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Immobilized $P2X_2$ purinergic receptor stationary phase for chromatographic determination of pharmacological properties and drug screening

Cleber A. Trujillo [a], Paromita Majumder [a], Fernando A. Gonzalez [b], Ruin Moaddel [c], Henning Ulrich [a,*]

[a] *Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo 05508-900, Brazil*
[b] *Department of Chemistry, University of Puerto Rico, San Juan PR 00931-3346, USA*
[c] *Gerontology Research Center, National Institute on Aging, National Institutes of Health, Baltimore 21224-6825, USA*

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Abstract

The purinergic receptor signaling system plays an important role in communication between cells in the nervous system and opens new opportunities for screening of potential drugs. Our objective was to explore the pharmacological properties and establish a new methodology for ligand screening for the $P2X_2$ receptor, which has been developed by the combinatorial library approach Systematic Evolution of Ligands by Exponential enrichment (SELEX). To this end, membranes of 1321N1 cells stably transfected with rat $P2X_2$ receptors were resuspended in 2% cholate detergent and subsequently coupled onto an immobilized artificial membrane (IAM). The IAM–cholate–$P2X_2$ mixture was then dialyzed, centrifuged and packed into a FPLC column. Equilibrium binding to the receptor and competition between ATP and the purinergic antagonists suramin and 2′3′-*O*-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate (TNP-ATP) were analyzed by a chromatographic assay using ^{32}P alpha ATP as a radioligand. Our data indicate that suramin does not compete with ATP for the ligand binding site and TNP-ATP is a competitive antagonist, confirming previous studies [C.A. Trujillo, A.A. Nery, A.H. Martins, P. Majumder, F.A. Gonzalez, H. Ulrich, Biochemistry 45 (2006) 224–233]. In addition, we demonstrate that this assay can be used in *in vitro* selection procedures for RNA aptamers binding to $P2X_2$ receptors. The results demonstrate that the receptor can be immobilized in a stable format and reused over an extended period of time, facilitating the exploration of ligand–receptor interactions and screening of combinatorial pools for possible ligands.

Keywords: Purinergic receptor; Affinity chromatography; Drug screening; SELEX; Aptamer

1. Introduction

The effects of purine nucleotides acting through P2 receptors have been studied in many biological systems, and it is now clearly established that endogenous ATP acts as an extracellular signaling molecule and neurotransmitter [1–4]. Plasma membrane nucleotide P2 receptors are subdivided into two families, namely the ionotropic purinergic receptors (P2X-ligand gated ion channels) and the metabotropic purinergic receptors (P2Y-G-protein coupled receptors). They are currently under extensive investigation as potential therapeutic targets in drug development (reviewed in Refs. [5–9]). To date, seven mammalian ionotropic ($P2X_{1–7}$) and eight metabotropic ($P2Y_{1,2,4,6,11–14}$) purinergic receptor subtypes have been identified by molecular cloning [10–12]. There has been an increasing interest in developing potent and selective agonists and antagonists for purinergic receptor subtypes. For instance, novel analogues of suramin, one of the most important, non-selective inhibitors of purinergic receptors, have been reported to act as potent $P2X_1$ receptor-selective antagonists [13]. The ATP analogue TNP-ATP (2′3′-O-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate) has been developed as a selective high affinity antagonist of $P2X_2$ and $P2X_3$ receptor subtypes [14].

A key element in drug development programs is the measurement of the binding affinities, the specificities of lead drug candidates to their target proteins and to correctly determine

* Corresponding author. Tel.: +55 11 3091 3810x223; fax: +55 11 3815 5579.
E-mail address: henning@iq.usp.br (H. Ulrich).

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whether these compounds are competitive or noncompetitive inhibitors. In this context, Dunn and Blakeley [15] were the first to report that suramin antagonized P2 receptor activity by competing with ATP for the ligand-binding site, while Burgard et al. [14] identified TNP-ATP as a competitive antagonist. However, our laboratory has predicted a new mechanism for $P2X_2$ receptor inhibition by these two ligands. Based on whole-cell recording patch-clamp experiments, binding assays with transfected 1321N1 living cells and a mathematical model for receptor kinetics, a noncompetitive inhibition mechanism of the rat recombinant $P2X_2$ receptor by suramin was concluded and the competitive inhibition of the receptor by TNP-ATP confirmed [16] (Fig. 1).

Attempting to support the inhibition mechanism of suramin and TNP-ATP proposed by Trujillo et al. [16] and to establish a new methodology for screening of ligands and inhibitors for the $P2X_2$ receptor, which have been identified by the combinatorial RNA library approach systematic evolution of ligands by exponential enrichment (SELEX), we have developed a chromatographic assay using stationary phases containing immobilized $P2X_2$ receptors as affinity matrix.

The SELEX technique introduced in parallel by Tuerk and Gold [17] and Ellington and Szostak [18], uses an oligonucleotide-based combinatorial library containing a vast number (around 10^{13}) of different sequences and structural motifs for the *in vitro* selection of DNA or RNA molecules with binding specificity to a desired target. Functional DNA or RNA molecules, also denominated as aptamers, have been identified as specific ligands to a variety of different target molecules including nucleotides, biologically active peptides and soluble proteins, and complex targets such as membrane bound receptors, erythrocyte surfaces and entire cells (reviewed in Ref. [19]). In many cases, these aptamers produced desired biological effects such as the inhibition of receptor function or cell-adhesion events [20,21] (Fig. 2).

In view of the lack of selective inhibitors for purinergic receptors, the identification of RNA aptamers binding specifically to P2X receptor subtypes, may provide an important tool for the investigation of physiological functions of these receptors. Reiterative *in vitro* selection cycles can be coupled to a high-throughput screening chromatographic method for the identification of RNA aptamers with desired biological activity, thereby accelerating the process and decreasing costs in the search for possible drug candidates. Moreover, we have developed liquid chromatographic stationary phases containing immobilized $P2X_2$ receptors on an immobilized artificial membrane (IAM) as an alternative method to classical radioligand-receptor binding assays [22,23].

Recent studies have demonstrated that this technique can be used to immobilize various types of transmembrane proteins including transporters [24], carriers [25] and receptors such as nicotinic acetylcholine receptors [26]. In every case, the proteins

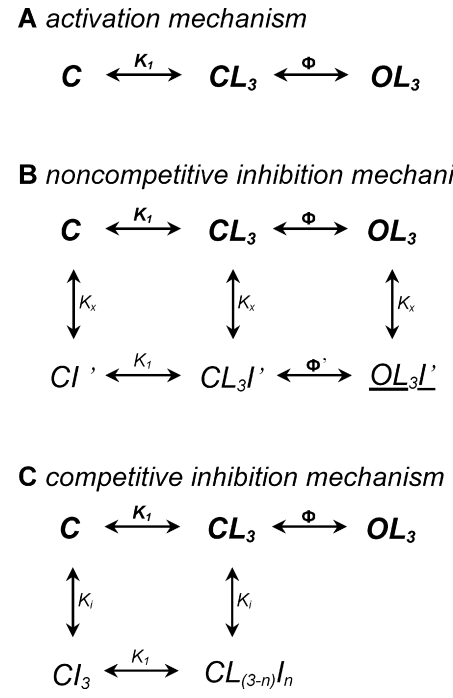

Fig. 1. Model for $P2X_2$ receptors kinetics, according to Refs. [16,44]. (A) Activation mechanism for the channel opening: C represents the non-desensitized (active) receptor and L the neurotransmitter ATP, the subscript indicates the number of receptor-bound ATP molecules prior to the channel opening. O is the open-channel form of the receptor. K_1 is the dissociation constant of the agonist ATP from the receptor site controlling the channel-opening equilibrium constant Φ. (B) Noncompetitive inhibition mechanism of suramin: I' represents the noncompetitive inhibitor. The channel-opening equilibrium constant in the presence of suramin Φ' is decreased, resulting in fewer channels in the open form ($\underline{OL_3}I'$). (C) Competitive inhibition mechanism of TNP-ATP: I represent the competitive inhibitor, K_i is the dissociation constant of the inhibitor from the receptor. The subscript denotes the number of TNP-ATP molecules bound to the ligand-binding site of the receptor.

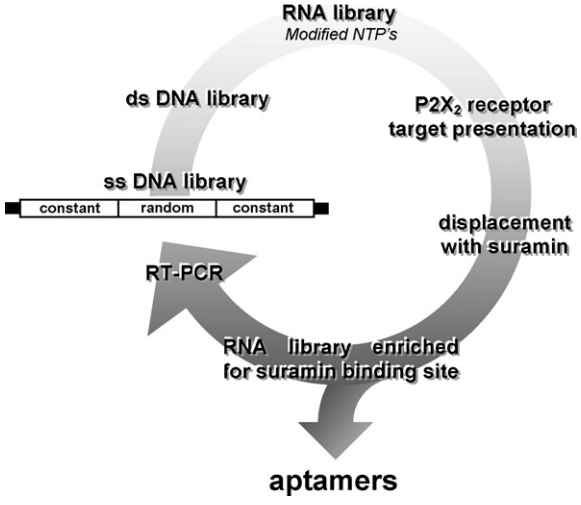

Fig. 2. Schematic representation of the systematic evolution of ligand by exponential enrichment (SELEX) technique used for the isolation of high-affinity RNA ligands to $P2X_2$ receptors. RNA aptamers were selected from partially randomized oligonucleotide libraries by reiterative *in vitro* selection rounds against $P2X_2$ receptor-containing cell membranes. RNA molecules bound to an allosteric site of the receptor were eluted in the presence of an excess of molar suramin over RNA concentration. Collected RNA target binders were reverse-transcribed to cDNA that then was amplified by polymerase chain reaction. Reiterative SELEX rounds were performed with increasing selection stringency to ensure the identification of the binders with the highest affinity. cDNAs obtained by reverse transcription from selected RNAs were isolated and DNA-sequenced for identification of individual aptamers. ss = single-stranded; ds = double-stranded; NTP = nucleoside-triphosphate.

retained the ability to bind ligands and were used to determine their pharmacological properties (reviewed in Ref. [27]). The results of these studies demonstrate that immobilized-receptors stationary phases can be used for rapid chromatographic or automated screening for the isolation and identification of lead compounds from complex biological mixtures.

The *in vitro* selection of aptamers depends on cyclic repetitive procedures and is normally time-consuming and not easily adaptable to high-throughput applications. However, in the last years, automated platforms have been developed to increase the throughput of aptamer selection *via* automation of the SELEX methodology. For instance, Cox et al. [28,29] established a reproducible protocol which is not prejudiced by any handling errors in order to carry out at least 10 automated SELEX cycles per day against a single target. The robotic workstation reported by Cox et al. [28,29] could perform eight selections in parallel and complete more than 10 rounds of selection in a couple of days. However, these estimates are not of general value as selection targets differ in their solubility and structural properties, making the selection of aptamers binding to membrane proteins more complex than of those directed against soluble targets [30]. In this context, the integration of IAM affinity chromatography in automated SELEX procedures may facilitate the characterization and development of aptamers as binding reagents for large biosensor arrays.

The $P2X_2$ receptor was successfully immobilized onto the IAM stationary phase and the resulting $P2X_2$-IAM column was fully characterized using [^{32}P]-ATP as the radiolabeled ligand and ATP, TNP-ATP and suramin as displacers.

2. Materials and methods

2.1. Material

Unless otherwise indicated, all reagents were purchased from Sigma and of the highest available quality. [α-^{32}P]-ATP (3000 Ci/mmol) was from GE Healthcare. Immobilized artificial membrane liquid chromatographic stationary phase (12 μm, 300 Å) was purchased from Regis Chemical Co. (Morton Grove, IL).

2.2. Cell culture and maintenance

Human 1321N1 astrocytoma cells expressing functional recombinant rat $P2X_2$ receptors [16] were grown in Dulbecco's modified Eagle's medium (DMEM, high glucose, Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Cultilab, Campinas, Brazil) in the presence of 100 IU/ml of penicillin, 100 μg/ml streptomycin, 0.5 mg/ml geneticin (Sigma) at 37 °C in a water-saturated atmosphere containing 5% CO_2. We have used 1321N1 cells transfected with $P2X_2$ receptors as a model for studying $P2X_2$ receptor inhibition, since these cells do not express any endogenous functional purinergic receptors [31,32].

2.3. Preparation of the $P2X_2$ receptor protein

Transfected 1321N1 cells were collected from cell culture flask by treatment with phosphate buffer saline (PBS)–2 mM EDTA. Cell lysis was started by sonication (3 × 10 pulses at 30%) for separation of cell fragments and nuclei in the presence of protease inhibitors at 0 °C. The material was centrifuged for 15 min at 3000 × *g* and 4 °C, and the supernatant was collected. The supernatant was ultracentrifuged for 1.5 h at 100,000 × *g* and 4 °C, and the resulting pellet was resuspended in 50 mM HEPES, pH 7.4, containing 2% cholate detergent and protease inhibitors. The mixture was stirred for 12 h at 4 °C and subsequently centrifuged at 35,000 *g* for 20 min and the supernatant containing $P2X_2$-cholate solution was collected.

2.4. Immobilization of the $P2X_2$ on IAM stationary phase

The IAM liquid chromatographic stationary phase was used as support for receptor coupling. The detergent solution containing $P2X_2$ receptor was mixed with 200 mg of dried IAM and gently stirred for 1 h at 25 °C, transferred into dialysis tubing and dialyzed for 48 h at 4 °C using the Slide-A-Lyser Dialysis Cassete 10000 MWCO kit (Pierce) against 2 × 1 L of HEPES (50 mM, pH 7.4) containing 5 mM EDTA, 100 mM NaCl, 0.1 mM $CaCl_2$ and protease inhibitors. The resulting mixture was centrifuged for 3 min at 700 × *g* and 4 °C, and the supernatant was discarded. The pellet containing $P2X_2$-IAM was washed with HEPES (50 mM, pH 7.4) and centrifuged, followed by collection of the solid phase.

2.5. Chromatographic studies

The $P2X_2$-IAM (200 mg) was packed into a GE Healthcare HR 5/2 glass column (5 mm i.d. × 2 cm) and connected to a FPLC pump (GE Healthcare). The mobile phase consisted of HEPES (50 mM, pH 7.4) containing protease inhibitors. The column was equilibrated at a flow rate of 0.4 ml/min at room temperature. A frontal sample of 50 ml of 500 pM [^{32}P]-ATP was applied to the $P2X_2$ receptor column. Elution profiles were obtained by fractionating the column eluate into samples of each 0.4 ml volume. Eluted radioactivity was quantified by scintillation counting. Chromatographic elution profiles were smoothed using Prism 4.0 (GraphPad) software.

2.6. Western blotting

Forty micrograms of membrane proteins from ultracentrifugation were mixed with SDS-PAGE sample buffer containing 0.05% (v/v) β-mercaptoethanol, heated for 5 min to 95 °C and centrifuged for 2 min at 12,000 × *g*. The supernatant was separated on 10% SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes (Transblot, 0.45 μm, Bio-Rad). No retention of protein aggregates was observed in the stacking gel. The membranes were incubated with a blocking solution containing 3% non-fat milk powder in PBS-T (PBS/0.05% Tween 20) for 30 min at room temperature, followed by overnight incubation at 4 °C with 1/250 diluted goat polyclonal anti-$P2X_2$ antibody (Santa Cruz Biotechnology, Heidelberg, Germany) in the same blocking solution. Following three washes with PBS-T, membranes were incubated for 2 h at room temperature with peroxidase-conjugated anti-goat IgG secondary antibody

(1/100) (Santa Cruz Biotechnology). Reactions were developed by using the ECL plus kit (Pierce) according to the instructions provided by the manufacturer.

2.7. Radioligand-receptor binding assay

Equilibrium binding of [^{32}P]-ATP (130 pM) to $P2X_2$-rich plasma membranes was measured by using a filtration assay. This methodology was generally adapted from procedures described by Michel et al. [33] and modified to enhance specific binding to cells expressing rat $P2X_2$ receptors. Briefly, 50 μg of membrane preparation resuspended in 1 mM EDTA, 50 mM HEPES and 0.01% Triton X-100 at pH 7.4 was incubated for 2 h at room temperature with [^{32}P]-ATP in the absence or presence of increasing concentrations of the purinergic receptor antagonists suramin or TNP-ATP to determine whether suramin, TNP-ATP and [^{32}P]-ATP compete for the same binding site. Following incubation, receptor- bound and free ligands were separated by rapid filtration through Whatman GF/F filters (Whatman, Middlesex, UK) pre-wetted with solubilization buffer. The filters were dried and then placed in vials containing 2 ml of scintillation liquid for determination of retained radioactivity using a liquid scintillation counter (Beckman-Coulter, Fullerton, CA). Specific binding was defined as the difference between total binding in the presence of the radioligand alone and nonspecific binding determined by using a molar excess of unlabeled competitor over radioligand concentration. Protein concentration was determined as described by Bradford [34].

2.8. SELEX procedure

Three cycles of *in vitro* selection for enrichment of high-affinity RNA ligands to $P2X_2$ receptors were carried out according to published protocols [20,35] (Fig. 2). Briefly, the RNA pools used for selection were *in vitro* transcribed from a combinatorial pool of double-stranded DNA templates, each of them consisting of 108 nucleotides with a 40 nucleotide random region which was flanked on both sites by constant regions. A T7 promoter site was incorporated in one of the constant regions. *In vitro* transcription reactions were set up in the presence of 20 μg of the combinatorial DNA pool, 1000 U of T7 RNA polymerase, 50 μCi of [α-^{32}P] ATP, 1 mM of each purine (2′-OH-ATP and -GTP) and 3 mM of each pyrimidine (2′-fluoro-deoxyuridine-5′-triphosphate (2-F′-dUTP) and 2′-fluoro-2′-deoxycytidine-5′-triphosphate (2′-F′-dCTP) (TriLink BioTechnologies, San Diego, CA), to provide stability of the transcripts against nuclease activity [36].

The resulting internally [^{32}P]-labeled RNA pool used in these selections was quantified, diluted to a final concentration of 50 μM in the 1321N1 external buffer (PBS, pH 7.4). The RNA pool was heated at 65 °C for 15 min and left to cool to room temperature for 30 min to allow for proper secondary structure formation. The membrane-bound receptor fraction was added to give a final concentration of 300 nM ATP-binding sites at $P2X_2$ receptors. The reaction mixture was incubated at room temperature for 40 min and then passed through nitrocellulose filters by using a 96-well Minifold filtration apparatus (Whatman Schleicher & Schuell). $P2X_2$ receptor-bound RNA molecules were retained, whereas unbound RNA molecules passed through the filters as flow through. Receptor-bound RNA molecules were eluted in the presence of 1-mM suramin. The eluted RNA pool was purified by phenol–chloroform extraction and concentrated by ethanol precipitation. The DNA pool needed for the next selection round was restored by reverse transcription-PCR. The amplified DNA pool was *in vitro*-transcribed to RNA for the next round of *in vitro* selection.

2.9. Determination of receptor–ligand binding by a gel-shift assay

RNA pools from SELEX cycles 3 and 4 were internally radiolabelled during *in vitro* selection reactions as detailed above. The [^{32}P]-RNA molecules were purified from unincorporated [^{32}P]-ATP by denaturing polyacrylamide gel electrophoresis, diluted to a final concentration of 30 μM in 1321N1 extracellular buffer containing 0.3 mg/ml yeast t-RNA and denaturated at 65 °C for 15 min. The [^{32}P]-RNA/t-RNA mixture was left to cool to room temperature for 30 min to allow for proper secondary structure formation and then incubated with 1321N1 cell membranes containing 100 nM $P2X_2$ receptor proteins in a total volume of 40 μl of 1321N1 external buffer. Following incubation for 40 min at room temperature glycerol and bromphenol blue were added to final concentrations (w/v) of 5 and 0.04%, respectively. The mixture was loaded onto a 3% native polyacrylamide gel which was cast and run in TBE (89 mM Tris; 89 mM boric acid; 2 mM EDTA) adjusted to pH 7.4. Electrophoresis was carried out at 7 V/cm until the bromphenol dye has migrated to the bottom of the gel. The gel was removed and imaged by using a phosphor-imaging plate (Fuji, Tokyo, Japan). The imaging plate was scanned by using a Storm PhosphorImage (GE Healthcare) and relative band intensities were quantified by using the Imagequant software from.

2.10. Electrophysiological measurements

Whole-cell recording experiments in living 1321N1 cells expressing the $P2X_2$ receptor were done to evaluate the activity of the selected RNA pools in a physiological background. The measurements were carried out as described by Trujillo et al. [16]. Briefly, a cell in the whole-cell recording configuration was placed in front of the porthole of a U-tube (Hamilton, Reno, NV) [37,38]. The flow rate of solutions emerging from the flow device, containing ATP with or without RNA SELEX-pool 5, was typically 1 cm/s. The induced whole-cell currents were amplified by using an Axopatch 200B amplifier (Molecular Devices, Sunnyvaley, CA) and analyzed by using the pCLAMP 8.1 (Molecular Devices) and Origin Microcal (Northampton, MA) software packages.

3. Results and discussion

In this work, we demonstrate that the recombinant $P2X_2$ receptor can be solubilized and then immobilized on an IAM liquid chromatography stationary phase column maintaining

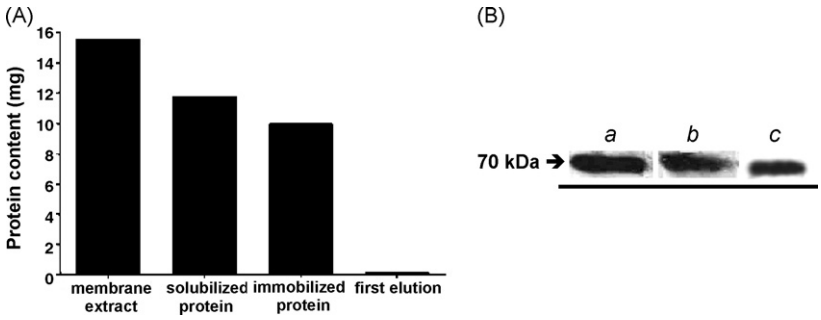

Fig. 3. Detection and quantification of the $P2X_2$ receptor protein during immobilization in the IAM stationary phase. (A) Determination of protein content in different stages of $P2X_2$ immobilization was done by using the Bradford method [34]. Protein content only decreased slightly in immobilization stages. Note that no proteins were detected in the first elution phase showing the efficacy of receptor-coupling to the column as continuous solution flow did not affect immobilized proteins. (B) Western Blot analysis of plasma membrane protein extracts (a), membrane fractions solubilized by 2% cholate (b) and immobilized protein in the IAM stationary phase (c) from 1321N1 cells stably transfected with recombinant rat $P2X_2$ receptors. Reactions were developed using the ECL-chemiluminescence kit (Pierce) followed by exposure to a Kodak X-Omat film.

its pharmacological properties. Using this column in affinity chromatographic experiments, we have verified the binding equilibrium of two purinergic receptor antagonists: TNP-ATP and suramin. Moreover, we have used the column to perform one cycle of *in vitro* selection for enrichment of RNA molecules binding to $P2X_2$ receptors.

3.1. Detection and quantification of the $P2X_2$ receptor protein during immobilization in the IAM stationary phase

Aliquots of total membrane extract, solubilized proteins, immobilized protein on IAM and fractions of first column elution were used for monitoring possible loss of protein during stages of the $P2X_2$ receptor solubilization and immobilization (Fig. 3A). The amount of protein only decreased slightly during these procedures and approximately 50 mg of protein per gram IAM particles were immobilized. In addition, no loss of protein was observed from the $P2X_2$-IAM column as determined by the Bradford protein quantification assay [34], when 60 column volumes of mobile phase had run through the column at 0.4 ml/min, showing the efficacy of receptor-coupling to the column [27].

The presence of the $P2X_2$ receptor throughout the immobilization process was determined using Western Blot analysis; the receptor protein was present in membrane protein preparation from 1321N1 cells expressing $P2X_2$ receptors (a) and in the supernatant of membrane fraction solubilization (b), as well in the column resin following coupling (c), as confirmed by recognition of a 70 kDa protein by the polyclonal anti-$P2X_2$ antibody (Fig. 3B).

The ability of the immobilized receptors to bind known $P2X_2$ receptor ligands was determined using a [^{32}P]-ATP filter-binding assay protocol designed for cell membrane homogenates [33]. In the displacement chromatographic assay, specific binding of [^{32}P]-ATP on the $P2X_2$-IAM support was $97 \pm 1\%$ of total with 4.0 pmol receptor/g of immobilized protein. No specific binding of [^{32}P]-ATP was found to native IAM particles.

3.2. Filter radiobinding assay

A radioligand binding assay was employed to determine whether the purinergic receptor ligands ATP, suramin, TNP-ATP and [^{32}P]-ATP (200,000 cpm, 130 pM) compete for the same binding sites on the receptor. As demonstrated in Fig. 4, increasing concentrations of ATP (10–1000 μM) displaced [^{32}P]-ATP from the ATP-binding site of the receptor in a dose-dependent manner. The purinergic receptor antagonist TNP-ATP (100 μM) potently inhibited binding of [^{32}P]-ATP to the $P2X_2$ receptor. This result confirms the competitive inhibition mechanism of

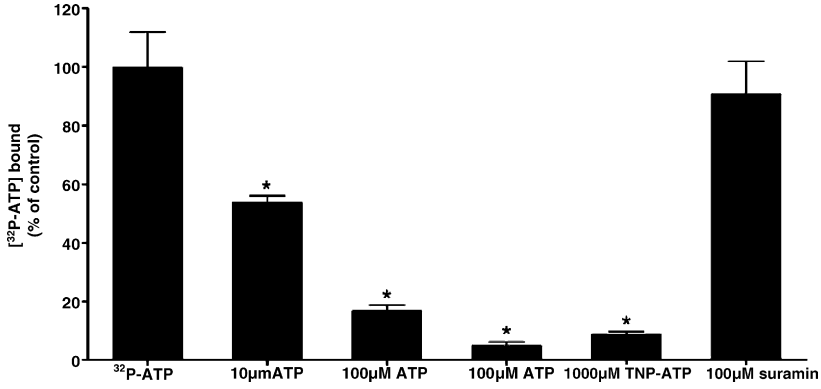

Fig. 4. Displacement of [^{32}P]-ATP from recombinant rat $P2X_2$ receptors expressed in 1321N1 cells using ATP, suramin and TNP-ATP as competitors. Experimental conditions are detailed in Section 2. All measurements were done in triplicates. Note the displacement of [α^{32}P]-ATP in the presence of different concentrations of ATP, TNP-ATP and no interference of suramin with [α^{32}P]-ATP receptor binding ($^{*}p < 0.05$).

$P2X_2$ receptors by TNP-ATP in membrane extract of transfected 1321N1 cells (Fig. 1C).

However, no displacement of receptor-bound [^{32}P]-ATP was observed in the presence of suramin (100 μM) indicating that ATP and suramin do not compete for the same binding site. This result is in agreement with already published studies based on whole-cell recording measurements and receptor–radioligand binding studies with living cells [16], supporting the noncompetitive mechanism of inhibition of the $P2X_2$ receptor by suramin.

3.3. Chromatographic displacement experiments

The chromatographic profile of [^{32}P]-ATP elution from the $P2X_2$ column is presented in Fig. 5. For chromatographic displacement studies, increasing concentrations of unlabeled ATP ranging from 10 to 1000 μM were added preceding an injection of an excess of [^{32}P]-ATP (~1,200,000 cpm) to the mobile phase, and the ligand elution are given as maximal peak values (Fig. 5A). Consistent displacement peaks of [^{32}P]-ATP in dose-dependent manner throughout the concentration range of ATP were observed. This result was in agreement with data obtained in the filter-binding assay using ATP as a competitor.

The profile of [^{32}P]-ATP elution from the $P2X_2$-IAM stationary phase, induced by application of 100 μM ATP preceding equilibration of the column with [α^{32}P]-ATP in the presence of 1 mM unlabelled ATP, was recorded to verify for unspecific binding of ATP to the $P2X_2$ receptor immobilized in IAM stationary phase (Fig. 5B). Assuming saturation of ATP-binding sites during equilibration of the receptor column with 1 mM of unlabeled ATP, the failure of 100 μM ATP to induce additional elution of [^{32}P]-ATP indicates the absence of unspecific binding of [^{32}P]-ATP to the column.

In order to determine whether the purinergic receptor antagonists suramin and TNP-ATP competed with [^{32}P]-ATP for receptor binding, 100 μM concentrations of the compounds were injected following pre-equilibration of the column with

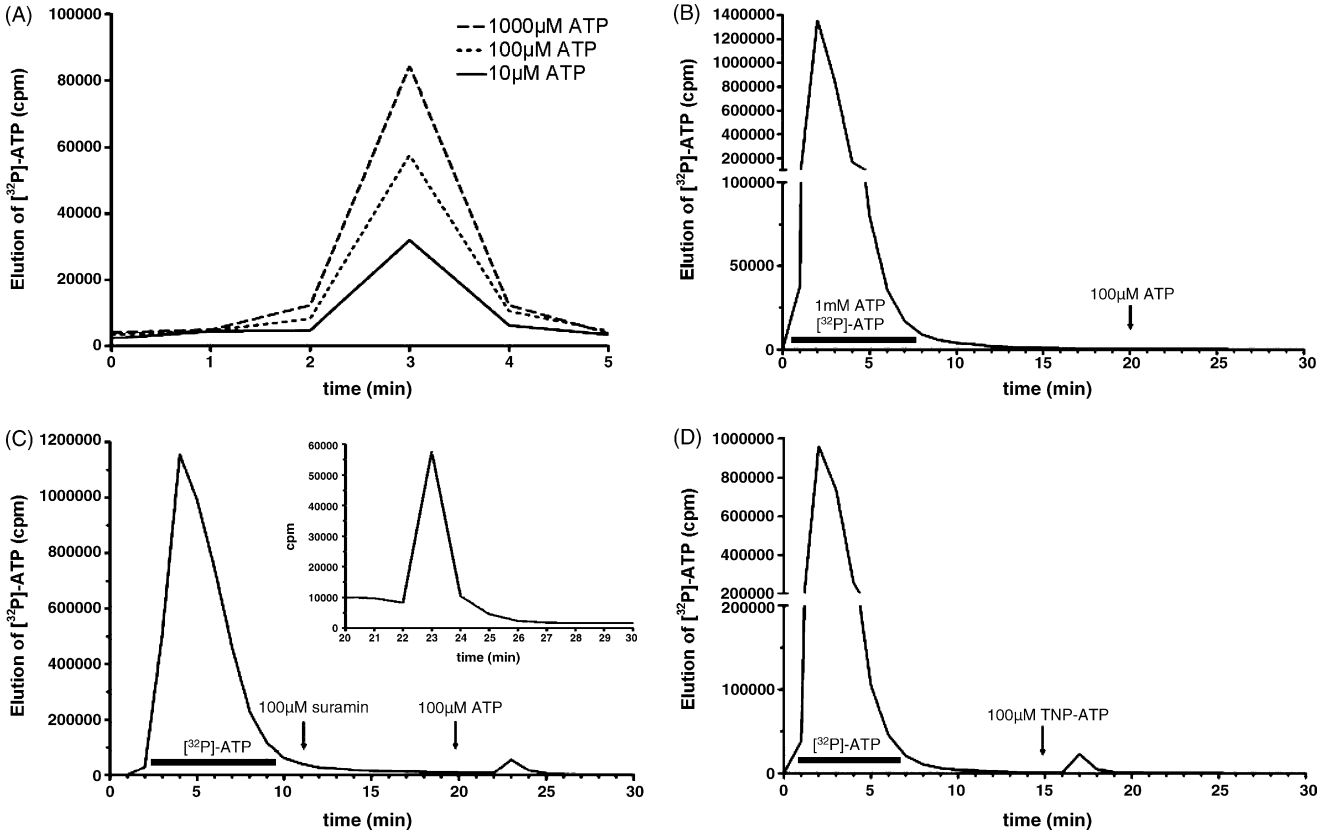

Fig. 5. Elution profiles of [^{32}P]-ATP in affinity chromatography with the $P2X_2$ receptor immobilized to a column. The mobile phase consisted of HEPES (50 mM with protease inhibitors, pH 7.4). Application of samples and isocratic elution was carried out at 25 °C with a flow-rate of 0.4 ml/min. Eluates were fractionated in 1 min time periods. (A) Elution of [^{32}P]-ATP following injection of 10–1000 μM unlabeled ATP. The receptor column was loaded with an excess concentration of [^{32}P]-ATP (~1,200,000 cpm) and the column flow through monitored until no further decrease of radioactivity in the eluate could be detected. At this time point (0 min) increasing concentrations of unlabeled ATP were injected and the amount of displaced [^{32}P]-ATP from the $P2X_2$ receptor column determined in the eluate. (B) Elution profile induced by application of 100 μM ATP preceding an injection of a saturating concentration of 1 mM ATP and [α^{32}P]-ATP to verify for unspecific binding of ATP to the $P2X_2$ receptor immobilized in IAM stationary phase. Note that no peak was observed after sample injection, demonstrating the absence of unspecific binding of [^{32}P]-ATP. (C) Elution profiles of [^{32}P]-ATP induced by 100 μM suramin and 100 μM ATP preceding an injection of a saturating concentration of [32P]-ATP to determine interference of suramin with the ATP binding site of the $P2X_2$ receptor. No competition between suramin and [^{32}P]-ATP $P2X_2$ receptor binding was observed, indicating a noncompetitive inhibition mechanism for suramin-receptor binding. The inset shows a magnified view of the [^{32}P]-ATP displacement peak induced by injection of 100 μM ATP. (D) Elution profile induced by application of 100 μM TNP-ATP preceding an injection of a saturating concentration of [α^{32}P]-ATP to determine equilibrium binding of TNP-ATP to the ATP binding site of $P2X_2$ receptor. The displacement of [^{32}P]-ATP by TNP-ATP supports a competitive inhibition mechanism.

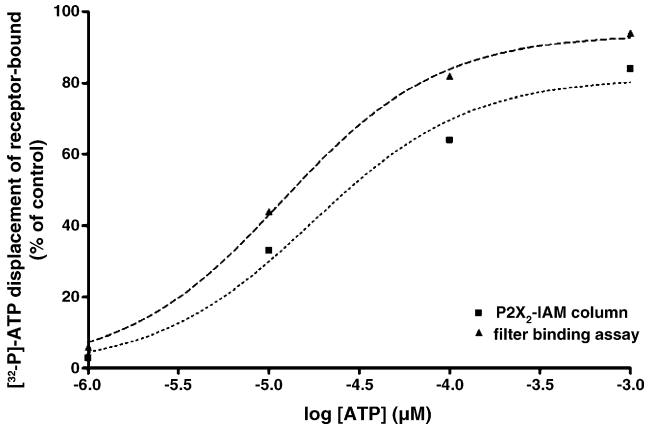

Fig. 6. Comparison of chromatographic displacement using $P2X_2$ receptor-IAM column and filter binding assay. (▲) Filter binding assay: displacement of [α^{32}P]-ATP (in cpm) in the presence of an increasing concentrations of ATP (1–1000 μM). The curve ranged from 4 to 82% of control values obtained in the absence of competitor. The concentration of unlabeled ATP necessary to displace [^{32}P]-ATP from its binding site on the receptor (IC_{50}) was equal to 12 ± 4 μM ($R^2 = 0.99$). (■) Chromatographic elution of the $P2X_2$-IAM column: the curve represents elution peaks from 3 to 94% compared to those obtained in excess of unlabeled ATP which is supposed to elute all receptor-bound [^{32}P]-ATP. The IC_{50} of [^{32}P]-ATP receptor binding was equal to 17.0 ± 3.5 μM ($R^2 = 0.98$).

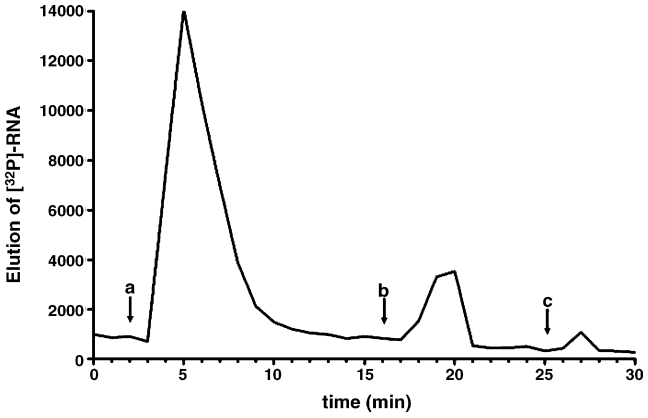

Fig. 7. Elution profiles of a [^{32}P]-SELEX RNA pool in affinity chromatography with the $P2X_2$-immobilized receptor column. (a) Approximately 80,000 cpm of [^{32}P]-RNA pool following three initial cycles of SELEX against $P2X_2$ receptors (SELEX 3 RNA pool) was injected for chromatographic selection and elution of [^{32}P]-labeled RNA ligands bound to the $P2X_2$ receptor. (b) Elution profile of the [^{32}P]-SELEX 3 RNA pool following the injection of 1-mM of suramin. (c) Elution profile of the [^{32}P]-SELEX 3 RNA pool following an injection of 100-μM ATP. Note that two peaks of liberation of [^{32}P]-labeled RNA were observed, supporting the inhibition mechanism proposed by Trujillo et al. [16].

[^{32}P]-ATP (Fig. 5C and D). Injection of suramin to the mobile phase did not displace receptor-bound [^{32}P]-ATP, (Fig. 5C). This observation indicates a noncompetitive inhibition mechanism in which suramin binds to an allosteric site on the receptor independent from the ATP-binding site. Injection of ATP as a control experiment resulted in [^{32}P]-ATP elution (see the inset of Fig. 5C for a magnified view) demonstrating the functionality of the $P2X_2$-receptor IAM column. However, TNP-ATP effectively eluted column-bound [^{32}P]-ATP indicating a competitive mechanism for [^{32}P]-ATP- and TNP-ATP-receptor binding. The conclusions obtained by chromatographic characterization of suramin-and TNP-ATP-receptor binding are consistent with results obtained in the filter binding assay results (Fig. 6). Our results support a noncompetitive inhibition mechanism of the rat recombinant $P2X_2$ receptor by suramin, confirm the competitive inhibition by TNP-ATP, and corroborate with the model for $P2X_2$ receptor inhibition [16].

In order to determine if the filtration assay and the chromatographic determination of $P2X_2$ receptor binding revealed qualitatively different results, dose-dependency of radioligand receptor binding obtained by chromatography with the $P2X_2$ receptor-IAM column was compared to that observed by the filtration-assay binding method (Fig. 6). No significant difference in the concentration of unlabeled ATP necessary to displace [^{32}P]-ATP from its binding site between both methods was detected: IC_{50} values were, 12 ± 4 μM for filtration-binding assay and 17.0 ± 3.5 μM for chromatographic elution.

3.4. Chromatographic in vitro selection of a [^{32}P]-SELEX RNA pool using a $P2X_2$-immobilized receptor column assay

RNA and DNA aptamers, identified by *in vitro* selection procedures from combinatorial oligonucleotide libraries, are promising tools for molecular target recognition in diagnostic applications, and, in particular, they can be used as immobilized ligands in separation technologies [19,39]. Aptamers are capable of specific molecular recognition, such as differentiating between protein isotypes and changes of the conformational state of the same protein, and have been immobilized in columns for target protein capture in affinity chromatography [39–41]. However, it is the first time that the $P2X_2$ ionotropic receptor, which needs a membrane environment for proper functionality, was immobilized on the stationary phase instead of the aptamers.

The [^{32}P]-labeled RNA pool (80,000 cpm) following three initial cycles of SELEX using plasma membrane preparations containing $P2X_2$ receptor as targets was loaded on the $P2X_2$-IAM column for chromatographic selection of RNA molecules with binding affinity to the $P2X_2$ receptor (Fig. 7(a)). A displacement of the [^{32}P]-RNA pool peak from the column following the injection of 1 mM of suramin was detected, indicating that a saturating concentration of this antagonist competed with the RNA pool for binding to an allosteric binding site of the receptor (Fig. 7(b)). The elution profile of RNA molecules from [^{32}P]-SELEX 3 RNA pool following an injection of 100 μM of ATP is presented in Fig. 7(c). These results indicate that RNA molecules were selected from the heterogeneous mixture pool of possible $P2X_2$ receptor ligands that either bind to the ligand-binding site or to an allosteric site of the receptor.

3.5. Increase in binding specificity of the RNA pool to the $P2X_2$ receptor following one cycle of chromatographic selection

A gel-shift assay was used to verify if there was any improvement of the pool of RNA molecules (SELEX 4 pool RNA) in

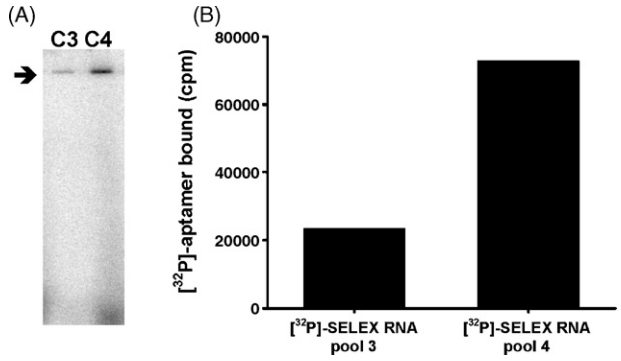

Fig. 8. Gel-shift binding assay. Gel-shift assays were performed to analyze the increase of [^{32}P]-SELEX RNA pool binding to the P2X$_2$ immobilized receptor after chromatographic selection. Experimental conditions are detailed in Section 2. (A) Gel-shift analysis of SELEX pool 3 (C3, before chromatographic selection) and SELEX pool 4 (C4, after chromatographic selection) binding to P2X$_2$ receptor proteins. The arrow indicates the location of protein-bound [^{32}P]-RNA in the native polyacrylamide gel. Unbound [^{32}P]-RNA did not suffer any retention during gel-electrophoresis and migrated to the bottom of the gel. Relative quantification of [^{32}P]-RNA retained at the top of the gel was performed by phosphor-imaging. The intensity of the SELEX 4 RNA-P2X$_2$ receptor band corrected for background was 3-times higher than the band corresponding to the SELEX 3 RNA-P2X$_2$ receptor complex. The intensity of the SELEX 0 pool RNA band was less than the background signal (data not shown). Therefore no comparison with SELEX 0 pool RNA could be made. (B) Enrichment of RNA binding to P2X$_2$ receptor following chromatographic selection. The intensities of [^{32}P]-RNA-P2X$_2$ receptor bands in a gel-shift assay obtained by phosphor imaging were compared with the amount of protein-bound [^{32}P]-RNA of the third selection pool.

P2X$_2$ receptor binding after one cycle of chromatographic selection with SELEX 3 pool RNA (Fig. 8). The concentration of [^{32}P]-RNA ligand–receptor complexes retained at the top of the native polyacrylamide gels increased considerably, as detected by phosphorimaging (Fig. 8A). The chromatographic *in vitro* selection step for enrichment of RNA species in the mixture of RNA molecules in SELEX 3 pool RNA led to an increase of receptor binding by 300% (Fig. 8B). It may be noted that such an increase in receptor binding could not be observed in any of the three initial SELEX cycles based on immobilization of receptor–RNA ligand complexes on nitrocellulose filters.

The chromatographic selection step using the immobilized P2X$_2$ receptor as an affinity matrix may help to accelerate the process of identification of novel receptor ligands and inhibitors from combinatorial oligonucleotide libraries. This method would decrease the number of reiterative cycles needed to maximize receptor-binding affinity and specificity of the selected RNA pool, thereby diminishing time and costs of aptamer production.

3.6. Electrophysiology

Fig. 9 shows a representative whole-cell current measurement performed in presence of 100 μM ATP or 100 μM ATP and 1 μM RNA SELEX-pool 5, using the cell-flow technique [37,38]. The current obtained with 100 μM ATP alone reached a maximum value of 3800 pA within 80 ms, showing rapid receptor activation.

Fig. 9. Electrophysiological investigations of the effects of ATP and RNA SELEX-pool 5 on rat P2X$_2$ receptor activity in 1321N1 glial cells by whole-cell recording. All measurements were carried out at pH 7.4 and 25 °C, with a fixed membrane voltage of −60 mV, using a rapid chemical kinetic technique [37,38]. The observed whole-cell current is a representative measure for the number of receptors in the open-channel form in the presence of 100 μM ATP and reached a maximum value of 3800 pA (left panel). In the right panel, we show a representative measurement of the inhibition of the whole-cell current induced by 100 μM ATP in the presence of 1 μM of RNA SELEX-pool 5 (2160 pA). In these conditions, the selected RNA pool inhibited 43 ± 6% of ATP-induced whole-cell currents amplitudes.

The RNA SELEX-pool 5 alone did not activate P2X$_2$ receptors (data not shown). However, the selected RNA pool inhibited 43 ± 6% of ATP-induced whole-cell currents amplitudes when co-applied with the neurotransmitter (Fig. 9). These observations suggest that the RNA SELEX-pool, previously selected using the IAM-chromatography, inhibits P2X$_2$ receptor activity in a physiological background, corroborating with the idea that chromatographic procedures are suitable for target presentation of neurotransmitter receptors during *in vitro* selection of biologically active aptamers.

3.7. Stability and reproducibility of the P2X$_2$ receptor-IAM column

The P2X$_2$ receptor-IAM column was utilized continuously over a month, stored at 4 °C and then used again once per month to check for column stability. Elution profiles of [^{32}P]-ATP induced by the injection of 100 μM ATP were obtained in the periods of column usage, as shown in Fig. 10. In agreement with previous studies using the membrane proteins immobilized to IAM columns [42,43], the stability of the P2X$_2$ receptor following immobilization exceeded 6 months.

The reproducibility of elution profiles was confirmed by independent experiments, and the pharmacological properties of the receptor–ligand binding were consistent with the binding data obtained by the filtration assay, confirming the reliability and reproducibility of the P2X$_2$ receptor-IAM column. These columns may facilitate exploration of ligand–receptor interactions and their pharmacological properties. In addition, receptor-based chromatography is a powerful tool for the rapid screening of combinatorial pools of possible ligands for lead compounds for drug development. In this approach, chromatographic retention times can be used to sort a mixture of compounds according to their relative binding affinities. Selected molecules can be immediately identified by online injection of eluates into mass spectrometers.

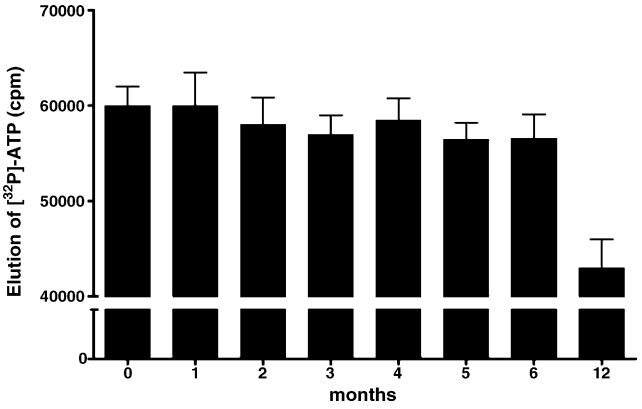

Fig. 10. Determination of the stability of the $P2X_2$ receptor-IAM column by analyzing elution profiles of [^{32}P]-ATP after injection of 100 μM of ATP following increasing time of column storage. An excess concentration of [^{32}P]-ATP (∼1,200,000 cpm) was applied to the $P2X_2$ receptor column followed by washing of the column until all unbound [^{32}P]-ATP was eluted. Elution of receptor-bound [^{32}P]-ATP was initiated by injection of 100 μM of unlabeled ATP. In agreement with other studies [42,43] the stability of the column exceeded 6 months.

4. Conclusion

Our data indicate that membranes from the 1321N1 cell lines stably transfected with rat $P2X_2$ receptor have been successfully immobilized onto the IAM stationary phase, creating $P2X_2$ receptor-IAM stationary phases. These observations also demonstrate that the column containing this stationary phase can be used to determine pharmacological properties.

The knowledge of the pharmacological properties of the $P2X_2$ receptor and the mechanism by which these receptors are activated and inhibited as a function of neurotransmitter and inhibitor concentrations, may contribute to understanding the process of signaling in the nervous system and the development of therapies for disorders associated with purinergic receptor dysfunction.

In this context, the demand for novel therapeutic agents acting on disease-causing or related protein functions has turned the SELEX technique into a promising approach for drug discovery, taking into account that aptamers can be evolved against almost every target. In this situation, the immobilized receptor-IAM column becomes a new tool for automation of aptamer selection, shortening the time and costs of aptamer production. This approach offers the opportunity of developing affinity-based detection systems, which can be applied to the area of analytical chemistry, and the use of aptamers can be expanded beyond clinical diagnostics, pharmaceutical or biochemical fields.

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