Dynaflow® Application Note

Sophisticated Characterization of P2X7 Antagonists - Flexibility and consistency of the Dynaflow Pro II system

Ion channel:
P2X7

Cell type:
HEK293

Chip type:
DF-16

Data courtesy of Evotec, San Francisco, CA

Allows detailed characterisation of agonist/antagonist kinetics

Resolves functional differences between the actions of closely related antagonists

Provides insight into structure-function relationships

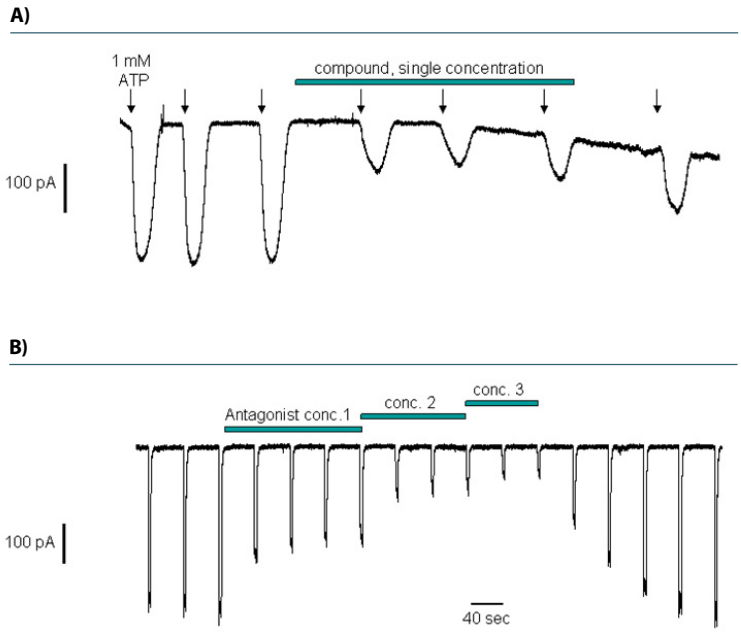
Methods

The P2X7 receptor belongs to the family of ligand gated ion channels which open in response to extracellular ATP. The different members of the purinergic receptor family display vast differences in response kinetics (*1*). The P2X7 receptor has several unusual characteristics that distinguish it from other purinergic ligand-gated channels: It requires high (mM) levels of ATP to open and does not desensitize. Extended activation results in the appearance of a large non-selective pore, and the affinity of the receptor is modulated by extracellular cations (Na^+, Ca^{2+} and Mg^{2+}). P2X7 knock-out mice attenuate disease severity in models of arthritis and neuropathic pain, finding specific antagonists to this receptor is therefore of great interest. The Dynaflow system was used in a patch clamp based study of these receptors. The system offers rapid solution exchange, stable and extended recordings, and it minimizes desensitization and run-down. It also uses very small volumes of reagents.

Results

Dynaflow allows recording of fast dose-response curves with intermittent wash. Repetitive stimulations of the same cell provide an excellent basis for the analysis of the reproducibilityof the current response. P2X7 currents were evoked by 2 seconds exposure to 1mM ATP followed by exposure to saline. Highly consistent current responses, sharp onset and immediate end of the response demonstrate the reliability of the compound delivery. ATP evoked currents were inhibited in the presence of increasing concentrations of antagonist, followed by complete recovery. The strong affinity of the antagonist to the receptor is shown by the slow recovery of the ATP evoked current after exposure to the 3rd concentration of the antagonist.

Figure 1



Inward currents elicited by repetitive exposure to extracellular ATP (1mM), time scale for A and B identical.
A) *Gravity driven perfusion (controlled by a 8-port-valve): ATP exposure followed by ATP exposure in the presence of antagonist at one concentration.*
B) *Compound delivery system: Dynaflow Pro II Platform using a 16 channel chip, the DF-16. ATP exposure for 2s followed by intermittent wash periods either by saline or saline containing increasing concentrations of antagonist. For the trace shown a single cell has been exposed to 10 of the fluid compartments of the 16 channels available in a DF-16 chip. The dose-re-sponse experiment was completed in about 8min. The following 4min were used to determine the binding properties of the antagonist in greater detail. After exposure to the antagonist (conc.3) was completed a series of ATP stimulations, interdigitated with saline, showed the reactivation of the current response.*

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P2X7 Antagonist Characteristics

RN-1 and RN-2 are both potent antagonists as determined by binding studies, IL-1b release and electrophysiology. However, closer examination with the Dynaflow Pro II system reveals differences in the function of the two compounds. The antagonist RN-1 showed slow on-rate of the block, but resulted in a complete inhibition of the ATP evoked current. The antagonist RN-2 had a fast on-rate but only evoked a partial inhibition of the ATP evoked current. For comparison, the P2X7 antagonist decavanadate ($V_{10}O_{28}^{6-}$) is shown. Decavanadate is described as a reversible and competitive antagonist of the P2X7 receptor (2). Decavanadate, applied together with ATP, resulted in a complete block of the inward current. RN-1 applied together with the same concentration of ATP only resulted in a partial block. Adding decavanadate to the partially

Binding site analysis

Binding data indicate that RN-1 and RN-2 may bind to the same binding site but that this site is different from the ATP binding site. When the two antagonists were applied together, the ATP activated current is partially blocked by RN-2, consistent with an allosteric mechanism. RN-2 completely prevented any further reduction of the remaining ATP current, indicating that the binding site for RN-1 was already occupied by RN-2. Extended washing was necessary to remove RN-2 and allow RN-1 to inhibit ATP induced current again (Fig. 3). This result is consistent with the binding data that suggests RN-1 and RN-2 occupy the same allosteric site.

Conclusion

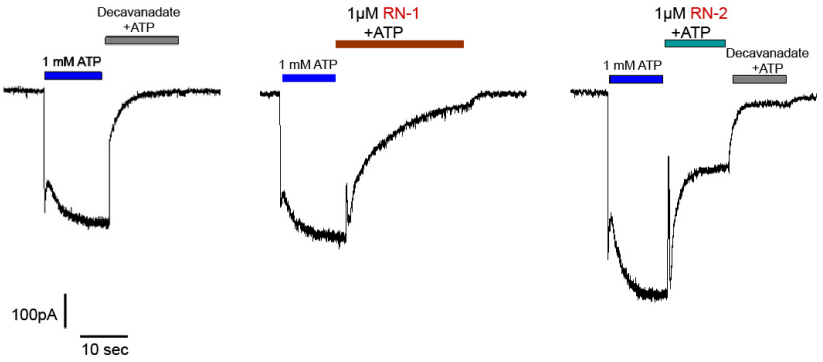
The Dynaflow system provides a quick and efficient way to deliver a series of different compounds or combinations of compounds to a single cell over a period of time. The system gives hour-long stable recordings, rapid solution exchange, minimized run-down, experimental flexibility, and the possibility to resolve mechanisms of action of agonists and antagonists. In this study the two P2X7 antagonists RN-1 and RN-2 were characterized. RN-1 was shown to produce complete block while RN-2 only produced partial block. RN-1 and RN-2 are non-competitive with respect to ATP binding and activity but appear to occupy the same allosteric binding pocket.

References

1. North RA. Molecular physiology of P2X receptors. Physiol Rev. 2002 82(4):1013-67.

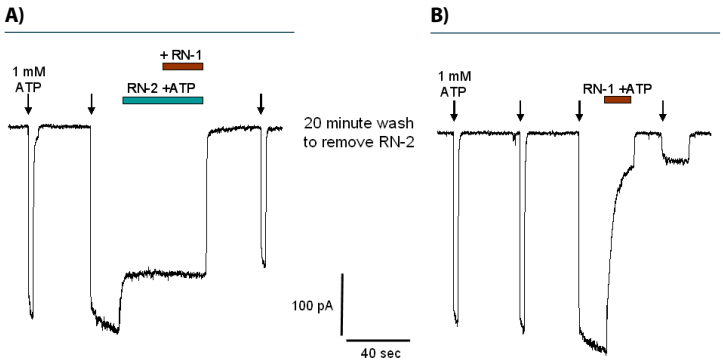
2. Michel AD, Xing M, Thompson KM, Jones CA, Humphrey PP. Decavanadate, a P2X receptor antagonist, and its use to study ligand interactions with P2X7 receptors.Eur J Pharmacol. 2006 534(1-3):19-29.

Figure 2



Characterization of two antagonists, RN-1 and RN-2, compared to the well known P2X7 antagonist decavanadate. Current was evoked by exposure to ATP (1mM) and antagonist was coapplied as shown by the bars.

Figure 3



Allosteric binding mechanism of RN-1 and RN-2.
***A)** Control response elicited by 1mM ATP, followed by a second application of ATP. During the second ATP application the partial antagonist RN-2 was applied. This resulted in a partial inhibition. Additional RN-1 perfusion did not reduce the inward current further. **B)** After a wash out time of 20min two control applications of ATP showed complete recovery of the ATP evoked current. Following direct coapplication of ATP and RN-1 the inward current was almost completely blocked.*

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