SEVERAL P2X RECEPTOR ANTAGONISTS, INCLUDING NF279 AND PPADS SUPPRESS GABA RESPONSES IN ISOLATED HIPPOCAMPAL NEURONS.

Storozhuk, M.1,2; Yatsenko, N.1; Nikolaenko, L.1; Krishtal, O.1,2
1A.A. Bogomoletz Institute of Physiology, National Academy of Sciences of Ukraine
2State Key Laboratory of Molecular and Cellular Biology; Bogomoletz st. 4, Kiev, Ukraine
*maksim@biph.kiev.ua

Abstract
Pharmacological analysis is one of the major tools in physiology. Therefore, it is important to be aware of non-specific effects of receptor agonists/antagonists. Beside other questions, pharmacological analysis is widely used to study a question of corelease of different neurotransmitters. Because concentration of transmitter in synaptic cleft is rather high, and only competitive antagonists are often available, high concentration of the antagonists are required to block the receptor of interest, making question of specificity even more important. Considering important earlier observation (Nakazawa et al., 1995) regarding suppressing side-effect of broad spectrum antagonist of P2X-receptors suramin on currents evoked by exogenous GABA application (GABA-currents), we studied effects of two other antagonists NF279 (8,8'-[Carbonylbis(imino-4,1-phenylenecarboxylimino-4,1-phenylenecarboxylimino)] bis-1,3,5-naphthalenetrisulfonic acid hexasodium salt) and PPADS (pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid) on GABA-currents in isolated hippocampal neurons. We found that NF279 (20 µM) and PPADS (50 µM) decreased GABA currents: NF279 to ~ 62% ; PPADS to ~ 71% respectively. Additionally, we have confirmed earlier observation regarding suppressing effect of suramin (20 µM) on GABA-currents. We conclude that interpretation of data obtained using relatively high concentrations of NF279, PPADS and suramin for determining the functional role P2X receptor in complex neural networks should be careful. Our results are also essential for improving the selectivity of P2X-receptor antagonists versus GABA-receptors.

Key words: P2X receptors, GABA-receptors, residual currents, corelease, suramin, NF279, PPAD
Introduction
In spite of advances in molecular biology, pharmacological analysis is still one of the major analytical tools in physiology. Therefore, it is important to be aware of non-specific effects of receptor agonists/antagonists. Beside other questions, pharmacological analysis is widely used to study a question of corelease of different neurotransmitters, in particular that of ATP and GABA. Because concentration of transmitter in synaptic cleft is rather high, and only competitive antagonists are often available, high concentration of the antagonists are required to block the receptor of interest. Indeed, suramin at 30 µM and PPADS at 50 µM were used to address question of GABA and ATP corelease in spinal [1] and lateral hypothalamic neurons [2]. Even higher concentrations of these antagonists (1 mM of suramin and 1 mM of PPADS) were used to study fast synaptic transmission mediated by P2X receptors in CA3 pyramidal cells [3].

Considering important earlier observation [4] regarding suppressing side-effect of broad spectrum antagonist of P2X-receptors suramin on currents evoked by exogenous GABA application (GABA-currents), we studied effects of two other antagonists NF279 and PPADS on GABA-currents in isolated hippocampal neurons.

Methods

Animals
Albino Wistar rat pups were housed with their dams under a constant 12/12 h light/dark cycle at 22–24°C in the institutional animal facility and removed from the litter no more than half an hour before sacrifice. All procedures used in this study were approved by the Animal Care Committee of Bogomoletz Institute of Physiology and conform to the Guidelines of the National Institutes of Health on the care and use of animals.

For studying responses evoked by application of exogenous GABA (GABA-responses) we used isolated hippocampal neurons.

Cell preparation
Wistar rats (12-17 d old age) were decapitated under ether anesthesia, and the hippocampus was removed and cut into slices (300-500 µm) in a solution containing the following (in mM): 150 NaCl, 5 KCl, 1.25 NaH₂PO₄., 26 NaHCO₃, 1.1 MgCl₂, and 10 glucose, pH 7.4. Then, the slices were incubated for 10 min hippocampal at 32°C with 0.5 mg/ml of protease (type XXIII) from Aspergillus oryzae.

Single pyramidal cells from CA1 and CA3 stratum pyramidale layers were isolated by vibrodissociation locally in the stratum pyramidale.

After isolation, the cells were usually suitable for recordings for 2-4 h. Throughout the entire procedure, the solutions with the slices were continuously saturated with 95% O₂ and 5% CO₂ gas mixture to maintain pH 7.4.

Current recordings
GABA-activated currents in isolated neurons were induced by the step application of agonists in the “concentration-clamp” mode [4], using the computerized Pharma-Robot set-up (Pharma-Robot, Kiev, Ukraine). This equipment allows a complete change of saline within 15 ms. Transmembrane currents were recorded using a conventional patch-clamp technique in the whole-cell configuration. The intracellular solution contained the following (in mM): 100 CsF, 35 CsCl, 10 HEPES, 10 EGTA, 5 Mg-ATP, and 0.5 GTP, pH 7.2. The composition of extracellular solution was as follows (in mM): 130 NaCl, 2 CaCl₂, 5 KCl, 1 MgCl₂, 10 HEPES-NaOH, 10 Glucose, pH 7.4. GABA responses were recorded with a 2 min interval. P2X antagonists used in this study: 8,8’-[Carbonylbis[imino-4,1-phenylene carbonylimino-4,1-phenylene carbonylimino]]bis-1,3,5-naphthalenetrisulfonic acid hexasodium salt (NF279, Tocris), pyridoxal phosphate-6-azophenyl-2’,4’-disulfonic acid (PPADS) and suramin (8,8’-[Carbonylbis[imino-3,1-phenylenecarboxylimino-4-methyl-3,1-phenylene]carboxylimino]]di(1,3,5-naphthalenetrisulfonic acid). Experiments were done at room temperature (20°-22°C). Unless noted otherwise, chemicals were obtained from SIGMA. All potentials indicated were corrected for measured liquid junction potentials as suggested earlier [5]. Digitized currents were analyzed using ANDATRA software kindly provided by Yaroslav Boychuk (A.A. Bogomoletz Institute of Physiology, Kiev, Ukraine).

The data are presented as a mean ± S.E.M, Student’s t-test was used for statistical comparisons.

Results
As already mentioned, suppressing side-effect of broad spectrum antagonist of P2X-receptors suramin on currents evoked by exogenous GABA application (GABA-currents) in hippocampal cell cultures has been previously reported [6]. We first verified whether this side effect is also present in acutely isolated hippocampal neurons. We found that preincubation in 20 µM of suramin substantially reduces the amplitudes of GABA-currents (not
illustrated). On average GABA currents were reduced to 41.9 ± 2.9 % of control (n=4; P<0.01; paired t-test). The effect was reversible upon suramin washout (94.1 ± 4.7 % of control).

We next examined whether some other often used antagonists of P2X receptor have similar effects on GABA-currents. Namely, we studied effects of NF279 (20 µM), and PPADS (50 µM). We found that NF279 (20 µM) reduced GABA currents to 61.8 ± 6.5 % (n=5; P<0.01; paired t-test). Results from a typical experiment are shown in Fig. 1. The effect was reversible upon washout (93.2 ± 8.46 % of control).

Like suramin (20 µM) and NF279 (20 µM), PPADS (50 µM) in our experiments also reduced GABA currents, however, in contrast to the above-mentioned effects, no apparent restoration was observed during 4-minute washout (Fig. 2). On average, PPADS (50 µM) reduced GABA currents to 70.9 ± 4.7 % (n=6; P<0.01; paired t-test). After 4-minute washout the amplitude of GABA currents was still to 72.9 ± 4.9 %. Lack of GABA-currents restoration upon PPADS washout in our experiments is probably due to slowly-reversible manner of action of this antagonist reported earlier [7].

Discussion

‘ATP has long been known as the main source of energy in living cells. However, a further fundamental role of ATP was later identified, namely that extracellular ATP serves as a messenger for fast intercellular communications via binding to and activation of a set of membrane proteins termed P2X receptors.’ [8] In spite of large achievements, revealing the latter role of ATP in different structures is still in progress, thus knowledge about selectivity of available pharmacological tools, used to address this question is important. In this work we extended previous conclusion regarding suppressing effect of suramin on GABA-currents made for hippocampal cell in culture (6) to acutely isolated hippocampal neurons. Additionally, we report that two other antagonists of P2X receptors also affect GABA-currents in isolated hippocampal neurons. Namely, NF279 (20 µM) and PPADS (50 µM) have noticeable effects on GABA-currents. Our results regarding inhibitory effect of PPADS on GABA-currents are in line with earlier report of Donato et al. 2008 [9]. In addition to effect of PPADS (30 µM) on the frequency of miniature GABAergic synaptic currents these authors also observed smaller (14%) but significant decrease of miniature synaptic currents amplitude. The latter kind of effects is generally thought to reflect postsynaptic receptor properties. While the concentrations of NF279 are and PPADS we used in our experiments are rather high, they are relevant for addressing question of ATP corelease. Indeed, because of competitive mechanism of action of these antagonists on P2X receptors and high concentration of transmitter (ATP) in synaptic cleft, they have to be used at high concentrations. For instance, PPADS at 50 µM was used to address question of GABA and ATP corelease [1], [2]. Even higher concentrations of these antagonists (1 mM of suramin and 1 mM of PPADS) were used to study fast synaptic transmission mediated by P2X receptors in CA3 pyramidal cells [3]. It has been previously found that mechanism of suramin effect on GABA receptors is rather non-competitive, since GABA-currents evoked by different concentrations of the agonist were decreased to a similar degree in suramin presence [6]. We think that this also quite likely to be the case for NF279 and PPADS because of chemical similarity of these antagonists to suramin. Nevertheless the question about exact mechanism of action needs further investigation. In particular, possibility that the effects we observed in our experiments are mediated by subcellular interactions between P2X and GABA receptor channels cannot be excluded. In this regard we should mention that negative cross talk between GABA and cationic P2X receptors has been previously reported in myenteric [10] and DRG neurons [11]. Nevertheless, regardless of exact mechanism of action, our finding is important in context of addressing physiological importance of P2X receptors in neuronal networks using higher concentrations of these antagonists. Indeed, considering that it has been shown previously that ATP is coreleased with the inhibitory neurotransmitter GABA by dorsal horn [1] and lateral hypothalamic neurons [2] and activates postsynaptic P2X receptors, our initial aim was to test whether it could be also a case for synaptic responses evoked by stimulation of GABAergic hippocampal neurons. We addressed this question using pharmacological approach in hippocampal cell cultures (Storozhuk et. unpublished observations). We found that: 1) suramin suppresses ‘residual’ synaptic currents at GABAergic synapses; 2) NF279 (20 µM) and PPADS (50 µM) decrease synaptic GABAergic responses. While these results alone suggest involvement of endogeneous ATP and P2X-receptors in mediation or modulation of synaptic responses in hippocampal cell cultures, this interpretation of the results becomes questionable if considered together with results we obtained on
isolated hippocampal neurons. Especially, considering that quantitatively suppressing effects of NF279 and PPADS on GABA PSCs in cell cultures and GABA-currents in isolated neurons are rather similar. GABA currents and GABA PSCs were decreased: to ~ 62% and ~ 66 % of control in presence NF279; to ~ 77% and to ~ 70% in presence of PPADS.

**Conclusion**
Interpretation of the results obtained using relatively high concentrations of suramin, NF279 and PPADS for determining the functional role P2X receptor in complex neural networks should be careful. Our results are also essential for improving the selectivity of P2X- receptor antagonists versus GABAA-receptors.

**Acknowledgments**
This work was partially supported by grant DFFD F 46.2/001.

**References**
Figure 1. NF279 decreases responses evoked by exogenous GABA application. Effect of NF279 (20 µM) on GABA-currents. GABA (100 µM) was applied every 2 minutes. For the responses with NF279, the compound was present before and during GABA-application. Vh=-70 mV. Upper panel - examples of original current traces. Lower panel - graph summarizing results of the experiments.

Figure 2. PPADS suppresses responses evoked by exogenous GABA application. Effect of PPADS (50 µM) on GABA-currents. GABA (100 µM) was applied every 2 minutes. For the responses with PPADS, the compound was present before and during GABA-application. Vh=-70 mV. Upper panel - examples of original current traces. Lower panel - graph summarizing results of the experiments.