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Artículo original

Glutamate determinations using Amplex Red Glutamic Acid Assay are affected by P2X agonist BzATP

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ABSTRACT

Amplex Red (AR) reagent is the most stable and sensitive fluorogenic substrate known for horseradish peroxidase (HRP). Thus, in the presence of hydrogen peroxide (H_2O_2), AR (a nonfluorescent compound) is converted to resorufin (a strongly fluorescing product) by the action of HRP. In the last years, multiple assays have been developed using this reagent to quantify a diverse assortment of analyses and also to detect the activity of many different enzymes. We recently showed that BzATP, agonist of several ionotropic nucleotide receptors (P2X), interfered with Amplex Red oxidation catalyzed by HRP. In the present work we reported that glutamate determinations using Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit are also strongly interfered by BzATP. These data are really interesting because demonstrate that resorufin fluorescence, in the presence of BzATP, is not proportional to glutamate concentration and, therefore, it must not be used as method to measure glutamate concentration.

Key words: Amplex Red.—Glutamate.—P2X.—BzATP.—Interferences.

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RESUMEN

Las determinaciones de glutamato utilizando Amplex Red Glutamic Acid Assay están afectadas por el agonista P2X BzATP

El compuesto Amplex Red (AR) es el sustrato fluorogénico para la peroxidasa de rábano (HRP) más estable y sensible. Así, en presencia de peróxido de hidrógeno (H_2O_2), el AR (un compuesto no fluorescente) es convertido en resorufina (un producto fuertemente fluorescente) por la acción de la HRP. En los últimos años se han realizado muchos ensayos utilizando este compuesto para cuantificar un variado surtido de análisis, así como para detectar la actividad de muchas y variadas enzimas. Recientemente hemos demostrado que el BzATP, un agonista de varios receptores ionotrópicos de nucleótidos (P2X), interfiere con la oxidación del Amplex Red catalizada por la HRP. En el presente trabajo mostramos que las determinaciones de glutamato utilizando el kit Amplex Red Glutamic Acid/Glutamate Oxidase Assay están fuertemente interferidas por el BzATP. Estos datos son realmente interesantes porque demuestran que la fluorescencia de la resorufina, en presencia del BzATP, no es proporcional a la concentración de glutamato y, por lo tanto, no debe ser utilizada como un método para medir la concentración de glutamato.

Palabras clave: Amplex Red.—Glutamato.—P2X.—BzATP.—Interferencias.

INTRODUCTION

Amplex Red (AR) reagent is the most stable and sensitive fluorogenic substrate known for horseradish peroxidase (1). A variety of novel fluorogenic and chromogenic assays for enzymes that produce hydrogen peroxide has been developed using this compound. Thus, these coupled assays permit the ultrasensitive quantitation of a diverse assortment of analytes, including glucose, galactose, cholesterol, glutamic acid, xanthine (or hypoxanthine), uric acid, choline, acetylcholine and hydrogen peroxide, as well as to detect the activity of many different enzymes (e.g. phospholipase D, phosphatidylcholine-specific phospholipase C, sphingomyelinase, ...) (Table 1).

We recently have reported that the precision of such determinations are affected by the presence in the medium of the ATP analog, BzATP (2'-3'-o-(4-benzoylbenzoyl)-adenosine 5'-triphosphate), an agonist of the ionotropic P2 receptors, $P2X_1$, $P2X_2$,

$P2X_3$ and $P2X_7$ (2, 3). Thus, we showed that AR, in the absence of H_2O_2, was quickly oxidized when BzATP was present. Furthermore, we obtained first evidences that glutamate determinations using Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit were also strongly interfered by BzATP by using individual measurements in a fluorescence emission spectra (LS55 spectrofluorimeter from Perkin Elmer). In the present work we showed how this interference can lead to misinterpret the results and, therefore, to hypothesize effects that do not correlate with the ones mediated through ionotropic nucleotide receptors (P2X) in biological systems.

TABLE 1. *Examples of Amplex Red-based assays. The limits of detection of the corresponding metabolites or enzymatic activities are shown*

Metabolite	*Enzymatic Activity*
Amplex Red Catalase Assay Kit	50 mU/ mL
Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit	1.2 x10^{-5} U/mL
Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit 10 nM	40 µU/mL
Amplex Red Glucose/Glucose Oxidase Assay Kit 3 µM	0.05 mU/mL
Amplex Red Galactose/Galactose Oxidase Assay Kit 4 µM	2 mU/mL
Amplex Red Cholesterol Assay Kit 5 ng/mL	
Amplex Red Acetylcholine/Acetylcholinesterase Assay Kit 0.3 µM	0.002 U/mL
Amplex Red Uric Acid/Uricase Assay Kit 20 nM	0.2 mU/mL
Amplex Red Xanthine/Xanthine Oxidase Assay Kit 200 nM	0.1 mU/mL

METHODS

Culture of granule cells

All experiments carried out at the Universidad Complutense of Madrid followed the guidelines of the International Council for Laboratory Animal Science (ICLAS). Cerebellar cultures were

prepared following procedures described by León *et al.* (2006) (4). Cerebella from three Wistar rat pups (P7) were removed aseptically, washed once in Earl's balanced salt solution (EBSS; Gibco BRL), cut into small pieces and transferred to a screw cap tube. Tissue fragments were allowed to settle, excess EBSS was aspirated, and 4 mL of EBSS containing 100 U/mL DNAse (Worthington, Lake Wood NJ), 2.5 mM $CaCl_2$ and 2.5 mM $MgCl_2$ were added. Sixty-five units of papain (Worthington) were added after it was preactivated (30 min at 37º C) in 1 mL of EBSS. Air in the tube was displaced with 95% O_2-5% CO_2 and the tube was incubated at 37º C for 40 minutes on a shaking platform. Undigested fragments were allowed to settle and the supernatant was centrifuged at 208 g for 5 min. The pellet was resuspended in 3 mL of EBSS containing 4.5 mg of ovomucoid protease inhibitor layered onto an albumin cushion, which consisted of 3 mL of EBSS containing ovomucoid protease inhibitor (Worthington) and ovoalbumin (Worthington) at 15 mg/mL each, and the resuspended pellet was centrifuged at 172 g for 5 min. The resulting pellet was finally resuspended in Neurobasal medium (Gibco BRL) and the cell number and viability was assessed. The dissociated cells were plated at a final density of 0.1-0.3 X 10^6 cells/cm^2 on poly-L-lysine-coated glass coverslips or 24-wells plates (2 cm^2 surface area/well) in Neurobasal medium supplemented with B27 (Gibco, BRL), 100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.25 µg/mL amphotericin B (Sigma), 21 mM KCl and 2 mM glutamine (Sigma), and were maintained in a humidified incubator at 37º C in 5 % CO_2. After 24 hours Neurobasal medium was replaced by new fresh medium containing 100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.25 µg/mL amphotericin B, 21 mM KCl and 0.5 mM glutamine. The culture medium was replaced every 3 days afterwards.

Glutamate release experiments

For the glutamate release experiments, cerebellar granule neurons were plated at a final density of 0.3 X 10^6 cells/cm^2 on 24-wells plates (2 cm^2 surface area/well) pre-coated with 0.1 mg/mL poly-L-lysine (Biochrom AG, Berlin). 7 to 14 days-old cells were washed twice and incubate for 1 hour at 37º C with Locke's solution. 5 minutes before

to start the experiment, Locke's solution was changed by a new one containing 50 μM PDC in order to block the glutamate reuptake systems. Then, granule neurons were stimulated for 1 min with BzATP (100 nM-200 μM) in Locke's solution without Mg^{2+}. All stimulations were performed in the presence of 3 U/ml of ADA in order to avoid the presence of adenosine in the medium. Finally, the medium was aspired and the glutamate content was measured using the Amplex Red Glutamic Acid Assay Kit (Molecular Probes) according to the protocol provided by the manufacturer. The resorfurin fluorescence was detected with a BMG Labtechnologies 96 plate reader using excitation filter at 544 nM and emission filter at 590 nM.

Analyses of membrane pore formation

To analyze wheter BzATP induced pore formation, cultured granule cells (14 days old) were exposed for 5 min with BzATP (without Mg^{2+}) in presence of 2 μM YO-PRO-1, the cell-impermeant dye, that becomes fluorescent when interacting with DNA. Changes in fluorescence were continuosly monitored using a fluorescence microscopy setting excitation wavelength at 490 nm and emission at 510 nm. At the end of each experiment positive control was carried out making permeable granule cell with 10% triton X-100.

Statistical analysis

Data are presented as mean ± S.E.M. of al least 3 independent experiments. Comparisons between experimental samples and controls were carried out using Student's t-test . Dose-response curve showing the effect of BzATP on glutamate release were fitted using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California.

RESULTS AND DISCUSSION

Previous works have shown that $P2X_7$ receptor activation, and also reported for $P2X_4$, $P2X_2$, $P2X_2/P2X_3$, can lead to open a large pore through which glutamate can reach the extracellular medium (5, 6). Since $P2X_7$ is highly expressed in granule neurons where distributed, mainly, along neuronal fibers (7) we decided to measure glutamate content in the extracellular media following $P2X_7$ receptor activation in order to test whether $P2X_7$ receptor could also open a pore in these cells. Thus, granule neurons growth in culture for 14 days were stimulated with BzATP for 1 minute and the glutamate content was measured using the Amplex Red Glutamic Acid Assay Kit (Molecular Probes) according to the protocol provided by the manufacturer.

The quantification of glutamate using the Amplex® Red Glutamic Acid/Glutamic Oxidase Assay Kit provides an ultrasentive method for detecting glutamic levels as low as 10 nM. We also tried to measure the glutamate release utilising the enzyme-linked fluorometric assay based on glutamate dehydrogenase activity and the changes in the fluorescence of NADPH (8), but the glutamate content in the media was under the limits of detection by this technique (data not shown). As it can be observed in Figure 1 the principle of the assay is based

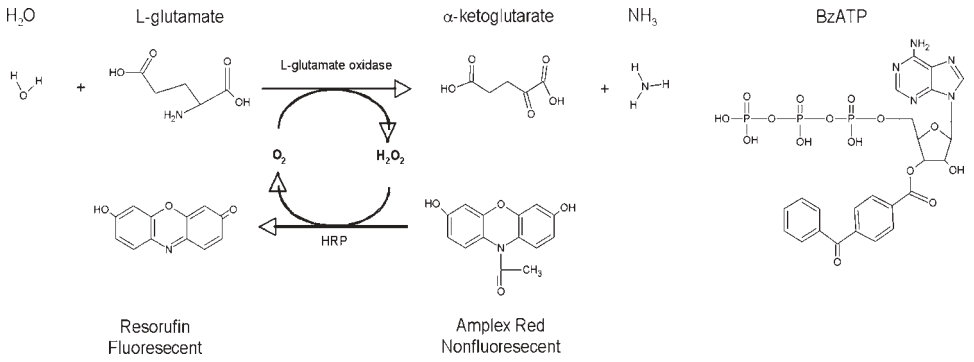

FIGURE 1. ***Principle of coupled enzymatic assays using Amplex Red reagent.*** *Oxidation of L-Glutamate by glutamate oxidase results in generation of H_2O_2, which is coupled to conversion of the Amplex Red reagent to fluorescent resorufin by HRP. The detection scheme shown here is used in Amplex® Red Glutamic Acid/Glutamate Oxidase Assay Kit.*

in the oxidation of L-glutamic acid by glutamate oxidase to produce α-ketoglutarate, NH_3 and H_2O_2. In a subsequent reaction H_2O_2 is reduced by horseradish peroxidase (HRP) using Amplex red reagent (10-acetyl-3, 7 dihidroxiphenoxazine) as an electron donor which is transformed to resorufin, a fluorescent compound that has excitation/emission maxima of ~540/585 nm.

Following the indications of the protocol, a working solution, containing 50 µM AR, 0.125 U/ml HRP, 0.04 U/ml L-glutamate oxidase, 0.25 U/ml L-glutamate pyruvate transaminase and 100 µM L-alanine in 100 mM Tris-HCl, pH 7.5 buffer, was prepared. Next, several concentrations of L-Glu (100 nM- 4 µM) were added and incubated at 37° C for 30 min. Finally, resorufin fluorescence was detected with a BMG Labtechnologies 96 plate reader using excitation filter at 544 nM and emission filter at 590 nM. As it can be observed in Figure 2, the resorufin fluorescence was glutamate concentration-dependent in the range assayed.

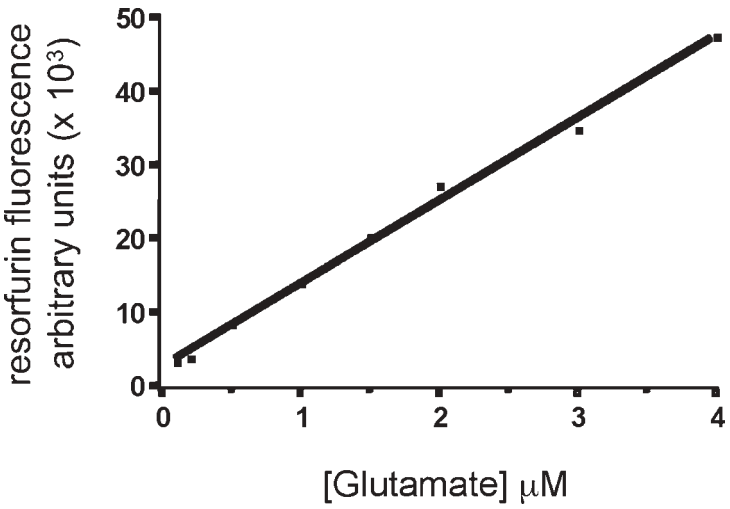

FIGURE 2. ***Detection of L-glutamic acid using the Amplex® Red reagent-based assay.*** *Each reaction contained 50 µM Amplex® Red reagent, 0.125 U/mL HRP, 0.04 U/mL L-glutamate oxidase, 0.25 U/mL L-glutamate-pyruvate transaminase, 100 µM L-alanine and the indicated amount of L-glutamic acid in 1X reaction buffer. Reactions were incubated at 37° C. After 30 minutes, resorfurin fluorescence was detected with a BMG Labtechnologies 96 plate reader using excitation filter at 544 nM and emission filter at 590 nM.*

Once we knew that resorufin fluorescence was dose-dependent in the range nano-micromolar of glutamate, we decided to investigate if extracellular glutamate could increase in response to pore formation after $P2X_7$ receptor activation. For that purpose, granule neurons (14 days old) were stimulated for 1 min with several concentrations of BzATP (100 nM-200 µM), in absence of magnesium, since this ion has been shown to inhibit $P2X_7$ receptor (2). Next, medium was taken and the glutamate content was measured using Amplex® Red Glutamic Acid/Glutamic Oxidase Assay Kit. As it can be observed in Figure 3a, BzATP induced an increase in resorufin fluorescence that was dose-dependent. Initially, these data seems to indicate that BzATP induced pore formation through which glutamate reached extracellular medium since the profile of glutamate release did not fit to a saturation kinetic. To verify this hypothesis, granule neurons were stimulated with BzATP and ATP in presence of YO-PRO-1 as indicated in methods section. The lack of variations in the YO-PRO-1 fluorescence after granule cell exposure to BzATP (100 µM) or ATP (100 µM) for 5 minutes discarded the possibility that prolonged activation of $P2X_7$ could induce pore formation in the plasma membrane of granule neurons (Figure 3b). Therefore, the non-saturable profile of the dose-response curve was caused by the presence of BzATP in the incubation medium and not by a lineal glutamate release through pore.

In a recent work (9) we showed that BzATP, and other molecules that contain 4-benzoylbenzoyl groups in their structure, interfere in the AR oxidation reaction catalyzed by HRP. Thus, BzATP enhances the AR oxidation mediated by HRP in the absence of externally added H_2O_2. This effect is produced by benzophenone group of BzATP molecule since other ATP agonists did not reproduce BzATP interferences (9). Therefore, we decided to investigate if resorufin fluorescence increase observed in Figure 3 could be a consequence of BzATP interference on Amplex Red oxidation. For that purpose, a working solution containing 50 µM AR, 0.125 U/ml HRP, 0.04 U/ml L-glutamate oxidase, 0.25 U/ml L-glutamate pyruvate transaminase and 100 µM L-alanine was prepared following the protocol of Amplex® Red Glutamic Acid/Glutamic Oxidase Assay Kit. This working solution was supplemented with 0.1 µM, 0.5 µM and 1 µM L-Glu respectively and incubated for 30 min at 37º C. Resorufin

fluorescence showed the following values, showed in Figure 4: Glu 0.1 µM, 909 ± 128 a.u. (n = 3); Glu 0.5 µM, 2713 ± 347 a.u. (n = 3); Glu 1 µM, 5621 ± 257 a.u. (n = 3). All these values were significantly enhanced in presence of 10 µM BzATP (Glu 0.1 µM, 1851 ± 304 a.u., n = 3, p < 0.01; Glu 0.5 µM 4362 ± 294 a.u., n = 3, p < 0.01; Glu 1 µM, 806551 ± 407 a.u., n = 3, p < 0.01) and 100 µM BzATP (Glu 0.1 µM, 5904 ± 935 a.u., p < 0.001; Glu 0.5 µM 8216 ± 1103 a.u., p < 0.001; Glu 1 µM 12371 ± 461 a.u., p < 0.001).

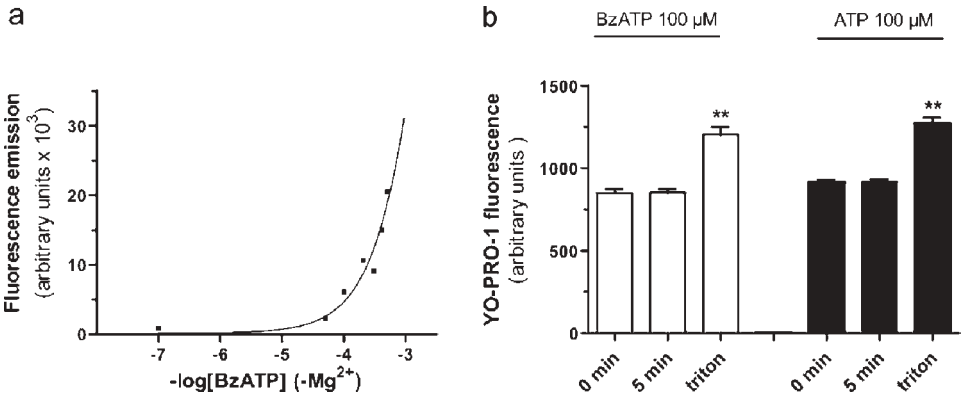

FIGURE 3. ***(a)*** *BzATP interferes with Amplex® Red Glutamic Acid/Glutamic Oxidase Assay Kit. Granule neurons (14 days old) were stimulated for 1 min with several concentrations of BzATP (100 nM-200 µM), in absence of magnesium, and the medium was taken and the glutamate content was measured using Amplex® Red Glutamic Acid/Glutamic Oxidase Assay Kit. As it can be observed in this graph, BzATP induced an increase in resorufin fluorescence that was dose-dependent.* ***(b)*** *Graph show that neither BzATP nor ATP stimulation induce pore formation in granule neurons in culture. As positive control, granule neurons were permeabilized with triton and an increase in YOPRO fluorescence could be detected.*

Data reported in this work confirm that BzATP interferes the measurement of glutamate using Amplex® Red Glutamic Acid/ Glutamic Oxidase Assay Kit. Thus, the measurement of glutamate in the media after stimulation with BzATP seems to indicate that P2X induced pore formation through which glutamate reached extracellular medium. However, the resorufin fluorescence was not due to an increase in glutamate content but BzATP interfered on Amplex Red oxidation catalyzed by HRP. These data are really

interesting because demonstrate that resorufin fluorescence, in the presence of BzATP, is not proportional to glutamate concentration and, therefore, it must not be used as method to measure glutamate concentration. This recommendation is applicable to the large variety of assays that also used Amplex Red for quantitating specific biological compounds (cholesterol, galactose, glucose, …) or enzymes (monoamine oxidase, PLD, …).

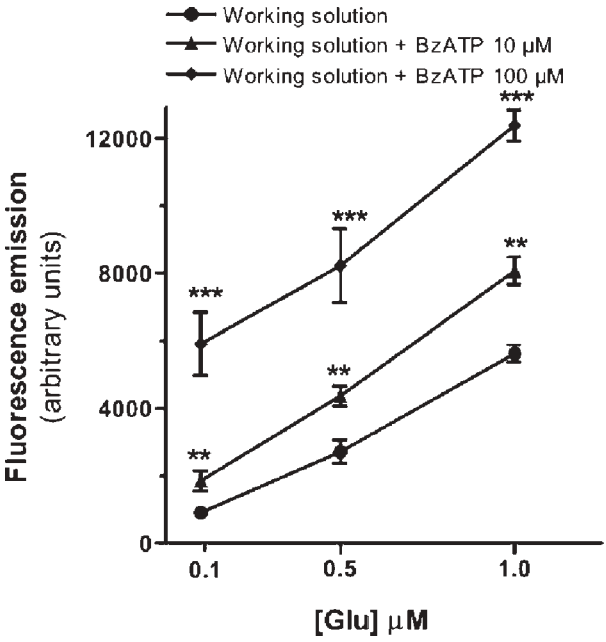

FIGURE 4. ***Glutamate determination using Amplex® Red Glutamic Acid/ Glutamic Oxidase Assay Kit in absence or presence of BzATP.*** *A working solution containing 100 µM Amplex® Red reagent, 0.25 U/mL HRP, 0.08 U/mL L-glutamate oxidase, 0.5 U/mL L-glutamate-pyruvate transaminase, and 200 µM L-alanine was supplemented with different glutamate concentrations (0.1, 0.5 and 1 mM) alone or with BzATP (10 and 100 mM). After incubation at 37º C, resorufin fluorescence was measured as indicated in methods section.*

In that sense, in a recent report Kukley *et al.* (10) showed that catabolism of BzATP, and subsequent adenosine A_1 receptor activation, depressed field potentials (fEPSP) at hippocampal synapses. This conclusion was reached analysing BzATP hydrolysis

by diphosphohydrolases and nucleotidases and measuring the amount of inorganic phosphate released with amplex red based-Pi Per Phosphate Assay Kit. However, they incubated together BzATP and HRP to know the phosphate concentration. Thus, in agreement with the results obtained in this work it could be possible that resorfurin fluorescence resulted affected by BzATP and not corresponded, therefore, completely to BzATP catabolism. Similarly, Parvathenani *et al.* (11) studied the role played by $P2X_7$ receptor in superoxide production in primary microglia. Thus, superoxide (O^-_2) was measured indirectly through the detection of hydrogen peroxide (H_2O_2) by using an Amplex Red based assay. Thus, microglia cells were stimulated with BzATP in a 96-well plate containing 0.2 units/mL horseradish peroxidase and 50 µM Amplex Red. It is relevant to indicate that Amplex Red and BzATP were incubated together. Accordingly to the results reported in this work where HRP-mediated Amplex Red oxidation, in presence of BzATP, is not proportional to H_2O_2 formed, it seems logical to hypothesize that resorufin fluorescence detected by Parvathenani and co-workers are not completely due to superoxide production but BzATP-mediated resorufin production could also be involved.

In summary, the measurement of glutamate concentration by Amplex® Red Glutamic Acid/Glutamic Oxidase Assay Kit results altered when BzATP is present in the medium. Thus, Amplex Red oxidation catalyzed by HRP is significantly enhanced by BzATP, even in absence of H_2O_2. Taking into account that Amplex Red oxidation by HRP is a shared reaction in Amplex Red-based assays, extra caution is needed to take when these assays are used with BzATP in order to avoid misinterpretations of the results.

ACKNOWLEDGMENTS

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