INTRODUCTION

Ionotropic purinergic receptors (P2X receptors) are ligand-gated ion channels that are activated by the binding of ATP at their extracellular side. In mammals, seven subunit isoforms (P2X1 to P2X7) have been identified (Coddou, Yan, Obsil, Huidobro-Toro, & Stojilkovic, 2011; Schmid & Evans, 2019; Surprenant & North, 2009) which form homotrimeric or multiple heterotrimeric channels. P2X receptors are expressed in a great variety of tissues and they are involved in highly diverse physiological and pathophysiological processes like pain, inflammation, or synaptic transmission. The channels are trimers and structural information on several of their isoforms is available. In contrast, the cooperation of the subunits in the activation process is poorly understood. We synthesized a novel fluorescent ATP derivative, 2-[DY-547P1]-AET-ATP (fATP) to unravel the complex activation process in P2X2 and mutated P2X2 H319K channels with enhanced apparent affinity by characterizing the relation between ligand binding and activation gating. fATP is a full agonist with respect to ATP that reports the degree of binding by bright fluorescence. For quantifying the binding, a fast automated algorithm was employed on human embryonic kidney cell culture images. The concentrations of half maximum occupancy and activation as well as the respective Hill coefficients were determined. All Hill coefficients exceeded unity, even at an occupancy <10%, suggesting cooperativity of the binding even for the first and second binding step. fATP shows promise for continuative functional studies on other purinergic receptors and, beyond, any other ATP-binding proteins.
pain, inflammation, taste or synaptic transmission (Hausmann, Kless, & Schmalzing, 2015).

Information about PXR receptor function is predominantly based on electrophysiological measurements. The apparent affinity (EC50) for ATP considerably varies among the isoforms, ranging from low micromolar to millimolar concentrations. Another difference between the seven isoforms is the speed of desensitization, that is, the rate at which the channel activity decays in the continued presence of ATP: While homomeric P2X1 and P2X3 channels desensitize with time constants of tens of milliseconds, P2X2, P2X4, P2X5, and P2X7 channels desensitize only in the range of many seconds or minutes or not at all (Bhargava, Nicke, & Rettinger, 2013; Coddou et al., 2011; North & Surprenant, 2000). If channels are opened, they generate a permeability for the cations Ca2+, Na+, and K+ ions (Egan, Samways, & Li, 2006). To obtain information about how P2X receptors work, electrophysiological approaches have been extensively complemented by molecular biological modifications but also by other methods as the substituted-cysteine accessibility method to identify differences in solvent accessibility (Egan, Haines, & Voigt, 1998; Krakun, Chaptal, Abramson, & Khakh, 2010), voltage-clamp fluorometry (to study the kinetics of conformational changes (Lorinczi et al., 2012) or inter-subunit cross-linking of cysteine residues to modify conformational changes (Kowalski et al., 2014).

The crystal structure of zebrafish P2X4 (zfP2X4) has proven the trimeric nature of the channels (Hattori & Gouaux, 2012; Kawate, Michel, Birdsong, & Gouaux, 2009), thereby confirming earlier functional studies based on concentration-response relationships (Ding & Sachs, 1999), cross-linking (Nicke et al., 1998), and disulfide bond formation between engineered cysteines (Jiang et al., 2003). The crystal structure also revealed that the molecular architecture of a single subunit resembles that of a dolphin. The transmembrane domains TM1 and TM2 form the tail and the ensemble of the three TM2 helices in a channel builds the pore (Hattori & Gouaux, 2012; Kawate et al., 2009). The TM1 helices are positioned laterally with respect to the pore. In the large ectodomain, the dolphin body is organized in a β-sheet structure with lateral fenestration sites where ions can enter the central vestibule. From the body four structurally flexible domains branch, including head, dorsal fin, left and right flipper. These regions are involved in the binding of ATP and transmitting this signal to the pore. The structure is completed by the intracellular N- and C-termini which are resolved in a structure of the human P2X3 subunit (Mansoor et al., 2016). These tails form an intracellular cap that essentially influences channel kinetics, at least for this isoform.

The three ATP binding sites in a trimeric channel are located at subunit interfaces of neighboring subunits on the extracellular side. By site-directed mutagenesis and electrophysiology, several amino acids were identified to be involved in ATP binding, including in zfP2X4 the basic residues (K69, K71, R290, K308), aromatic (F183, F289), and polar residues (T184, N288). These residues form a pocket within an intersubunit cavity that is surrounded by the head and left flipper of one subunit and the dorsal fin of another one. The binding of ATP to P2X1 channels has been studied in the context of desensitization by means of a fluorescent ATP derivative Alexa Fluor® 647 adenosine 5’triphosphate (Alexa-647-ATP) which is a full agonist (Bhargava et al., 2013).

Knowledge of the crystal structure of zfP2X4 in both the ATP-free closed and the ATP-bound open state has also paved the way to perform new functional studies on understanding how the ATP binding is transmitted within the channel to the pore opening. To address the question how many subunits are required for channel activation the results are contradictory: On the one hand, in P2X2 channels, built of concatenated subunits with defined numbers of active binding sites, less than three subunits were shown to be required for activation (Stelmashenko et al., 2012) which was confirmed by a later study, specifying that it is two subunits which are required (Keceli & Kubo, 2014). On the other hand, using voltage-clamp fluorometry to study conformational changes in P2X1 receptors, differences in the time courses of labeled subunits between receptors containing two or three binding sites were identified, supporting the notion that all three subunits are involved in channel activation (Fryatt, Dayl, Cullis, Schmid, & Evans, 2016). Also Hill coefficients in concentration-activation relationships of various P2X channels exceeding 2 (Ding & Sachs, 1999; Jiang et al., 2003; Stelmashenko et al., 2012) argue for a relevant involvement of three subunits in the activation gating. For transmitting ATP binding to the pore opening a central role has been identified for the β-14 sheet which connects the ATP binding site on the upper end with the pore forming TM2 helix on the lower end in a subunit. This β-14 sheet interacts with the β-1 sheet of the adjacent subunit and undergoes a rotation in opposite direction after ATP binding, pulls the TM2 helix and thereby opens the channel (Du, Dong, & Zhou, 2012). Concerning the interaction of the subunits, it has been suggested that each intersubunit ATP-binding signal is transmitted first along the same subunit until residue D315 along the domain contributing K308 to the β-14 sheet and from there the signal spreads equally to all three subunits towards the pore, suggesting that in the last step the subunits contribute equally and independent to channel activation (Keceli & Kubo, 2014). It has also been proposed that the gating of P2X receptors can be subdivided into five key steps: ATP binding, tightening of the binding jaw, flexing of the lower body regions, expansion of the lateral fenestrations, and pore opening (Habermacher, Dunning, Chataigneau, & Grutter, 2016).

Despite an enormously growing insight into structure and function of P2X receptors over the past two decades, many questions remain open, including the type of cooperativity of the subunits, the exact and mutual effects between ligand binding and activation gating as well as the concentration-binding relationship itself which does not necessarily superimpose with the concentration-activation relationship of a receptor (Colquhoun, 1998). A relevant gain of information can be achieved if it is possible to monitor the ligand binding together with the activation gating, as performed previously in cyclic nucleotide-gated and hyperpolarization activated cyclic nucleotide-modulated channels by
confocal patch-clamp fluorometry (Biskup et al., 2007; Kusch et al., 2010, 2012). For these channels this enabled us to substantiate Hidden Markov Models describing the channel gating in considerable detail. A major prerequisite for this type of strategy is to make available a fluorescent ligand that is a full agonist with respect to ATP, has a reasonable potency to activate the receptor, is bright enough to do optical recording and does not have any other negative side effects. Herein we designed and synthesized a novel fluorescent ATP derivative in which the dye DY-547P1 was coupled to the 2-position of the adenine ring. This position was chosen because of the promising property of 2-MeS-ATP to be a full agonist for P2X2-receptors with an enhanced potency compared to ATP (Coddou et al., 2011). This is in contrast to ribose-labeled ATP derivatives. We studied binding by an optical approach and the binding-induced activation of P2X2 channels by electrophysiology. Our novel fluorescent ligand fATP bears great potential for functional studies on any other purinergic receptor and, beyond this, on any other protein binding ATP.

2 | METHODS

2.1 | Synthesis of fATP

The synthesis of the novel fluorescent ATP derivative 2-[DY-547P1]-AET-ATP (fATP) is described in Supplementary Methods.

2.2 | Molecular biology

Rat P2X2 (pP2X2) in pcDNA5/FRT/TO (RRID: Addgene_137071) was kindly provided by Günther Schmalzing (RWTH Aachen University, Aachen, Germany). The mutation H319K (RRID: Addgene_137072) was introduced with the QuickChange site-directed mutagenesis (Stratagene) using a primer containing the modified base triplet with Phusion polymerase followed by DPN1 digestion. The obtained clones were verified by restriction analysis and sequencing.

2.3 | Cell culture

HEK293 cell lines containing an inducible promoter (Flp-In-T-Rex 293; Invitrogen #R78007) were cultured in minimum essential medium (MEM) supplemented with 10% fetal calf serum, non-essential amino acids (Gibco) and antibiotics according to the manufacturer’s instructions. The cell line is not listed as a commonly misidentified cell line by the International Cell Line Authentication Committee and was used without further authentication. For stable cell lines, Flp-In-T-Rex 293 cells were transfected using the calcium phosphate method with a mixture of plasmids (0.5 µg pcDNA5/FRT/TO P2X2 (RRID: AC line CVCL_YJ33) or P2X2H319K (RRID: AC line CVCL_YJ34) and 1.5 µg pOG44). After hycromycine B treatment stable clones were selected and cultured until a passage number of approximately 20.

Cells were seeded on glass coverslips for electrophysiological measurements and used 24–48 hr after tetracycline induction. For binding measurements cells were plated on chambered glass coverslips (ThermoFisher Scientific) coated with poly-L-lysine (Sigma-Aldrich) and measured 48 hr after induction. This study was not pre-registered and no institutional ethical approval was required. Cell lines and plasmids will be shared upon reasonable request.

2.4 | Confocal microscopy

For detection of ligand binding the cells were incubated with the fluorescent ligand 2-[DY-547P1]-AET-ATP (fATP). The bulk-solution was counter-stained with a red fluorescence dye Dy647 (Dyomics; 1 μM) showing only low non-specific binding. Automated data analysis was implemented in IgorPro (Wavemetrics). For data import the Lsmreader.pxp by Stephen R. Ikeda (NIH) was used. Each confocal voxel contains both the signal from bound ligand as well as signal from ligands in the bulk solution. This additional signal, for example, the fraction of bulk solution inside a voxel, varies from pixel to pixel and can hardly be described theoretically. At low expression level or at concentrations in nano-molar and above, this background signal is not negligible. Therefore a scaled difference image between the images of labeled ligand and reference fluorophore will show the pure binding signal according to.

\[
\text{binding} = \text{ligand} - \text{reference} = l_{\text{high}} - r_{\text{low}} = \frac{l_{\text{high}} - l_{\text{low}}}{r_{\text{high}} - r_{\text{low}}} \tag{1}
\]

Binding, ligand, and reference are the pixel-wise image signal. \(l_{\text{low}}, l_{\text{high}}, r_{\text{low}}, r_{\text{high}}\) are the average signal of bulk and cell-interior signals for the ligand and the reference dye, respectively. \(r_{\text{low}}, r_{\text{high}}\) were determined as the two main peaks of intensity histograms of the reference image. \(l_{\text{low}}, l_{\text{high}}\) were determined by calculating the mean intensity of the pixels in the ligand channel, for which the reference signal was \(r_{\text{low}} < 3,000 < r_{\text{low}} + 3000\) and \(r_{\text{high}} > 3,000 < r_{\text{high}} + 3000\). As these values can be determined without manual interactions, the approach can be easily automated.

As substantial noise was observed within the bulk solution as well as inside the cells, the cell-bulk interfaces were selected using a Sobel operator (Sobel, 1990) on the reference fluorophore signal. This mask signal was further filtered to remove speckles and to adjust the width of the considered edges and finally an appropriate threshold was applied. Note that only cell-bulk boundaries and not cell-cell boundaries are included in this approach.

For cells not expressing the receptor or having excess of competing unlabeled ligand, a small systematic error, that is, a negative apparent binding-signal was observed. This signal is linearly dependent on the concentration of the labeled ligand. It is therefore likely due to the non-gaussian-nature of the intensity distribution or...
differences in the detectors for fluorescence and ligand channels, as it was influenced by the mode of calculation of \( I_{\text{low} \rightarrow \text{high}} \), (e.g., mean or median). To compensate the error, appropriate negative controls were measured and subtracted. Binding measurements and analysis were performed by different persons.

The reference dye DY647 was purchased from Dyomics GmbH, Jena, Germany.

2.5 | Electrophysiology

Current recording were performed with a standard patch-clamp technique (Hamill, Marty, Neher, Sakmann, & Sigworth, 1981) in the whole-cell recording. The patch pipettes were pulled from borosilicate glass (ID 1.0 mm, outer diameter 2.0 mm; VITROCOM) using a micropipette puller (P-2000; Sutter Instrument). The pipettes were filled with intracellular solution containing (mM) 142 NaCl, 1.2bis(o-aminophenoxy)ethan-N,N’,N’-tetraacetic acid, 5 EGTA and 10 HEPES, pH 7.4. The pipette resistance was 2.5 to 6.0 MΩ. The bath solution contained (mM) 142 NaCl, 10 EGTA, 10 HEPES, and 10 Glucose, pH 7.4. For recording the cells were lifted from the chamber bottom by the patch pipette and positioned in front of the outlet of the theta-glass pipette.

Solutions switches were carried out with a theta-glass pipette (inner diameter ~100 μm, World Precision Instruments), mounted on a piezo device, or with a three-barrel glass (inner diameter ~600 μm, Warner Instruments) controlled by a step motor (SF-77B, Warner Instruments). The speed of the laminar solution flow out of the barrels was estimated to be 2–5 cm/s. In case of the Theta-glass pipette one barrel contained control solution and the other was connected to a solution selector (Vici Valco Instruments) which allowed us to apply different test solutions by exchanging the solution during the interval of applying the control solution. The speed of the switch around a whole cell was estimated to be below 10 ms by switching between different salt solutions. This speed is sufficiently slow to keep the cell mechanically stable and sufficiently fast with respect to the slow time courses of current activation and deactivation.

Saturation of activation was determined with ATP at 100 μM for wt P2X2 and 1 μM for P2X2 H319K. The currents were recorded with an Axopatch 200B or HEKA EPC 10 amplifier in combination with the ISO3 hard and software or Patchmaster software hard and software, respectively. The sampling rate was 2 to 10 kHz and the recordings were on-line filtered at 1 and 2.9 kHz using a 4-pole Bessel filter. The currents were recorded at a constant holding potential of ~50 mV.

2.6 | Quantification and statistical analysis

Concentration-activation relationships were constructed from the maximum currents during a solution application. Measurements with rundown of the current of more than 10% were excluded from the analysis. These current amplitudes were normalized with respect to the current at saturating ATP (wt 100 μM; H319K 1 μM in each individual cell) and the resulting data points were fitted with

\[
\frac{I}{I_{\text{max}}} = \frac{1}{1 + \frac{[\text{ATP}]_{\text{EC}_{50}}}{[\text{ATP}]}}^{H_{\text{ATP}}},
\]

the Origin 8.5.1® software using none linear curve fitting routine with statistical weighting. \( I \) is the actual current amplitude and \( I_{\text{max}} \) the maximum current amplitude at saturating ATP. \( \text{EC}_{50} \) is the ligand concentration generating half maximum current and \( H_{\text{ATP}} \) the respective Hill coefficient. [X] is the actual concentration of either ATP or fATP to be tested.

Concentration-binding relationships for fATP were fitted according to

\[
\frac{F}{F_{\text{max}}} = \frac{1}{1 + \frac{[\text{BC}_{50}/[\text{fATP}]^{a}}{[\text{BC}_{50}/[\text{fATP}]^{a}}. \]

\( F \) is the actual relative fluorescence intensity and \( F_{\text{max}} \) the maximum relative fluorescence intensity at the saturating fATP concentration of 10 μM. \( \text{BC}_{50} \) is the ligand concentration generating half maximum binding, \( H_{\text{fATP}} \) the respective Hill coefficient and [fATP] the actual concentration to be tested.

Based on the values of \( \text{BC}_{50} \) of \( H_{\text{ATP}} \) for fATP, the respective values for ATP were determined by displacing 1,000 nM fATP at the receptors by variable ATP concentrations. For a simple system consisting of a receptor, \( R \), with \( n \) binding sites, and ligands, \( L \), in excess, the fraction of liganded receptors, \([L,R]\), can be described by the Hill equation.

\[
[L,R] = \frac{1}{1 + \frac{[\text{BC}_{50}/[L]]^{H_{L}}}. \]

where \( \text{BC}_{50} \) is the ligand concentration producing half maximum occupation at the receptor and \( H \) is the Hill coefficient which may significantly differ from \( n \). \( H \) is influenced by the number of binding sites and also by possible cooperativity effects but it provides a lower limit for the number of bound ligands. In case that two ligands compete for the same receptor, the situation can be illustrated as shown by the scheme in Figure 4b. The two coupled binding reactions can be described by the Gaddum equation proposed already decades ago previously (Colquhoun, 2006; Gaddum, 1957; Kenakin, 2017). After adaptation to our notation, the Gaddum equation reads.

\[
[\text{ATP},R] = \frac{1 + \left( \frac{[\text{BC}_{50}/[\text{ATP}]}{[\text{BC}_{50}/[\text{ATP}]} \right]^{H_{\text{ATP}}}^{H_{\text{ATP}}} + 1}}, \]

where \([\text{ATP}],[\text{fATP}],[\text{BC}_{50}/\text{ATP}],[\text{BC}_{50}/\text{fATP}]^{a}\) are the concentrations of ATP and fATP as well as the half occupation concentrations, respectively. \( H_{\text{fATP}} \) the Hill coefficients for binding of ATP and fATP, respectively. Hence, inserting the values obtained for \( \text{BC}_{50} \) and \( \text{fATP} \) in Equation (6) for \( \text{BC}_{50}/\text{ATP} \) and \( \text{fATP} \) for ATP by fitting the experimental data for ATP (Figure 4a).
To determine the Hill coefficient at the lowest measurable ligand concentrations where both binding and gating are <0.1, the limiting slope was calculated (Patneau & Mayer, 1990; Wahl, Madsen, Banke, Krosgaard-Larsen, & Schousboe, 1996) from log-log plots and fitting a straight line through the data points <0.1 (Figure 3b). The slope determined in this way reports the Hill coefficient directly because in the limit of $[\text{fATP}] \to 0$ Equations (2) and (3) reduce to

$$\log \left( \frac{I}{I_{\text{max}}} \right) = H_b \log \left( \left[ \text{fATP} \right] \right) - H_b \log \left( \text{EC}_{50} \right)$$  \hspace{1cm} (6)

and

$$\log \left( \frac{F}{F_{\text{max}}} \right) = H_b \log \left( \left[ \text{fATP} \right] \right) - H_b \log \left( \text{BC}_{50} \right)$$  \hspace{1cm} (7)

respectively.

Experimental data are given as mean ± SEM.

2.7 | Structural modeling

The homotrimeric rat P2X2 structure (UniProt accession ID: P49653) was generated by homology modeling, using the 2.77 Å X-ray crystal structure of the human P2X3 ion channel in the ATP-bound open state (Protein Data Bank [PDB]: 5SVK) as a template (Mansoor et al., 2016). The modeling was carried out by the SWISS-MODEL server (Blasini et al., 2014) based on a target-template alignment with a sequence identity of 50.7% and coverage of 75% using residues 13–370 of a channel subunit. The quality of the model was validated by the MolProbity server (Chen et al., 2010), yielding an overall MolProbity score of 1.35.

3 | RESULTS

3.1 | Chemical synthesis of fATP

We synthesized the novel fluorescent ATP derivative 2-[DY-547P1]:AET-ATP by coupling the dye DY-547P1 to the 2-position of the purine ring via an amino-ethyl-thio spacer (Figure 1a). The compound is termed from hereon ‘fATP’. The chemical synthesis is described in detail in Supplementary Methods.

3.2 | The fluorescent ATP-derivative fATP is an agonist with similar efficacy as ATP for P2X2 channels

P2X2 receptors belong to the group of slowly or non-desensitizing P2X receptors. Expressing the channels in HEK293 cells, building the whole-cell configuration with the patch-clamp technique, lifting the cell and positioning it in front of the outlet of the theta-glass application pipette allowed us to generate solution jumps at the cell below 10 ms (see Methods). The maximum amplitude of the current signals elicited with steps by either fATP or ATP (Figure 1b) was normalized with respect to the maximum current amplitude at saturating ATP (100 µM for wt P2X2 and 1 µM for P2X2 H319K) and the data points were plotted as function of the respective ligand concentration (Figure 1c). Fitting the data points with Equation (2) yielded that the apparent affinity with fATP was more than an order of magnitude below that with ATP.

Determining the concentration-binding relationship requires to measure the binding of fATP at saturating concentrations to refer the binding at all other concentrations to this value. Using wt P2X2 receptors this is impossible because at concentrations above ~30 µM it becomes critical to delineate the specific binding of the bound labelled ligands from freely diffusing labelled ligands in the bath solution (Biskup et al., 2007). Moreover even if possible, the required high concentrations of the labelled ligand would become exceedingly expensive. We therefore decided to repeat the measurements in a mutant with significantly increased apparent affinity with the idea that also the binding is respectively tighter. We chose the H319K mutation in P2X2 channels which was reported to increase the apparent affinity 40-fold with respect to wt channels (Clyne, LaPointe, & Hume, 2002). Histidine 319, unique in P2X2 receptors and located in the upper part of the β-14 linker, is responsible for the positive modulating effect of protons on ATP induced currents. This effect can be constitutively mimicked by the mutation H319K.

Similar to published results (Clyne et al., 2002) the concentration-activation relationships for P2X2 H319K channels has significantly shifted with ATP to lower concentrations by about 35-fold yielding an EC$_{50}$ value of 34.2 nM (Figure 1d) compared to 1.2 µM in wt channels. Accordingly, the concentration-activation relationship with fATP has been similarly shifted by a factor of 10.5 compared to 10.4 µM in wt channels. Hence, P2X2 H319K channels are appropriate for studies on relating ligand binding to activation in P2X2 channel gating because activation is maximal at concentrations that are readily accessible with optical measurements.

3.3 | Binding of fATP to P2X2 H319K channels

Ligand binding was measured in cultured HEK293 cells attached to the bottom of chambered coverslips (Figure 2a). The cells were exposed to the test solutions containing the desired concentrations of fATP and the binding was quantified by exciting the dye moiety of the ligand at 543 nm using a HeNe laser. To distinguish the freely diffusing non-bound ligands from those bound to the channels, we followed the strategy used previously in confocal patch-clamp fluorometry (Biskup et al., 2007; Kusch et al., 2010) and added a second dye not coupled to a ligand to the test solution, in this case Dy647. This allowed us to label the space of the non-bound ligands and, thus, to determine the amount of bound ligands (Figure 2b–f; see Methods).

Herein, we measured ligand binding in ensembles of intact HEK293 cells instead in excised patches from Xenopus oocytes (Biskup et al., 2007; Kusch et al., 2010; Nache, Eick, Schulz, Schmauder, & Benndorf, 2013). This is because membrane patches...
of Xenopus oocytes showed significant non-specific binding of fATP which was not observed in HEK293 cells. However, the signal intensity in patches from HEK293 cells was significantly lower than that in patches from Xenopus oocytes and, thus, only measurements in ensembles of cells provided a sufficiently high signal intensity.

Typical images obtained for quantifying the binding of fATP to P2X2 H319K channels are shown in Figure 3a. In induced cells (P2X2 H319K (+)), an increase of the fATP concentration from 100 to 300 nM shows a clearly visible increase of staining at the membrane due to specific binding to the channels. Specificity is demonstrated by comparison with both non-induced cells (P2X2 H319K (−)) at 300 nM and 10 µM and also in induced cells in the presence of competing ATP added at the high concentration of 300 µM. The concentration-binding relationship for fATP to P2X2 H319K channels shows saturation below 10 µM (Figure 3b) and that the value for half maximum binding is closely similar to the EC_{50} value obtained for activation (c.f. Figure 1d).

Both the concentration-binding and the concentration-activation relationship required Hill coefficients larger than unity and are thus steeper than predicted by a Langmuir isotherm assuming a single binding site. This suggests not only for activation but also for ligand binding itself a cooperative action of the subunits. Furthermore, the binding curve saturates at lower concentrations than the activation curve (Figure 3b), which is typical for channels that are activated by the binding of more than one ligand (Colquhoun, 1998).

To consider the Hill coefficient separately at the lowest measurable ligand concentrations where both binding and gating are <0.1, we examined the limiting slope (Patneau & Mayer, 1990; Wahl et al., 1996) for both relationships by building log-log plots and fitting a straight line through the data points <0.1. The slope determined in this way reports the Hill coefficient directly because in the limit of [fATP]→0 Equations (2) and (3) reduce to Equations (6) and (7), respectively. The result is that both Hill coefficients still exceed unity and are also close to each other (Figure 3c), indicating that significant cooperativity is present already at very low degrees of occupancy, that is, for the first and second binding step.

3.4 | Binding of ATP to P2X2 H319K channels

Determination of the BC_{50} and H_a value for fATP allowed us also to determine the BC_{50} value for ATP itself by displacement experiments.
In the presence of 1,000 nM fATP the application of increasing ATP concentrations increasingly displaces fATP from the binding sites at the channels. The resulting concentration-displacement relationship (Figure 4a) was analyzed on the basis of the kinetic scheme for the two coupled binding reactions (Figure 4b). In this scheme the ligands ATP (A) and fATP (P1) compete for the same receptor site. Under equilibrium conditions, this scheme obeys Equation (5), which is in fact the Gaddum equation adapted to our notation (Colquhoun, 2006; Gaddum, 1957; Kenakin, 2017), and is also related to the Schild equation (Schild, 1957). Using the determined BC_{50} value for fATP as BC_{50ATP} and the determined Hill coefficient, H_{bATP}, Equation (5) was fitted to the data points yielding the indicated BC_{50fATP} and H_{bATP} value for ATP. It should be noted that the Hill coefficient was similar for the binding of ATP and fATP.

3.5 | Binding of fATP to wt P2X2 channels

We finally measured the binding of the novel fluorescent ATP derivative fATP on labeling wt P2X2 channels expressed in HEK293 cells (Figure 5a). Despite the fact that measurement of binding at 30 μM and higher becomes increasingly vague, below this concentration
the concentration-binding-relationship was obtained by assuming that the BC$_{50}$ and the EC$_{50}$ value are also similar as in H319K channels. Under this assumption the data points were scaled with respect to the data point at 10 $\mu$M which was set to 50% binding. Then Equation (3) was fitted with a fixed BC$_{50}$ value of 10 $\mu$M, yielding $H_b = 1.28$ (Figure 5b) which is similar to that in H319K channels.

4 | DISCUSSION

In this study, we synthesized and functionally characterized the novel fluorescent ATP derivative fATP regarding its agonistic effects on both P2X2 channels and the P2X2 H319K mutant with significantly enhanced apparent affinity for ATP. fATP proved to be a full agonist with respect to ATP that can emit bright fluorescence when bound to the channels. We used this compound to determine both the concentration-binding and the concentration-activation relationships at equilibrium conditions, which allowed us to relate ligand binding to activation gating and to determine the respective half maximum concentrations and Hill coefficients. In addition, the Hill coefficients were determined also at an occupancy <10% by the method of the limiting slope. The results suggest cooperative binding, already for the first and second binding step. Finally, the fluorescent fATP was also used to determine the occupancy of ATP at the binding sites by applying the Gaddum equation (Gaddum, 1957).

Together, the fluorescent ATP derivative fATP presented herein has properties that enabled for the first time to determine a concentration-binding relationship in activated P2X2 channels and, moreover, to prove cooperativity by binding on the basis of a Hill coefficient exceeding unity.

The fluorescent ATP derivative fATP designed and investigated herein has coupled the dye DY-547P1 via an aminoethylthio-linker to the 2-position of the adenine. Though with respect to ATP the fluorescent derivative fATP is a full agonist in P2X2 H319K channels, its potency is reduced by more than an order of magnitude. This reduced potency is presumably caused by the dye moiety and a steric
limitation in the binding pocket. It is possible that the relatively long
linker in fATP (see structure in Figure 1a) provides sufficient flexi-
bility to enable binding. Notably, we demonstrated that in fATP it is
indeed the binding that is disturbed (Figures 3b, 4a).

Two other ATP derivatives have been used previously to study
P2X channels. One derivative is Alexa-647-ATP in which the dye is
coupled to the 2',3' position of the ribose via an aminoethylcarba-
moyl-linker (Bhargava et al., 2013). It was applied to study strongly
desensitizing P2X1 channels and to a non-desensitizing P2X2-1 chi-
mera (Rettinger & Schmalzing, 2004). Alexa-647-ATP was used in this
report to elegantly study desensitization, as typical for P2X1 chan-
nels, with a half maximum concentration as low as 3 nM. In the other
fluorescent ATP derivative, BODIPY-TR ATP, the dye was coupled to
the ribose (Kowalski et al., 2014). It was used to study the agonist
induced movement of the binding jaw for ATP. The ability to monitor

the binding and gating with the fluorescent agonist allows for iden-
tifying amino acids not located in the binding pocked but influencing
access to it. It should be noted, that none of these ATP derivatives
could be used to determine a concentration-activation relationship
associated with channel activation, as performed herein for P2X2 re-
ceptors. We are not aware of any other fluorescent ATP derivatives
with similar efficacy that were used to study ligand binding in P2X2
channels.

Regarding the Hill coefficient of binding, $H_b$, determined herein
for H319K, we determined a value of 1.22. This value is in the order
of 1.09 observed for the Hill coefficient of activation, $H_a$. Under sim-
ilar experimental conditions as used herein, that is, in the absence
of divalent ions, these values were only some smaller than the Hill co-
efficient of 1.3 reported for P2X2 channels (Li, Silberberg, & Swartz,
2013). Notably, in the presence of millimolar Ca$^{2+}$ and Mg$^{2+}$, a much

![FIGURE 4](image1.png)

**FIGURE 4** Occupancy of ATP at P2X2 H319K channels. (a) Plot of the specific fluorescence intensity of fATP binding to P2X2 H319K
channels in HEK293 cells as function of the ATP concentration. fATP and ATP compete for the binding sites. The curve has been calculated
according to the scheme in b as described in Methods. Each data point was obtained from 3 to 5 images each containing about 100 cells.
Cells from at least three cultures were used. (b) Scheme describing the competitive binding of ATP and fATP to a binding site of a receptor $R$.
$BC_{\text{SO}_{\text{ATP}}}$ and $BC_{\text{SO}_{\text{fATP}}}$ are the respective ligand concentrations generating half maximum occupancy. $n$ is the number of binding ligands

![FIGURE 5](image2.png)

**FIGURE 5** Binding of fATP to wt P2X2 channels. (a) Representative confocal images of HEK293 cells expressing wt P2X2 channels. The
cells were exposed to 3 μM fATP. (b) Concentration-binding relationship. Based on the assumption that the $BC_{\text{SO}}$ and the $EC_{\text{SO}}$ value for
H319K channels are close by, we scaled the data points with respect to the data point at 10 μM which was set to 50% binding and fitted with
fixed $BC_{\text{SO}} = 10 \mu M$ the data points with Equation (3), yielding the Hill coefficient for binding $H_b = 1.28$. Each data point was obtained from 8
to 9 images each containing about 100 cells. Cells from at least three cultures were used
larger Hill coefficient of 2.3 was reported (Ding & Sachs, 1999), suggesting that the divalent ions critically influence the Hill coefficient. Furthermore, it should be noted that the Hill coefficients in the latter study were determined from single-channel recordings which leads a priori to steeper concentration activation relationships because single-channel recordings do not include the natural variability among the channels present in multichannel recordings.

Concerning the position of the H319 mutation, it has been outlined above that it is located in the \( \beta \)-14 linker and that it has been identified as pH sensor (Clyne et al., 2002). The major functional property of the H319K mutation in the context herein is a shift of steady-state activation and binding to lower concentrations by more than an order of magnitude (c.f. Figure 1c,d) and a slowed deactivation kinetics (c.f. Figure 1b) suggesting a slowed off-rate. In principle, this leftward shift can be explained by a modification of the binding site itself, a long-distance effect from a remote site, or a changed isomerization constant of a processes following the ligand binding (Colquhoun, 1998). The first option can be excluded because the H319K mutation is distant from the binding site. The second option, the long distance effect, is to consider here because the H319K mutation is located in the \( \beta \)-14 sheet which is known to be involved in the transmission pathway from the ATP binding to the pore (Keceli & Kubo, 2014). Notably, the distance from H319 to all three binding sites in a P2X2 channel is considerably long. Using a homology model based on the P2X3 structure (Mansoor et al., 2016) this distance was determined to be 31.8 Å to R290 in the binding site of the own subunit and 28.1 and 34.4 Å to the respective other two R290 residues (Figure 6). In the light that in wt P2X2 channels protons control the amount of positive charge of histidine 319, the conclusion is that the extracellular pH controls indeed the occupancy with ATP in the remote binding sites. At the second glance this is not so surprising because the \( \beta \)-14 sheet and the binding sites must functionally be intimately coupled to enable the control of channel activity. Therefore, the effect of H319 can be simply seen as a reciprocal control of the binding sites from the \( \beta \)-14 sheet, in some analogy to the reciprocal control of the occupancy in the cyclic nucleotide binding domains in hyperpolarization-activated cyclic nucleotide-modulated channel (isoform 2) (HCN2) channels by voltage-induced activation of the channel (Kusch et al., 2010).

This remote control of the occupancy is also not surprising from another point of view. Even in the very simple del Castillo-Katz scheme, consisting of a ligand bind step and a coupled step for a closed-open isomerization, the equilibrium of this closed-open isomerization can dramatically change the occupancy of the receptor at a constant dissociation constant for the binding step, that is, at constant affinity of the receptor (Colquhoun, 1998). Hence, the observed remote control of the occupancy of the ATP binding sites in our P2X2 H319K receptors is a good example for the complicated reciprocal relationship between ligand binding and channel gating.

Concerning the use of the Hill and Gaddum equation it should be emphasized that already the Hill equation is only an empirical equation to describe relationships between ligand concentration and either ligand binding or any ligand-induced effect. This means that any half-maximum concentrations determined by fits with the Hill equation are mechanistically baseless (Colquhoun, 1998, 2006). Nevertheless, the Hill equation provides a value for a concentration generating a half-maximum binding or activation effect, and with the Hill coefficient a measure for a lower limit of the number of ligands involved. Hence, Hill coefficients exceeding unity, as determined herein, unequivocally tell that a process is not controlled by the binding of a single ligand according to a Langmuir isotherm. And, notably, half maximum concentrations and Hill coefficients can be used for comparing effects, as done here, to compare the concentration dependence of ligand binding and gating: The Hill coefficients for binding and activation gating, f course, the mentioned limitations of the Hill equation are also inherent in the Gaddum equation (Gaddum, 1957) used herein, because this equation is based on the same prerequisites. Nevertheless, within this framework usage of this equation allowed us to estimate a half-maximum concentration for ATP binding to functional channels by displacement experiments with the fluorescent ligand fATP (c.f. Figure 5).

Whereas we have not tested fATP with other P2X subtypes yet, it is conceivable that our novel fluorescent ligand fATP can also be used with other P2X receptors. This would enable fast and safe optical
binding assays on living cells under physiological conditions, thereby avoiding any radioactive labelling or the need of membrane preparation and probe separations after reaching an equilibrium. It would be particularly interesting to see whether the mysterious process of desensitization could be further unravelled by identifying corresponding components of ligand binding at low ligand concentrations and, ideally, as function of time during the running desensitization process as well during its recovery. It seems to be also a good idea to test the binding performance of fATP in metabotropic purinergic P2Y receptors (von Kugelgen & Hoffmann, 2016). Another challenging perspective would be to extend the present studies to measure in P2X channels binding and activation in parallel, as performed previously with fluorescent cyclic nucleotides in HCN2 or cyclic nucleotide-gated channel A2channels (Kusch et al., 2010; Nache et al., 2013). Such combined measurements provide an enormous gain of information with respect to measure either binding or current alone and therefore allow to substantiate kinetic schemes for the activation gating in considerable detail (Biskup et al., 2007; Kusch et al., 2012).

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CONFLICT OF INTEREST
The author(s) declare no competing interests.

AUTHOR CONTRIBUTIONS
C.S. conducted the patch-clamp recordings and analyses and also did the molecular biology. RS did the optical measurements on ligand binding together with C.S. and wrote the analysis software. C.U. contributed with patch-clamp recordings. M.O. did the homology structures. F.S., A.S., and T.S. designed the fluorescent ATP derivative. F.S. performed the chemical synthesis. K.B. designed the study together with C.S. and wrote the manuscript.

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REFERENCES
Bharagava, Y., Nicke, A., & Rettinger, J. (2013). Validation of Alexa-647-ATP as a powerful tool to study P2X receptor ligand binding and desensitization. Biochemical and Biophysical Research Communications, 438, 295–300. https://doi.org/10.1016/j.bbrc.2013.07.058
Biasini, M., Bienert, S., Waterhouse, A., Arnold, K., Studer, G., Schmidt, T., ... Schwede, T. (2014). SWISS-MODEL: Modelling protein tertiary and quaternary structure using evolutionary information. Nucleic Acids Research, 42, W252-W258. https://doi.org/10.1093/nar/gku340
Biskup, C., Kusch, J., Schulz, E., Nache, V., Schwede, F., Lehmann, F., ... Benndorf, K. (2007). Relating ligand binding to activation gating in CNGA2 channels. Nature, 446, 440–443. https://doi.org/10.1038/ nature05596
Chen, V. B., Arendall, W. B. III, Headd, J. J., Keedy, D. A., Immormino, R. M., Kapral, G. J., ... Richardson, D. C. (2010). MolProbity: All-atom structure validation for macromolecular crystallography. Acta Crystallographica Section D, 66, 12–21.
Clyne, J. D., LaPointe, L. D., & Hume, R. I. (2002). The role of histidine residues in modulation of the rat P2X(2) purinoceptor by zinc and pH. Journal of Physiology, 539, 347–359.
Coddou, C., Yan, Z., Oobli, T., Huidobro-Toro, J. P., & Stoijilkovic, S. S. (2011). Activation and regulation of purinergic P2X receptor channels. Pharmacological Reviews, 63, 641–683. https://doi.org/10.1124/pr.110.003129
Colquhoun, D. (1998). Binding, gating, affinity and efficacy: The interpretation of structure-activity relationships for agonists and of the effects of mutating receptors. British Journal of Pharmacology, 125, 923–947. https://doi.org/10.1038/sj.bjp.0702164
Colquhoun, D. (2006). The quantitative analysis of drug-receptor interactions: A short history. Trends in Pharmacological Sciences, 27, 149–157. https://doi.org/10.1016/j.tips.2006.01.008
Ding, S., & Sachs, F. (1999). Single channel properties of P2X2 purinoceptors. Journal of General Physiology, 113, 695–720. https://doi.org/10.1085/jgp.113.5.695
Du, J., Dong, H., & Zhou, H. X. (2012). Gating mechanism of a P2X4 receptor developed from normal mode analysis and molecular dynamics simulations. Proceedings of the National Academy of Sciences of the United States of America, 109, 4140–4145. https://doi.org/10.1073/pnas.1119546109
Egan, T. M., Haines, W. R., & Voigt, M. M. (1998). A domain contributing to the ion channel of ATP-gated P2X2 receptors identified by the substituted cysteine accessibility method. Journal of Neuroscience, 18, 2350–2359.
Egan, T. M., Samways, D. S., & Li, Z. (2006). Biophysics of P2X receptors. Pflugers Archiv. European Journal of Physiology, 452, 501–512. https://doi.org/10.1007/s00424-006-0078-1
Fryatt, A. G., Dayl, S., Cullis, P. M., Schmid, R., & Evans, R. J. (2016). Mechanistic insights from resolving ligand-dependent kinetics of conformational changes at ATP-gated P2X1R ion channels. Scientific Reports, 6, 32918. https://doi.org/10.1038/srep32918
Gaddum, J. H. (1957). Theories of drug antagonism. Pharmacological Reviews, 9, 211–218.
Habermacher, C., Dunning, K., Chataigneau, T., & Gutter, T. (2016). Molecular structure and function of P2X receptors. Neuropharmacology, 104, 18–30. https://doi.org/10.1016/j.neuropharm.2015.07.032
Hamill, O. P., Marty, A., Neher, E., Sakmann, B., & Sigworth, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Archiv. European Journal of Physiology, 391, 85–100. https://doi.org/10.1007/BF00656997
Hattori, M., & Gouaux, E. (2012). Molecular mechanism of ATP binding and ion channel activation in P2X receptors. Nature, 485, 207–212. https://doi.org/10.1038/nature11010
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