British Journal of Pharmacology (1998) 124, 1–3
http://www.stockton-press.co.uk/bjp

SPECIAL REPORT

Competitive and selective antagonism of $P2Y_1$ receptors by N^6-methyl 2′-deoxyadenosine 3′,5′-bisphosphate

[1,3]José L. Boyer, [1]Arvind Mohanram, [2]Emidio Camaioni, [2]Kenneth A. Jacobson & [1]T. Kendall Harden

[1]Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, NC 27599-7365 and [2]Molecular Recognition Section, NIDDK, NIH, Bethesda, MD 20892-1008, U.S.A.

The antagonist activity of N^6-methyl 2′-deoxyadenosine 3′,5′-bisphosphate (N6MABP) has been examined at the phospholipase C-coupled $P2Y_1$ receptor of turkey erythrocyte membranes. N6MABP antagonized 2MeSATP-stimulated inositol phosphate hydrolysis with a potency approximately 20 fold greater than the previously studied parent molecule, adenosine 3′,5′-bisphosphate. The $P2Y_1$ receptor antagonism observed with N6MABP was competitive as revealed by Schild analysis ($pK_B = 6.99 \pm 0.13$). Whereas N6MABP was an antagonist at the human $P2Y_1$ receptor, no antagonist effect of N6MABP was observed at the human $P2Y_2$, human $P2Y_4$ or rat $P2Y_6$ receptors.

Keywords: $P2Y_1$ antagonist; $P2Y_1$ receptor; inositol lipid signalling; turkey erythrocytes; phospholipase C

Introduction Pharmacological classification of natively expressed G protein-coupled P2Y receptors and unambiguous definition of their physiological roles have been problematic due to lack of selective receptor antagonists. The available antagonists interact with non-receptor ATP-binding proteins and antagonize both P2Y and P2X receptors (Ziganshin *et al*., 1993; Bultmann & Starke, 1994; Harden *et al*., 1995; Charlton *et al*., 1996).

The turkey erythrocyte has been utilized as a model cell-free assay system for pharmacological and biochemical characterization of the $P2Y_1$ receptor and its associated signalling proteins (Boyer *et al*., 1996b). Using this test system we recently identified a group of molecules, the adenosine bisphosphates, that exhibit partial agonist/antagonist activities at the $P2Y_1$ receptor (Boyer *et al*., 1996a). To identify antagonist molecules that interact with higher affinity with the $P2Y_1$ receptor, a series of adenosine bisphosphate derivatives were synthesized with various adenine base- or ribose-substitutions (Camaioni *et al*., 1998). In this paper we describe the $P2Y_1$ receptor activity and P2Y receptor subtype specificity of one of the most promising of these molecules, N^6-methyl 2′-deoxyadenosine 3′,5′-bisphosphate (N6MABP or MRS2179).

Methods *Assay of P2Y receptor-promoted inositol lipid hydrolysis* Turkey erythrocytes were labelled with [^{3}H]-inositol and $P2Y_1$ receptor-promoted phospholipase C activity was determined in membranes prepared from these cells as previously described (Boyer *et al*., 1996a). Inositol phosphate accumulation in 1321N1 human astrocytoma cells expressing the human $P2Y_1$ receptor, the human $P2Y_2$ receptor, the human $P2Y_4$ receptor or the rat $P2Y_6$ receptor was determined as previously described (Boyer *et al*., 1997).

Chemical synthesis N6MABP was prepared as previously described (Camaioni *et al*., 1998). The chemical structure of the phosphorylated nucleoside was verified by use of ^{1}H-n.m.r. and ^{31}P-n.m.r. techniques, as well as by high resolution fast atomic bombardment mass spectroscopy.

[3]Author for correspondence at: Department of Pharmacology, CB#7365, Mary Ellen Jones Bldg, University of North Carolina, Chapel Hill, NC 27599-7365, U.S.A.

Results A series of second generation molecules was synthesized (Camaioni *et al*., 1998) based on our observation that adenosine bisphosphates are $P2Y_1$ receptor antagonists (Boyer *et al*., 1996a). Absence of a hydroxyl in the 2′ position of these molecules resulted in analogues that, in contrast to the originally studied adenosine bisphosphates, do not interact with A_1-adenosine receptors (data not shown). N6MABP (insert in Figure 1a) was one of the most promising of these molecules in preliminary studies, since no partial agonist activity was observed at the turkey erythrocyte membrane $P2Y_1$ receptor and the effect of the full agonist 2MeSATP was inhibited with an IC_{50} approximately 20 fold lower than that of the parent compound 2′-deoxyadenosine 3′,5′-bisphosphate (data not shown). The nature of the antagonism was determined by generating a series of concentration-effect curves for 2MeSATP for stimulation of phospholipase C in the presence of different concentrations of N6MABP. N6MABP caused a parallel shift to the right of the concentration-response curve for 2MeSATP (Figure 1a). In contrast to the effects of reactive blue-2, suramin and PPADS in the same assay system (Boyer *et al*., 1994), the effects of high concentrations of N6MABP were completely surmountable by high concentrations of 2MeSATP (Figure 1a). Schild analysis (Figure 1b) confirmed that the antagonism produced by N6MABP was competitive with a pK_b of 6.99 ± 0.13 ($n = 3$). Identical antagonist properties were observed with the positional isomer of N6MABP, N^6-methyl 3′-deoxyadenosine 2′,5′-bisphosphate (data not shown).

The selectivity of N6MABP was tested by examining its effects at the human $P2Y_1$, human $P2Y_2$, human $P2Y_4$ and rat $P2Y_6$ receptors (Figure 2). N6MABP completely blocked $P2Y_1$ receptor-promoted stimulation of phospholipase C activity induced by a near maximal concentration (100 nM) of 2MeSATP (Figure 2). This inhibition also was competitive (data not shown). In contrast, no significant inhibition was observed of the response to 100 nM UTP at the human $P2Y_2$ and $P2Y_4$ receptors, or to 100 nM UDP at the $P2Y_6$ receptor (Figure 2).

Discussion The data presented here illustrate that N6MABP is a high affinity competitive antagonist at the $P2Y_1$ receptor. This antagonist also is remarkably selective since it apparently does not block the three other cloned mammalian P2Y

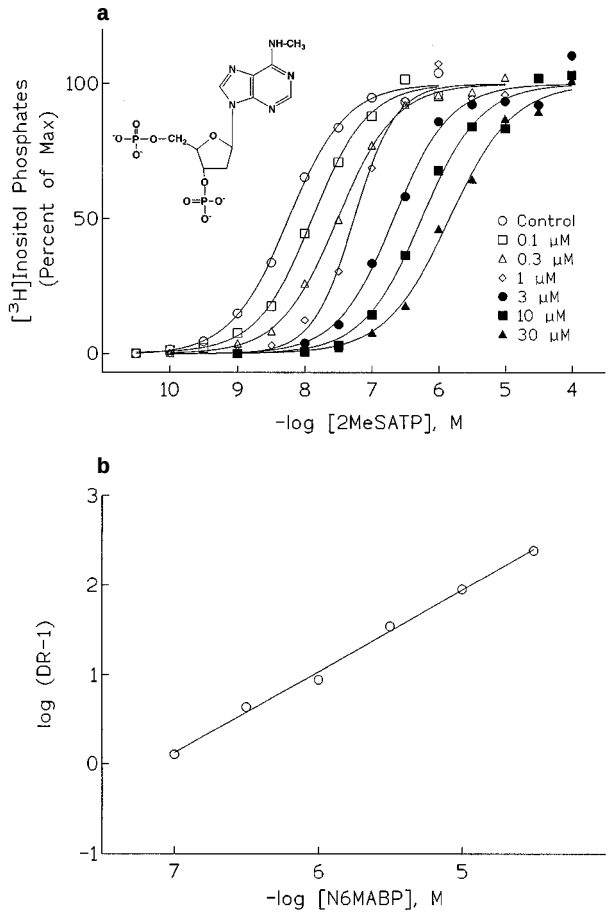

Figure 1 Competitive inhibition of 2MeSATP-promoted activation of $P2Y_1$ receptors by N6MABP. (a) [^{3}H]-inositol-labelled turkey erythrocyte membranes were incubated with the indicated concentrations of 2MeSATP in the presence or absence of the indicated concentrations of N6MABP (inset). (b) Schild regression analysis of data shown in (a). The data shown are the results from a representative experiment repeated three times to yield a mean pK_B of 6.99 ± 0.13 and Schild slope of 0.93 ± 0.05.

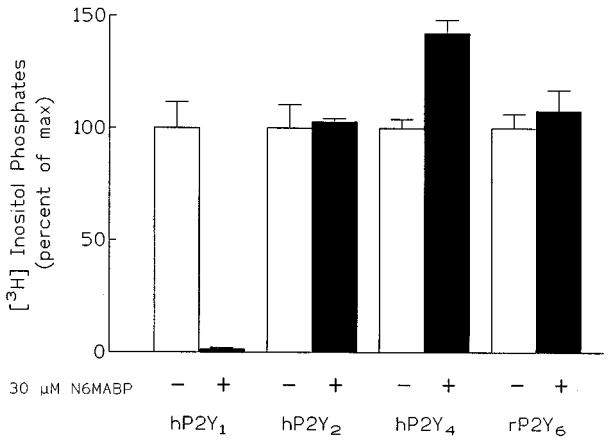

Figure 2 Effects of N6MABP on mammalian P2Y receptors stably expressed in 1321N1 human astrocytoma cells. [^{3}H]-inositol-labelled 1321N1 human astrocytoma cells stably expressing the human $P2Y_1$, human $P2Y_2$, human $P2Y_4$ or rat $P2Y_6$ receptors were incubated with 100 nM 2MeSATP (P2Y1), 100 nM UTP ($P2Y_2$ and $P2Y_4$), or 100 nM UDP ($P2Y_6$) in the absence or in the presence of 30 μM N6MABP. The data are from a representative experiment repeated three times.

receptors. The high affinity and receptor selectivity of N6MABP make it unique among the molecules now available for investigation of P2Y receptors. Although useful as P2 receptor antagonists, reactive blue 2 and suramin are well known to interact with many nucleotide binding proteins in addition to the P2 receptors, and they bind to multiple of the known P2 receptors (Harden *et al.*, 1995). Lambrecht and coworkers have provided an important advance by introducing PPADS (Lambrecht *et al.*, 1992), which apparently exhibits selectivity for P2 receptors over other types of proteins. However, PPADS also antagonizes both P2X and P2Y receptors, although it does exhibit selectivity for the $P2Y_1$ receptor over other P2Y receptor subtypes (Ziganshin *et al.*, 1993; Boyer *et al.*, 1994; Bultmann & Starke, 1994; Charlton *et al.*, 1996). A notable exception to the relatively non-selective P2 receptor antagonists is the molecule, 2-propylthio-D-β,γ-dichloromethylene ATP (ARL 67085), which is a selective antagonist of very high affinity at the Gi/adenylyl cyclase-linked P2Y receptor of platelets (Humphries *et al.*, 1995). This molecule does not block $P2Y_1$ receptors, which prominently distinguishes it from adenosine 3′,5′-bisphosphate which competitively blocks the $P2Y_1$ receptor but does not interact with the Gi/adenylyl cyclase-linked receptor of C6 rat glioma cells (Boyer *et al.*, 1996a).

A major advantage of N6MABP is that it is an adenine nucleotide derivative in a group of second generation molecules that have followed from the observation that phosphate-substitution in the 2′- or 3′-position results in loss of agonist activity at P2Y receptors without a coincident loss of receptor affinity. Discovery of antagonist activity in an adenine nucleotide derivative places us in position to take further advantage of the wide ranging structure activity studies that have preceded on adenine nucleotide derivatives at the $P2Y_1$ receptor. Moreover, the pharmacological selectivity and apparent affinity of the adenosine bisphosphate derivative demonstrated here should prove useful for pharmacological resolution of the $P2Y_1$ receptor in various mammalian tissues.

References

BOYER, J.L., ROMERO, T., SCHACHTER, J.B. & HARDEN, T.K. (1996a). Identification of competitive antagonists of the P2Y1 receptor. *Mol. Pharmacol.*, **50,** 1323–1329.

BOYER, J.L., SCHACHTER, J.B., SROMEK, S.M., PALMER, R.K., JACOBSON, K.A., NICHOLAS, R.A. & HARDEN, T.K. (1996b). Avian and human homologues of the P2Y1 receptor: pharmacological, signaling, and molecular properties. *Drug Dev. Res.*, **39,** 253–261.

BOYER, J.L., WALDO, G.L. & HARDEN, T.K. (1997). Molecular cloning and expression of an avian G protein-coupled P2Y receptor. *Mol. Pharmacol.*, **52,** 928–934.

BOYER, J.L., ZOHN, I., JACOBSON, K.A. & HARDEN, T.K. (1994). Differential effects of putative P2-purinoceptor antagonists on phospholipase C- and adenylyl cyclase-coupled P2Y-purinoceptors. *Br. J. Pharmacol.*, **113,** 614–620.

BULTMANN, R. & STARKE, K. (1994). P_2-purinoceptor antagonists discriminate three contraction-mediating receptors for ATP in rat vas deferens. *Naunyn Schmiedebergs Arch. Pharmacol.*, **349,** 74–80.

CAMAIONI, E., BOYER, J.L., MOHANRAM, A., HARDEN, T.K. & JACOBSON, K.A. (1998). Deoxyadenosine-bisphosphate derivatives as potent antagonists at P2Y1 receptors. *J. Med. Chem.,* **41,** 183–190.

CHARLTON, S.J., BROWN, C.A., WEISMAN, G.A., TURNER, J.T. & BOARDER, M.R. (1996). PPADS and Suramin as antagonists at cloned P2Y- and P2U-purinoceptors. *Br. J. Pharmacol,* **118,** 704–710.

HARDEN, T.K., BOYER, J.L. & NICHOLAS, R.A. (1995). P2-purinergic receptors: subtype-associated signaling responses and structure. *Annu. Rev. Pharmacol. Toxicol.,* **35,** 541–579.

HUMPHRIES, R.G., TOMLINSON, W., CLEGG, J.A., INGALL, A.H., KINDON, N.D. & LEFF, P. (1995). Pharmacological profile of the novel P2T-purinoceptor antagonist, FLP 67085 in vitro and in the anaesthetized rat in vivo. *Br. J. Pharmacol.,* **115,** 1110–1116.

LAMBRECHT, G., FRIEBE, T., GRIMM, U., WINDSCHEIF, U., BUNGARDT, E., HILDEBRANDT, C., BAUMERT, H., SPATZ-KUMBEL, G. & MUTSCHLER, E. (1992). PPADS, a novel functionally selective antagonist of P2 purinoceptor-mediated responses. *Eur. J. Pharmacol.,* **217,** 217–219.

ZIGASHIN, A.U., HOYLE, C.H.V., BO, H., LAMBRECHT, G., MUTSCHLER, E., BÄUMERT, H.G. & BURNSTOCK, G. (1993). PPADS selectivity antagonizes P_{2X}-purinoceptor-mediated responses in the rabbit urinary bladder. *Br. J. Pharmacol.,* **110,** 1491–1495.

(Received January 8, 1998
Accepted February 18, 1998)