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The Synthesis and Initial Characterization of an Immobilized Purinergic Receptor (P2Y₁) Liquid Chromatography Stationary Phase for Online Screening

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Adenine and uridine nucleotides play a variety of roles in the regulation of physiological responses, many of which are mediated through a family of transmembrane receptors, the purinergic receptors (P2 receptors) [1]. P2 receptors are separated into two subfamilies: the P2X receptors, which are ligand-gated ion channels and the P2Y receptors, which are G-protein coupled receptors (GPCRs). The P2Y family currently consists of 8 receptor subtypes (P2Y_{1,2,4,6} & 11–14) and P2Y antagonists, specifically antagonists of the P2Y₁ subtype, have been shown to be therapeutically useful as antithrombotic drugs [2] and may also play a role in nociception [3].

A variety of methods have been utilized to identify P2Y agonists and antagonists including inhibition of human platelet function [4] and yeast growth [5], intra-cellular calcium mobilization measured using a fluorescence-imaging plate reader (FLIPR) [5,6] and phospholipase C activation, measured by the accumulation of [³H]-inositol phosphates [5–8]. Membrane binding studies have been carried out using the selective P2Y₁ radiolabeled antagonists [³H]-MRS2279 [9] and [³²P]-MRS2500 [10] and membranes from cell lines expressing the human P2Y₁ receptor, Sf9 insect cells [8,9] and human 1321N1 astrocytoma cell line (1321N1 Cells) [5].

We now report an alternative approach to the determination of competitive agonists and antagonists of the P2Y₁ receptor, the P2Y₁-Immobilized Artificial Membrane (P2Y₁-IAM) liquid chromatography stationary phase for use in frontal affinity chromatography. This technique is based upon the previous synthesis and characterization of IAM phases containing membrane fragments from cell lines expressing the GPCRs μ and κ opioid receptors [11] and β_2 -adrenergic receptor [12].

In the current study, 10⁷ 1321N1 Cells {provided by Dr. K. Harden, Department of Medicine, University of North Carolina} were homogenized for 3 × 30 s at the setting of 11 on a Model

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PT-2100 homogenizer (Kinematica AG, Luzern, Switzerland) in 10 ml of HEPES buffer [20 mM, pH 8.0] containing 500 mM NaCl, 5 mM 2-mercaptoethanol, 100 μ M benzamidine, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 50 μ g/ml TPCK, 100 μ g/ml PMSF and 100 μ M ATP (unless otherwise noted, all chemicals were purchased from Sigma-Aldrich). The homogenate was centrifuged at $700 \times g$ for 5 min, the pellet discarded and the supernatant centrifuged at $100,000 \times g$ for 30 min at 4°C. The resulting pellet was suspended in 10 ml of HEPES buffer [20 mM, pH 8.0] containing 2% (w/v) n-octyl- β -D-glucopyranoside 500 mM NaCl, 5 mM 2-mercaptoethanol, 100 μ M benzamidine, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 50 μ g/ml TPCK, 100 μ g/ml PMSF, 100 μ M ATP and 10% glycerol. The resulting mixture was rotated at 150 rpm using an orbit shaker for 18 h at 4°C, centrifuged at $100,000 \times g$ for 25 min, the supernatant mixed with 200 mg IAM particles (Regis Chemical Co.) and rotated at room temperature for 1 h at 150 rpm. The suspended particles were then dialyzed against HEPES buffer [20 mM, pH 8.0] containing 500 mM NaCl, 1 mM EDTA for 1 day, centrifuged for 3 min at 4 °C at $700 \times g$ and the pellet (P2Y₁-IAM) was washed with running buffer (Tris-HCl [10 mM, pH 7.5] containing 1 mM MgCl₂), centrifuged and packed into a HR 5/2 column (Amersham Pharmacia Biotech) to yield a 150 mm $\times$ 5 mm (ID) chromatographic bed. A 5 ml solution of 10 μ M ATP in running buffer was pumped through the column, followed by 35 ml of running buffer delivered at 0.2 ml/min before starting the frontal analysis experiments. The chromatographic experiments were carried out using a previously described chromatographic system that includes a radioflow detector and frontal displacement affinity chromatography techniques [11–13].

In the frontal affinity chromatography experiments conducted in this study, the observed retentions resulted from both specific and non-specific interactions with the immobilized cellular membranes. The initial relatively flat section of the chromatographic trace observed in frontal chromatography represents the binding of the test compound to specific and non-specific sites up to saturation, which is represented by the vertical breakthrough portion of the trace. Once the available binding sites are saturated, the chromatographic trace contains a plateau region, Fig 1. In this experimental approach, the chromatographic retention of a compound is determined at the half-height of the breakthrough curve [13]. In this study, the characterized P2Y₁ agonist [³H]-2-MeSADP was used as the marker ligand and increasing concentrations of 2-MeSADP 1 nM to 10 mM was added as a displacer (Figure 1a).

The data from the frontal chromatography studies were analyzed using a previously described method to determine binding affinities (K_d values) and the number of active binding sites (B_{max}) [13]. The calculated K_d for 2-MeSADP was 199.0 ± 4.5 nM, which is consistent with the K_d determined using membrane binding techniques, Table 1. The results demonstrate that the 1321N1 Cell membrane fragments were successfully immobilized on the IAM stationary phase and that the immobilized P2Y₁ receptor maintained its ability to bind 2-MeSADP.

The 1321N1 Cell membrane fragments were used as obtained and no attempt was made to isolate or purify the P2Y₁ receptor. Previous studies have demonstrated that the determination of the amount of immobilized protein is not an accurate means to follow the efficiency of the membrane immobilization and that the determination of B_{max} is a more effective approach, c.f. [13]. In this study, two P2Y₁-IAM columns were prepared and the calculated B_{max} values were 92 nmoles for the first column and 89 nmoles for the second, indicating that the approach produced reproducible columns.

The frontal chromatogram produced by [³H]-2-MeSADP [1 nM] was obtained before the beginning of each set of experiments and changes in the breakthrough volume was used as a measure of the stability of the columns, i.e. the retention of P2Y₁ binding capacity. Using this probe, the columns were stable for 50 chromatographic experiments.

N6-Methyl-2X-deoxyadenosine-3X,5X-bisphosphate (MRS-2179), a competitive inhibitor of P2Y₁ [4], and ATP, a P2Y₁ agonist [6,10] were added to the running buffer in increasing concentrations from 0.5 nM to 10 μ M. The calculated K_d values for these compounds were consistent with previously reported data, Table 1.

The selectivity of the immobilized P2Y₁ receptor was examined using the effect on the retention of [³H]-2-MeSADP of 10 μ M concentrations of ATP and NF-023, a selective P2X₁ antagonist [14] in the running buffer, Fig. 1B. The addition of ATP reduced the retention of [³H]-2-MeSADP indicating a competitive displacement of the marker. The addition of NF-023 had no effect, indicating that, under the experimental conditions, NF-023 did not compete with [³H]-2-MeSADP for binding at the P2Y₁, which is consistent with the literature [14].

The results of this study demonstrate that cellular membrane fragments obtained from an astrocytoma cell line expressing the P2Y₁ receptor can be immobilized on the IAM liquid chromatography stationary phase with retention of receptor binding activity. The development of the P2Y₁-IAM column expands the application of affinity chromatography-based online screening to another family of GPCRs and cell lines. It is of interest to note that with the β ₂-adrenergic and opioid receptor columns, it was necessary to add phosphatidylcholine lipids to the running buffer to stabilize the immobilized membranes [11,12], while this was not necessary with the P2Y₁-IAM column. It is not known whether this was due to the type of cell, i.e. astrocytoma versus Chinese hamster ovary (opioid) and HEK 293 (β ₂-adrenergic) and/or the receptor. This question is under investigation and will be reported elsewhere.

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References

1. Koles L, Furst S, Illes P. P2X and P2Y receptors as possible targets of therapeutic manipulations in CNS illnesses. *Drug News and Perspectives* 2005;18(2):85–101. [PubMed: 15883618]
2. Shankar H, Kunapuli SP. Is the P2Y₁ receptor a better target for antithrombotic drugs? *Drug Discovery Today: Therapeutic Strategies* 2005;2(3):285–290.
3. Gerevich Z, Illes P. P2Y receptors and pain transmission. *Purinergic Signaling* 2004;1:3–10.
4. Baurand A, Raboisson P, Freund M, Leon C, Cazenave J, Bourguignon J, Gachet C. Inhibition of platelet function by administration of MRS2179, a P2Y₁ receptor antagonist. *European Journal of Pharmacology* 2001;412:213–221. [PubMed: 11166284]
5. Niebauer RT, Gao ZG, Li B, Wess J, Jacobson KA. Signaling of the human P2Y₁ receptor measured by a yeast growth assay with comparisons to assays of phospholipase C and calcium mobilization in 1321N1 human astrocytoma cells. *Purinergic Signaling* 2005;1:241–247.
6. Palmer RK, Boyer JL, Schachter JB, Nicholas RA, Harden TK. Agonist Action of Adenosine Triphosphates at the Human P2Y₁ Receptor. *Molecular Pharmacology* 1998;54:1118–1123. [PubMed: 9855642]
7. Boyer JL, Romero-Avila T, Schachter JB, Harden TK. Identification of Competitive Antagonists of the P2Y₁ Receptor. *Molecular Pharmacology* 1996;50:1323–1329. [PubMed: 8913364]
8. Kim HS, Ohno M, Xu B, Kim HO, Choi Y, Ji XD, Maddileti S, Marquez VE, Harden TK, Jacobson KA. 2-Substitution of Adenine Nucleotide Analogues Containing a Bicyclo [3.1.0]hexane Ring System Locked in a Northern Conformation: Enhanced Potency as P2Y₁ Receptor Antagonists. *J Med Chem* 2003;46:4974–4987. [PubMed: 14584948]
9. Waldo GL, Corbitt J, Boyer JL, Ravi G, Kim HS, Ji X, Lacy J, Jacobson KA, Harden TK. *Mol Pharmacol* 2002;62:1249–1257. [PubMed: 12391289]
10. Houston D, Ohno M, Nicholas RA, Jacobson KA, Harden TK. [³²P] 2-iodo-N⁶-methyl-(N)-methanocarpa-2'-deoxyadenosine-3',5'-bisphosphate ([³²P]MRS2500), a novel radioligand for

quantification of native P2Y₁ receptors. *British Journal of Pharmacology* 2006;147:459–467. [PubMed: 16299552]

11. Beigi F, Wainer IW. Syntheses of Immobilized G Protein-Coupled Receptor Chromatographic Stationary Phases: Characterization of Immobilized μ and κ Opioid Receptors. *Anal Chem* 2003;75:4480–4485. [PubMed: 14632053]
12. Beigi F, Chakir K, Xiao R, Wainer IW. G-Protein-Coupled Receptor Chromatographic Stationary Phases. 2. Ligand-Induced Conformational Mobility in an Immobilized β_2 -Adrenergic Receptor. *Anal Chem* 2004;76:7187–7193. [PubMed: 15595859]
13. Moaddel R, Wainer IW. Development of immobilized membrane-based affinity columns for use in the online characterization of membrane bound proteins and for targeted affinity isolations. *Anal Chem* 2006;78:97–105.
14. Ziyal R, Ziganshin AU, Nickel P, Ardanuy U, Mutschler E, Lambrecht G, Burnstock G. Vasoconstrictor responses via P2X-receptors are selectively antagonized by NF023 in rabbit aorta and saphenous artery. *Br J Pharmacol* 1997;120:954–960. [PubMed: 9138704]

Figure 1.

a. The effect of the addition of increasing concentrations of unlabeled 2-MeSADP on the chromatographic retention of 1 nM $[^3\text{H}]\text{-2-MeSADP}$ (A) on the P2Y₁-IAM: (B) 0.1 μM unlabeled 2-MeSADP; (C) 0.5 μM unlabeled 2-MeSADP; (D) 1 μM unlabeled 2-MeSADP; (E) 10 μM unlabeled 2-MeSADP. **b.** The effect of the addition of 10 μM of NF023 (B) and 10 μM ATP (C) on the chromatographic retention of 0.5 nM of $[^3\text{H}]\text{-2-MeSADP}$ (A).

Table 1

Binding affinities were calculated by frontal affinity chromatography using the immobilized P2Y1 receptor columns, where the affinities are presented as K_d values $\pm$ the Standard Error of the Mean. The chromatographic results are compared to binding affinities reported in literature [6,10].

	2-MeSADP (nM)	MRS-2179 (nM)	ATP (μ M)
P2Y1-IAM	186.4 $\pm$ 85.7	199.0 $\pm$ 4.5	1.38 $\pm$ 0.94
Literature	50 $\pm$ 10[10]	117 $\pm$ 9 [10] 109 $\pm$ 7 [6]	14 $\pm$ 6 [10]