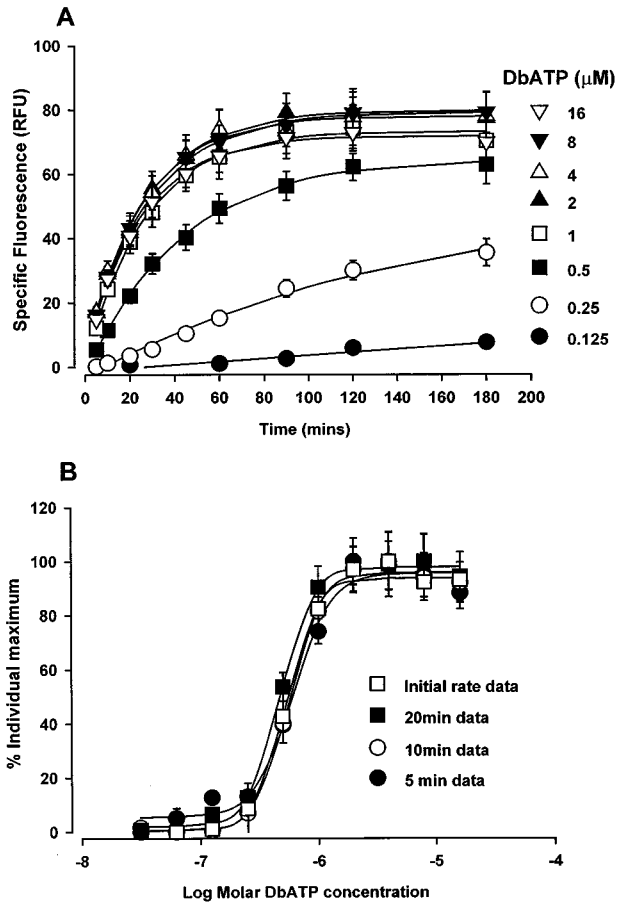

Figure 1 DbATP-stimulated increases in YO-PRO-1 fluorescence in HEK293 cells expressing the human recombinant P2X$_7$ receptor. (A) Specific YO-PRO-1 fluorescence, in relative fluorescence units (RFU), was determined at various times after addition of cell suspensions to a mixture of YO-PRO-1 and the various concentrations of DbATP. Basal fluorescence values were 11, 14, 19 and 24 RFU at 0, 60, 120 and 180 min, respectively, and were subtracted from the data. (B) Concentration-effect curves for DbATP-stimulated increases in YO-PRO-1 fluorescence in HEK293 cells expressing the human recombinant P2X$_7$ receptor were constructed using the increases in specific YO-PRO-1 fluorescence measured after 5, 10 and 20 min in A. These data are presented as a percentage of the maximum obtained at each time point. In addition the initial rates of DbATP-stimulated YO-PRO-1 accumulation were estimated from the data in A and have been presented as a percentage of the maximum initial rate. The data are the mean$\pm$s.e.mean of five experiments.

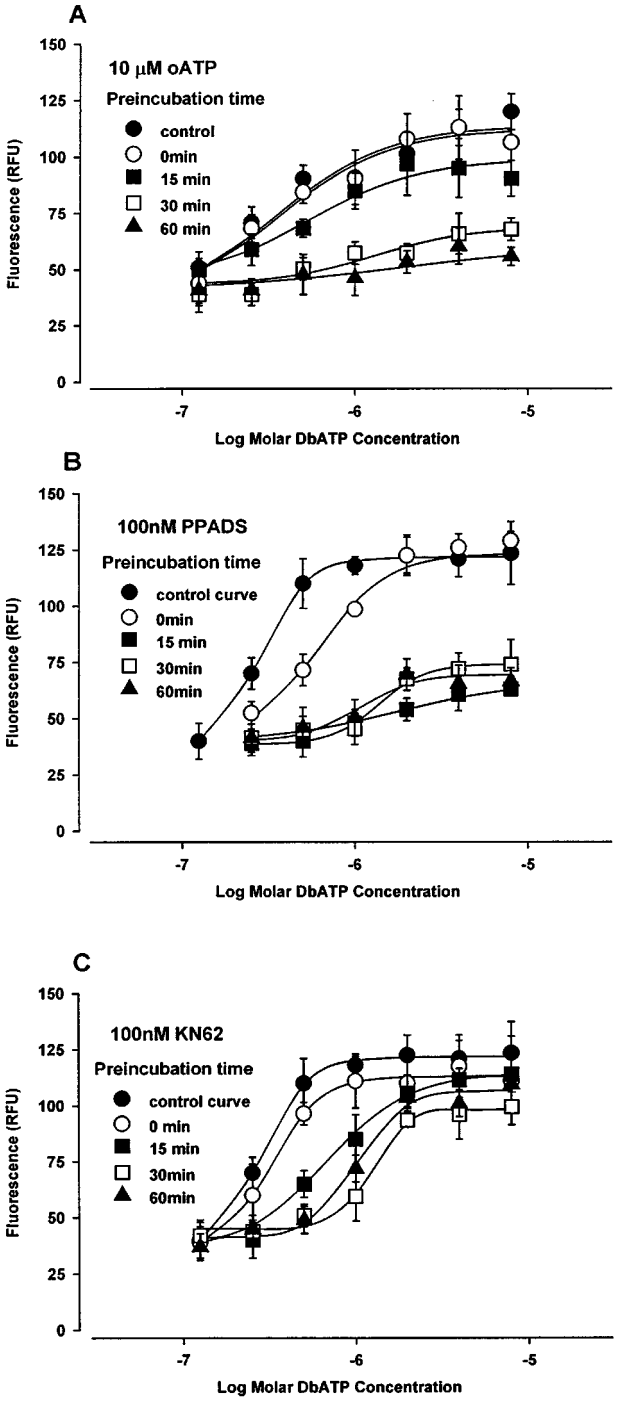

Figure 2 Effect of equilibration time on the ability of antagonists to block DbATP-stimulated increases in YO-PRO-1 fluorescence in HEK293 cells expressing the human recombinant P2X$_7$ receptor. Cells were pre-incubated for 0, 15, 30 or 60 min with 10 μM oATP (A), 100 nM PPADS (B) or 100 nM KN62 (C) before addition of DbATP and YO-PRO-1. After a further 20 min incubation YO-PRO-1 fluorescence, in relative fluorescence units (RFU), was measured. For clarity, the figure only shows the control CEC to DbATP determined for the 30 min pre-incubation study. Control CEC determined at 0, 15 and 60 min were not significantly different to the control CEC determined after a 30 min pre-incubation. The data are the mean$\pm$s.e.mean of four experiments.

The pEC_{50} values determined after 5, 10, 15, 20, and 30 min were 6.22 ± 0.03, 6.20 ± 0.03, 6.23 ± 0.05, 6.25 ± 0.03 and 6.30 ± 0.04 and these values were not significantly different from each other or from the pEC_{50} value of 6.27 calculated from initial rates (Figure 1B).

At the highest concentrations of DbATP examined, YO-PRO-1 accumulation was maximal after approximately 60 min. To determine if the rate-limiting step for DbATP-stimulated YO-PRO-1 accumulation was channel dilation to the 'large pore-form' of the receptor or cellular entry/permeation of YO-PRO-1, cells were pre-incubated with DbATP for 0, 30 or 60 min before addition of YO-PRO-1. The maximum initial rate was not affected by pre-incubation since $t_{1/2}$ values determined using a maximally effective concentration of DbATP (4 μM) were 19.1 ± 2.7, 17.3 ± 1.8 and 15 ± 2.6 min, respectively, following 0, 30 or 60 min pre-incubation with DbATP ($P > 0.05$; one way ANOVA followed by Tukey's post test). Furthermore, the pEC_{50} values calculated from measurement of initial rates following 0, 30 and 60 min pre-incubation periods with DbATP, were 5.96 ± 0.09, 6.04 ± 0.09 and 6.10 ± 0.13, respectively, and these were not significantly different from each other ($P > 0.05$; one way ANOVA followed by Tukey's post test).

Since pEC_{50} values obtained using these various methods were similar, in subsequent studies pEC_{50} values were estimated from the data obtained following a 20 min exposure of cells to DbATP and YO-PRO-1.

Effect of antagonist equilibration time

The effects of several of the antagonists were determined after various pre-incubation times (0, 15, 30 and 60 min) to determine the optimal antagonist equilibration period (Figure 2). In the absence of antagonist, concentration-effect curves for DbATP did not change significantly with time of incubation (data not shown). However the effects of the antagonists varied with pre-incubation time. This was particularly evident in the case of oATP (Figure 2A). Thus, without pre-incubation, oATP (10 μM) had little effect on the DbATP CEC but, with 15–60 min pre-incubation, 10 μM oATP produced a marked antagonist effect, although there was little difference between the antagonism produced after 30 or 60 min. A similar time-dependence of antagonist effect was evident with KN62 and PPADS (Figure 2B,C). Thus, for both compounds the antagonist effects were increased at 15 and 30 min compared to that determined with no pre-incubation period. Increasing the pre-incubation period to 60 min did not produce any further increase in antagonist effect and so a 30 min pre-incubation time was selected for all subsequent studies.

Antagonist effects on DbATP-stimulated YO-PRO-1 accumulation

As can be seen in Figure 3, oATP, P5P and PPADS produced concentration-related decreases in DbATP-stimu-

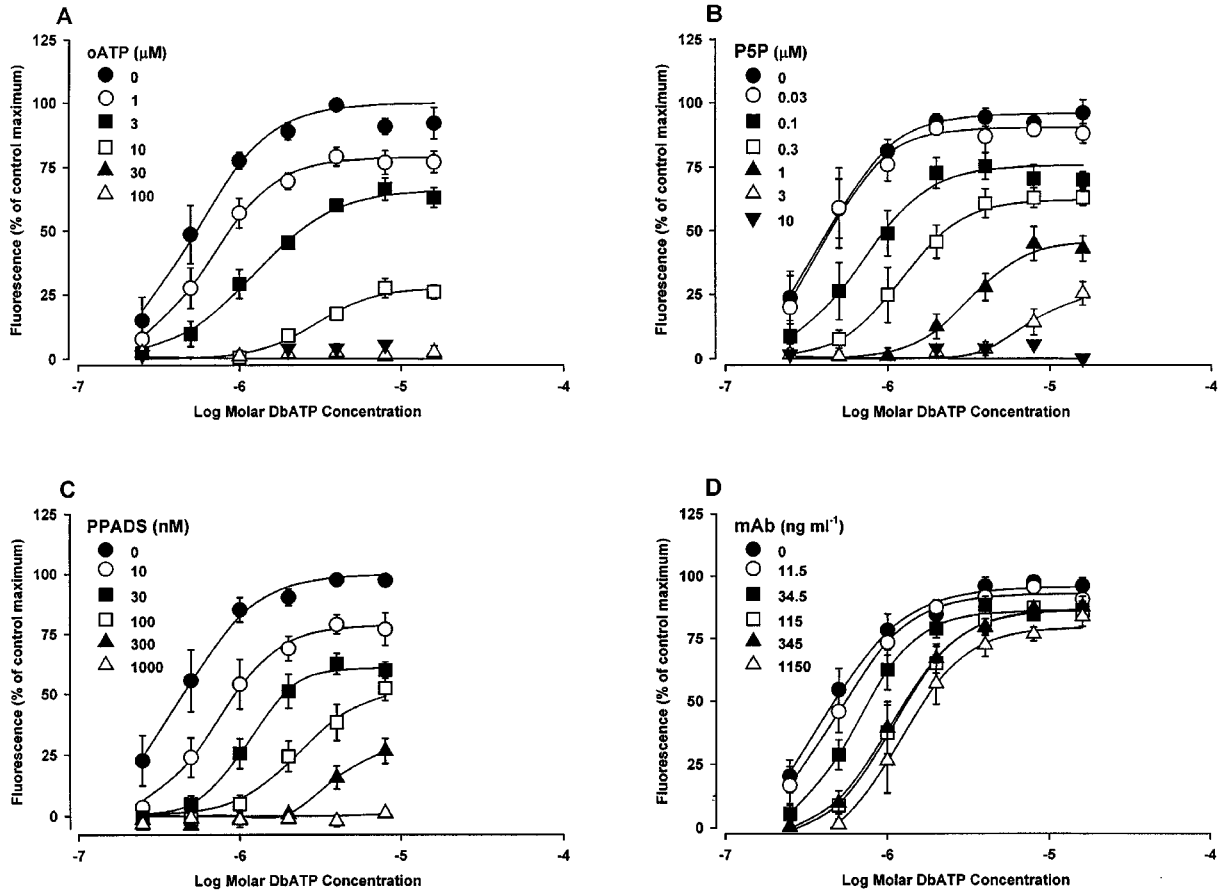

Figure 3 Effect of antagonists on DbATP-stimulated increases in YO-PRO-1 fluorescence in HEK293 cells expressing the human recombinant $P2X_7$ receptor. Cells were pre-incubated for 30 min with varying concentrations of oATP (A), P5P (B), PPADS (C) or a monoclonal antibody (mAb) to the human $P2X_7$ receptor (D), before addition of DbATP and YO-PRO-1. After a further 20 min incubation, YO-PRO-1 fluorescence was measured. The data represent specific increases in YO-PRO-1 fluorescence obtained by subtracting basal fluorescence levels obtained in the absence of DbATP. The data have been normalized to the control curve maximum for each series of experiments and are the mean $\pm$ s.e.mean of 3–6 experiments.

lated YO-PRO-1 accumulation, resulting in a reduction in the maximum response and a decrease in DbATP potency. In these studies oATP was left in contact with cells after its 30 min pre-incubation period. However, similar results were obtained when DbATP-stimulated YO-PRO-1 accumulation was determined in cells that had been pre-incubated with oATP for 30 min and then washed as described in the Methods (data not shown). Schild analysis of these data was not possible due to the marked reductions in maximum response.

Suramin could not be studied since it was fluorescent at the wavelengths used to measure YO-PRO-1 fluorescence. A monoclonal antibody to the $P2X_7$ receptor attenuated responses to DbATP but only produced a 3 fold decrease in the potency of DbATP (Figure 3D). Calmidazolium (0.1 – 1 μM) produced very similar effects to the monoclonal antibody (data not shown) and, at a concentration of 1 μM, produced a 2 fold decrease in the potency of DbATP with no effect on the maximum response. At concentrations of 3 μM and greater, calmidazolium caused an increase in YO-PRO-1 fluorescence, which was also observed in wild type HEK293 cells, suggesting that calmidazolium was causing cell death at these high concentrations.

KN62 produced concentration-related, rightward, shifts in the DbATP concentration-effect curve and also produced a small decrease in the maximum response (Figure 4A). Since the reductions in maximum response were not marked these data were examined using a Schild plot (Figure 4C). The pA_2 value for KN62 was 8.10 ± 0.09 but the Schild slope was less than unity (0.51 ± 0.03). At concentrations between 30 nM and 1 μM, KN04 (Figure 4B) produced similar effects to KN62 but was approximately 6 fold weaker (pA_2 7.31 ± 0.08; Schild slope $= 0.57 \pm 0.06$ using data points between 30 nM and 1 μM KN04). However, increasing the concentration of KN04 above 1 μM did not produce any further effect on the DbATP CEC.

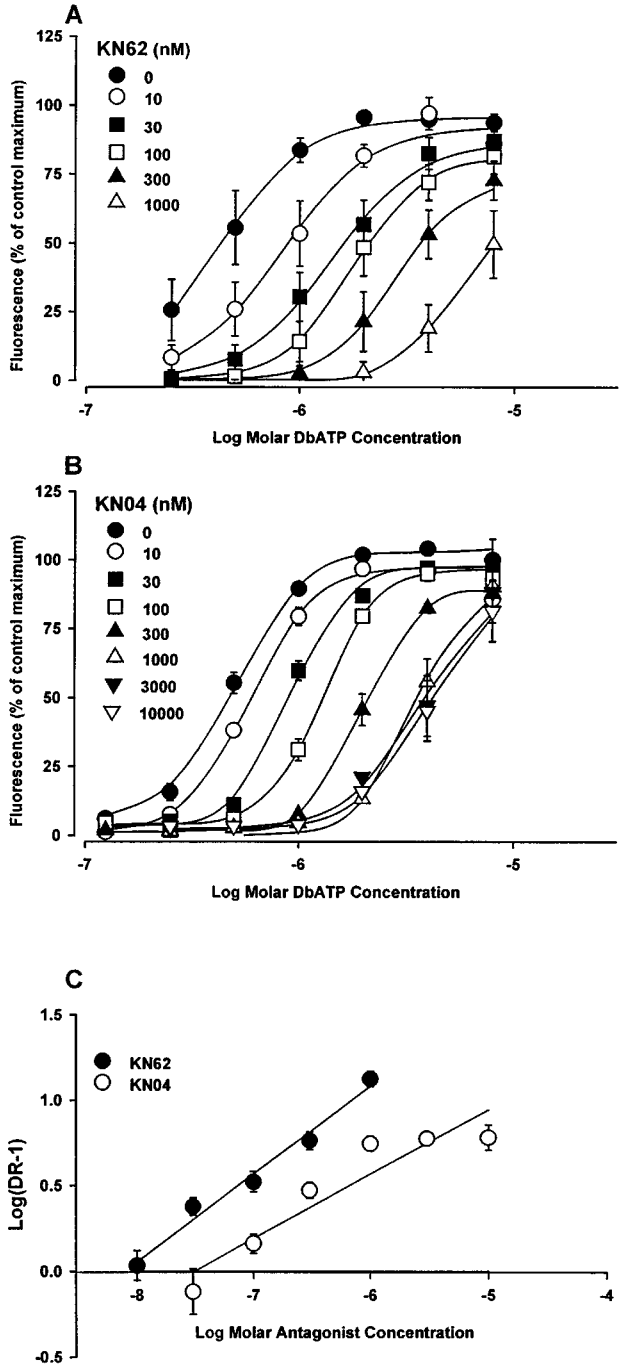

Figure 4 Effect of KN62 and KN04 on DbATP-stimulated increases in YO-PRO-1 fluorescence in HEK293 cells expressing the human recombinant $P2X_7$ receptor. Cells were pre-incubated for 30 min with varying concentrations of KN62 (A) or KN04 (B) before addition of DbATP and YO-PRO-1. After a further 20 min incubation, YO-PRO-1 fluorescence was measured. The data represent specific increases in YO-PRO-1 fluorescence obtained after subtraction of basal fluorescence obtained in the absence of DbATP. The data have been normalized to the control curve maximum for each series of experiments. (C) Schild plot of the data for KN04 and KN62. The solid lines in (C) represent best fit lines determined for the entire data set. The data are the mean $\pm$ s.e.mean of 3 – 6 experiments.

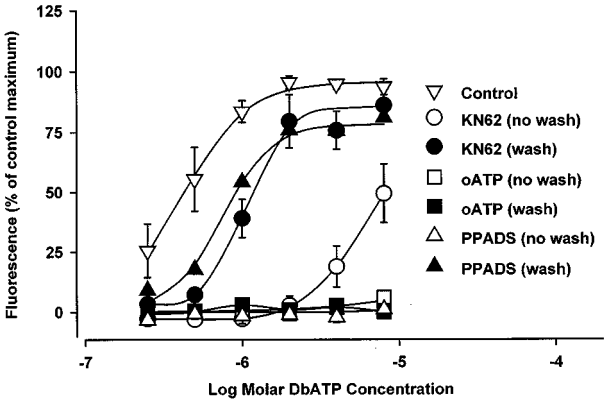

Figure 5 Reversibility of the inhibitory effects of PPADS, KN62 and oATP on DbATP-stimulated increases in YO-PRO-1 fluorescence in HEK293 cells expressing the human recombinant $P2X_7$ receptor. Cells were pre-incubated for 30 min with 1 μM PPADS, 100 μM oATP, 1 μM KN62 or assay buffer and subjected to extensive washing (wash), as described in the Methods, before addition of DbATP and YO-PRO-1. After a further 20 min, YO-PRO-1 fluorescence was measured. The data are specific changes in fluorescence intensity and are expressed as a percentage of the maximal response in control cells. For comparative purposes, the effects of antagonists are also shown in the absence of extensive washing (no wash). The data are the mean $\pm$ s.e.mean of four experiments. In the case of studies with KN62 and PPADS, the maximum responses to DbATP obtained after washout of the antagonist were significantly greater ($P<0.05$) than the respective maxima obtained in the continued presence of the antagonist, but were not significantly different ($P>0.05$) to the maximum obtained in control cells. The maximum response to DbATP after washout of oATP was not significantly different ($P>0.05$) to the maximum response obtained in the continued presence of oATP, but was significantly different ($P<0.05$) to the maximum response obtained in control cells. In all cases statistical significance was determined using a one way ANOVA with Tukey's post test.

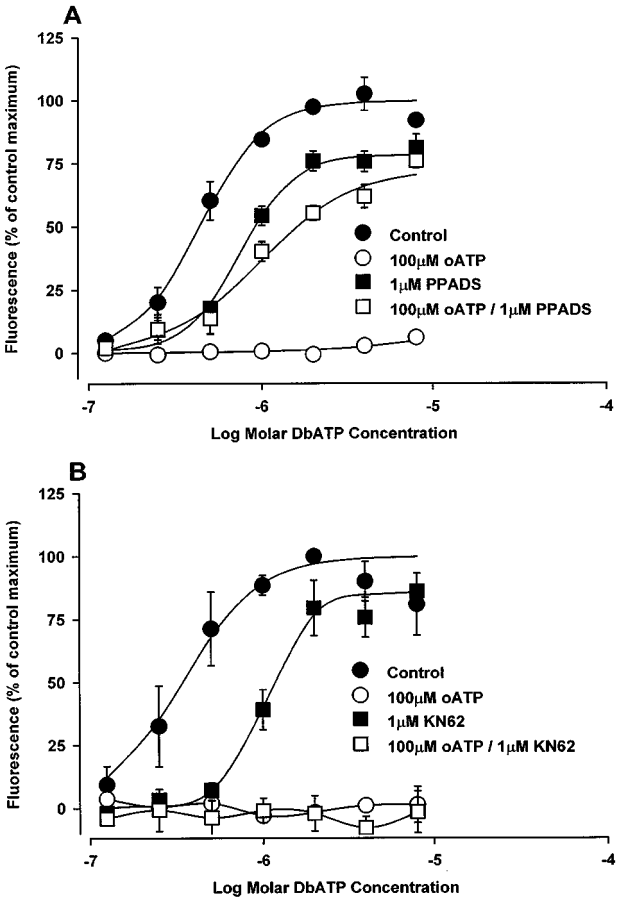

Figure 6 Effect of PPADS (A) and KN62 (B) on the ability of oATP to irreversibly block DbATP-stimulated increases in YO-PRO-1 fluorescence in HEK293 cells expressing the human recombinant $P2X_7$ receptor. Cells were pre-incubated for 15 min with either buffer, 1 μM PPADS or 1 μM KN62 before addition of 100 μM oATP or assay buffer. After a further 30 min cells were washed as described in the Methods before addition of DbATP and YO-PRO-1. After a further 20 min, YO-PRO-1 fluorescence was measured. The data represent specific changes in fluorescence intensity and have been expressed as a percentage of the maximal response in the control cells. The data are the mean$\pm$s.e.mean of four experiments. In A the maximum response to DbATP after washout of the combined mixture of PPADS and oATP (100 μM oATP/1 μM PPADS) was significantly greater ($P<0.05$) than the maximum response after washout of 100 μM oATP, but was not significantly different ($P>0.05$) to the maximum response obtained after washout of 1 μM PPADS. In B the maximum responses to DbATP after washout of the combined mixture of oATP and KN62 (100 μM oATP/1 μM KN62) was not significantly different ($P>0.05$) to the maximum response after washout of 100 μM oATP, but was significantly different ($P<0.05$) to the maximum response after washout of 1 μM KN62. In all cases statistical significance was determined using a one way ANOVA with Tukey's post test.

Reversibility of antagonist effects and P2 antagonist interactions with oATP

The effects of 1 μM PPADS, 1 μM KN62 and 1 μM P5P were partially reversible following washout (see Methods) whereas the effects of 100 μM oATP were not (Figure 5; data for PPADS and KN62). The effects of the human $P2X_7$ receptor monoclonal antibody (1150 ng ml^{-1}) did not reverse appreciably after a 30 min washout (data not shown). To determine if the antagonists were interacting with similar sites to those identified by oATP their ability to prevent the irreversible antagonism produced by oATP was examined. When PPADS (100 nM and 1 μM), P5P (1 μM) or suramin (1 and 10 μM) were pre-incubated with cells for 15 min before addition of oATP, they attenuated the irreversible antagonist effect of oATP whereas pre-incubation with KN62 (10–1000 nM) or KN04 (10–1000 nM) was without effect (Figure 6; data for 1 μM PPADS and 1 μM KN62).

Discussion

In this study we have shown that $P2X_7$ receptor function can be quantified by measurement of YO-PRO-1 accumulation and that this approach can be used to study antagonist interactions with the $P2X_7$ receptor.

DbATP caused a concentration- and time-dependent increase in YO-PRO-1 fluorescence in suspensions of HEK293 cells expressing the human $P2X_7$ receptor and a kinetic analysis of these data revealed that it was possible to construct complete CEC for DbATP and to estimate agonist potency using this approach. There have been few detailed reports on the effects of antagonists on the $P2X_7$ receptor and few studies have utilized adequate antagonist equilibration times (see below). Since it was possible to obtain full CEC for the ability of DbATP to stimulate YO-PRO-1 accumulation we have examined the effect of a number of P2 antagonists using this method.

PPADS, P5P, oATP, KN04 and KN62 were all found to be relatively potent antagonist of the human $P2X_7$ receptor, being active at nanomolar to low-micromolar concentrations. For KN62 and KN04 this is consistent with previous studies since these compounds have been shown to block a number of $P2X_7$ receptor-mediated effects at concentrations of 10–1000 nM (Blanchard *et al*., 1995; Gargett & Wiley, 1997; Humphreys *et al*., 1998, Chessell *et al*., 1998). In the case of PPADS and P5P the high potency was not expected since the original publication suggested the potency of PPADS to be approximately 50 μM (Rassendren *et al*., 1997). However, we found the kinetics of PPADS and P5P to be relatively slow, with the compounds requiring 15–30 min equilibration times, even at 37°C, and it is possible that such lengthy preincubations were not utilized in the previous studies.

Although PPADS and P5P were relatively potent compounds they did not behave as competitive antagonists since they produced a reduction in the maximum response and produced a slight decrease in DbATP potency. This response was similar to that observed with oATP which is an irreversible antagonist (Murgia *et al*., 1993) and might suggest that PPADS and P5P are also irreversible antagonists. Certainly, PPADS is a potent antagonist of $P2X_2$ receptors where its effects are thought to be irreversible and involve Schiff base formation (Buell *et al*., 1996). However, unlike oATP, the effects of both PPADS and P5P on the $P2X_7$ receptor were partially reversible following a 10–20 min washout period suggesting that these compounds are not irreversible antagonists. Nevertheless, since their effects were only slowly reversible it is likely that over the time course used to measure YO-PRO-1 accumulation, these compounds behaved as 'pseudo-irreversible' antagonists (Kennakin, 1993) and so reduced the maximum response.

In contrast to PPADS, P5P and oATP, the isoquinoline compounds, KN62 and KN04, did not produce any marked effect on the maximum response but instead produced a rightward shift in the DbATP CEC. The slope of the Schild plot for both compounds was low suggesting that they were not competitive antagonists. Furthermore, at concentrations in excess of 1 μM, KN04 did not produce any further shift in the concentration-effect curve to DbATP. This behaviour may

suggest that KN62 and KN04 are allosteric regulators of receptor function and would be consistent with the lack of effect of KN62 on oATP receptor inactivation (see below). However, it may also reflect limitations in the solubility of the compounds since KN62 is reported as being poorly soluble in physiological solutions at concentrations in excess of 1 – 10 μM (Seikagaku technical bulletin).

We have previously found that a monoclonal antibody to the human $P2X_7$ receptor is a potent antagonist of the human $P2X_7$ receptor producing a complete blockade of the inward currents evoked by DbATP in HEK293 cells expressing the human recombinant $P2X_7$ receptor (Buell *et al*., 1998; Chessell *et al*., 1999). Furthermore, the monoclonal antibody also antagonized DbATP-stimulated interleukin-1β release in THP-1 cells (Buell *et al*., 1998). In the present study we found that the antibody was able to attenuate DbATP-stimulated YO-PRO-1 accumulation and this was achieved over the same concentration range (100 – 1000 ng ml^{-1}) that was effective in blocking DbATP-stimulated currents or interleukin-1β release. However, the antagonism was not competitive and the maximal reduction in EC_{50} was only 3 fold. Calmidazolium has been shown to block DbATP-stimulated inward currents at the rat (Virginio *et al*., 1997) and human $P2X_7$ receptor (Chessell *et al*., 1998), but behaved in a similar manner to the monoclonal antibody producing only a small, 2 fold, decrease in the potency of DbATP at a concentration of 1 μM. Higher concentrations of calmidazolium increased YO-PRO-1 fluorescence possibly due to cell lysis.

The reason for the small shift in the DbATP concentration-effect curve produced by the monoclonal antibody and calmidazolium compared to their more profound effects observed in electrophysiological studies is not known, but these observations are consistent with previous reports. Thus, calmidazolium was more effective at blocking DbATP-induced currents at the rat $P2X_7$ receptor than at blocking DbATP-stimulated YO-PRO-1 accumulation (Virginio *et al*., 1997). Furthermore, the monoclonal antibody to the human $P2X_7$ receptor preferentially blocked DbATP-induced inward currents through the 'channel' form of the $P2X_7$ receptor in naïve cells, but was ineffective at blocking DbATP-induced currents in cells that had been repeatedly exposed to ATP to induce 'large-pore' formation (Chessell *et al*., 1999). One explanation of these data is that calmidazolium and the monoclonal antibody may possess different affinity for the putative 'channel' and 'large-pore' forms of the $P2X_7$ receptor (see Introduction). If this were the case then it is possible that only a small proportion of the $P2X_7$ receptors examined in the present study were present as the 'channel-form' so explaining the limited effects of calmidazolium and the monoclonal antibody. It is also conceivable that both agents, like KN62 and KN04, may function as allosteric regulators of the $P2X_7$ receptor (see below).

Finally, the mechanism of action of the various compounds was studied further by examining their interactions with oATP to determine if they were interacting at similar or distinct sites. oATP was an irreversible antagonist of the $P2X_7$ receptor and, given its close structural similarity to ATP, it seems reasonable to suppose that it interacts directly with the ATP binding site. PPADS, P5P and suramin were able to prevent the irreversible antagonist effects of oATP suggesting a common site of action. This may be the ATP binding site although the possibility that they bind to an allosteric site whose occupancy reduces the affinity of oATP, and so prevents its binding, cannot be ruled out. In contrast to P5P, PPADS and suramin, the isoquinoline compounds, KN62 and KN04, did not affect the receptor inactivation produced by oATP suggesting that their effects on the receptor are mediated through a site distinct from that recognized by oATP, P5P and PPADS. It may thus be possible that KN62 and KN04 interact at an allosteric site on the $P2X_7$ receptor which is able to prevent channel opening subsequent to ATP binding. Such an allosteric mechanism of action could also account for the failure of KN62 and KN04 to act as competitive antagonists of DbATP-stimulated YO-PRO-1 accumulation.

In summary, we have found that it is possible to quantify the effects of DbATP on the $P2X_7$ receptor by measurement of YO-PRO-1 accumulation and to determine the effects of P2 antagonists. We have found that these antagonists require relatively long equilibration times and, under appropriate conditions, that PPADS and P5P are potent and reversible antagonists of the $P2X_7$ receptor. We have also provided evidence that KN62 and KN04 may function as allosteric regulators of $P2X_7$ receptor function.

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