Br. J. Pharmac. (1981), **72**, 443–447

5′-N-ETHYLCARBOXAMIDOADENOSINE: A POTENT INHIBITOR OF HUMAN PLATELET AGGREGATION

N.J. CUSACK & S.M.O. HOURANI

Department of Pharmacology, University of London, King's College, Strand, London WC2R 2LS

1 5′-N-ethylcarboxamidoadenosine (NECA) is an adenosine analogue which is 22,900 times more potent than adenosine as a vasodilator. Adenosine and some of its analogues are also inhibitors of human platelet aggregation. NECA was tested for its effects on human platelets.

2 NECA (1 μM) inhibited human platelet aggregation induced by adenosine 5′-diphosphate (ADP), adrenaline, 5-hydroxytryptamine (5-HT) and thrombin more powerfully than adenosine. NECA was 5 to 10 times more potent than adenosine at inhibiting ADP- and adrenaline-induced aggregation.

3 NECA, like adenosine, caused dose-dependent increases in levels of platelet adenosine 3′,5′-cyclic monophosphate (cyclic AMP), which were competitively inhibited by theophylline, an adenosine antagonist.

4 These effects of NECA, like those of adenosine, were completely stereospecific as the L-enantiomer of NECA was inactive.

5 NECA did not interfere with the inhibition by ADP of prostaglandin E_1 (PGE_1)-stimulated adenylate cyclase.

6 NECA is the most potent analogue of adenosine tested so far on human platelets, and is the first example of a 5′ modification to retain affinity for the platelet adenosine receptor.

Introduction

Adenosine is a vasodilator, and also inhibits platelet aggregation apparently by acting at an external membrane receptor to increase levels of platelet adenosine 3′,5′-cyclic monophosphate (cyclic AMP) (Haslam & Rosson, 1975). Certain analogues of adenosine, generally those substituted at the C^2 or N^6 positions, retain activity as vasodilators (Dietmann, Birkenheier & Schaumann, 1970; Cobbin, Einstein & Maguire, 1974) and also inhibit platelet aggregation by raising levels of cyclic AMP (Kikugawa, Iizuka & Ichino, 1973a; Kikugawa, Suehiro & Ichino, 1973b; Haslam, Davidson & Desjardins, 1978).

Recently 5′-N-ethylcarboxamidoadenosine (NECA) (Figure 1) an analogue of adenosine substituted at the 5′ position, was reported to be an extraordinarily powerful vasodilator, 22,900 times more potent than adenosine (Raberger, Schütz & Kraupp, 1977). As it appeared that NECA might also be a potent inhibitor of platelet aggregation, its effects on platelet function were tested.

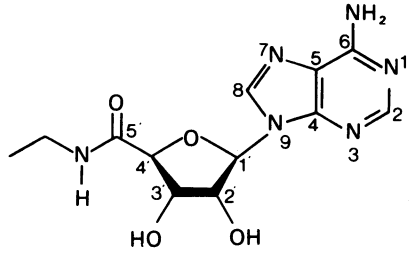

Figure 1 Structure of 5′-N-ethylcarboxamidoadenosine (NECA).

Methods

Aggregation studies

Human platelet-rich plasma was separated from citrated venous blood by centrifugation at 260 *g* for 20 min at room temperature. Aggregation was quantified photometrically (Michal & Born, 1971) as the maximal rate of increase in light transmission through a 1 ml sample of stirred platelet-rich plasma at 37°C on addition of aggregating agents. Adenosine and its analogues were tested for inhibitory activity by incubation with platelet-rich plasma at 37°C for 3 min before being challenged by aggregating agents.

Measurement of changes in cyclic AMP

Platelet-rich plasma was preincubated for 90 min at 37°C with purified [^{14}C]-adenine to label platelet

0007-1188/81/030443-05$01.00

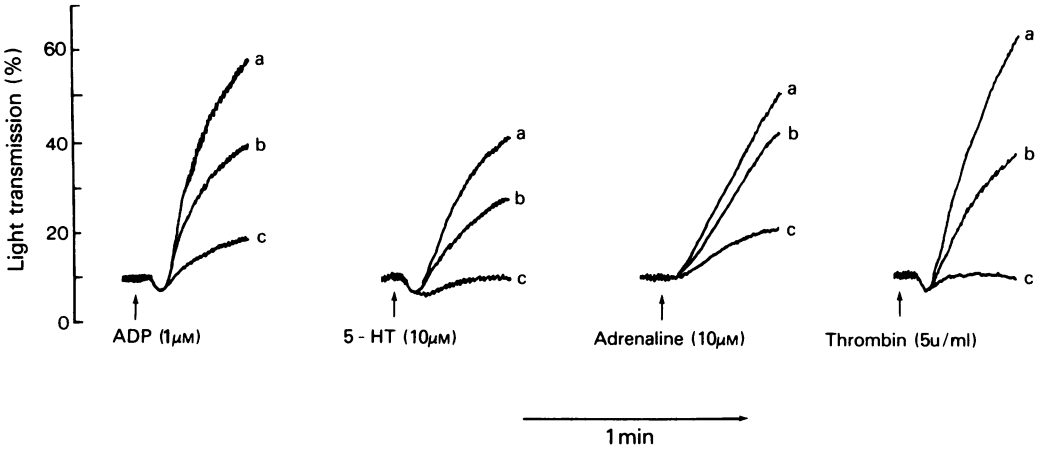

Figure 2 Inhibition of human platelet aggregation by NECA and adenosine. Platelet-rich plasma (1 ml) was incubated for 3 min at 37 °C with (a) no additions; (b) adenosine (1 μM); (c) NECA (1 μM) before addition of aggregating agents (at arrows).

adenine nucleotides (Haslam & Rosson, 1975). Aliquots (0.9 ml) were treated with solutions (0.1 ml) of the test compounds containing papaverine to inhibit phosphodiesterase. The reaction was terminated and cyclic AMP extracted by addition of 3 M perchloric acid (0.2 ml) containing [^{3}H]-cyclic AMP to estimate recovery. The samples were centrifuged and the supernatant (0.9 ml) applied to a column of AG50W ×8 [H^+] (1 ml). After elution with 1 mM KH_2PO_4 buffer (pH 7.3) the eluate was treated twice with freshly precipitated 0.25 M $BaSO_4$ (0.6 ml) and centrifuged. The supernatant (6 ml) was lyophilised and ^{14}C and ^{3}H were estimated by liquid scintillation counting.

Drugs

Adenosine 5'-diphosphate (ADP), 5-hydroxytryptamine (5-HT), adrenaline bitartrate, adenosine, theophylline, thrombin and papaverine hydrochloride were obtained from Sigma London. AG50W×8 [H^+] ion exchange resin was obtained from Bio-Rad Laboratories Ltd. Prostaglandin E_1 (PGE_1) was a generous gift from Dr D. Pike of the Upjohn Company in Kalamazoo, Michigan. [U-^{14}C]-adenine and [8-^{3}H]-cyclic AMP were obtained from the Radiochemical Centre, Amersham. L-Adenosine was prepared by the method of Acton, Ryan & Goodman (1964). NECA was synthesized from adenosine via isopropylidene adenosine 5'-carboxylic acid (Harman, Zenerosa & Gupta, 1969) as described by Prasad & Tietje (1978). 5'-N-Ethylcarboxamido-L-adenosine (L-NECA) was synthesized in an identical manner from L-adenosine.

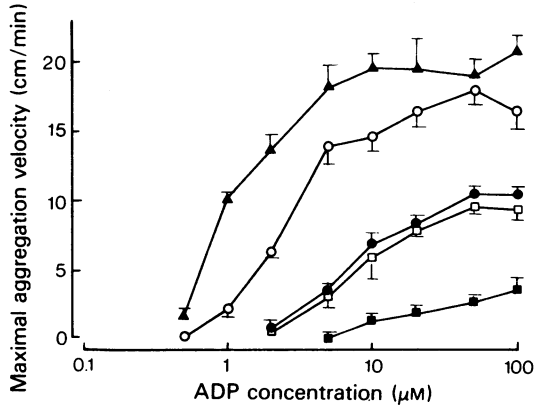

Figure 3 Inhibition of ADP-induced human platelet aggregation by NECA and adenosine after incubation with platelet-rich plasma for 3 min at 37°C; (▲) no additions; (○) adenosine (1 μM); (□) NECA (1 μM); (●) adenosine (10 μM); (■) NECA (10 μM). Each point is the mean of 3 observations. Vertical bars show s.e. mean.

Results

Effects of NECA on human platelet aggregation

NECA (1 μM) inhibited aggregation of human platelets induced by ADP, 5-HT, adrenaline and thrombin more powerfully than adenosine at the same concentration (Figure 2). Log dose-response curves to ADP showed that NECA, like adenosine, inhibited ADP-induced aggregation noncompetitively (Figure 3).

NECA was approximately 5 times more potent than adenosine as an inhibitor of aggregation induced by 5 μM ADP, with an IC_{50} value of 0.14 μM compared to an IC_{50} value of 0.72 μM for adenosine (Figure 4a). With 200 μM ADP, NECA was approximately 10 times more potent than adenosine, with an IC_{50} value of 1 μM compared to an IC_{50} value of 10 μM for adenosine (Figue 4b). The log dose-response curves for NECA and adenosine as inhibitors of aggregation induced by 200 μM ADP were not parallel, and only NECA approached 100% inhibition. NECA was approximately 10 times more potent than adenosine at inhibiting aggregation induced by adrenaline (200 μM) (results not shown). The enantiomer of NECA, L-NECA, (100 μM) was inactive as an inhibitor of platelet aggregation.

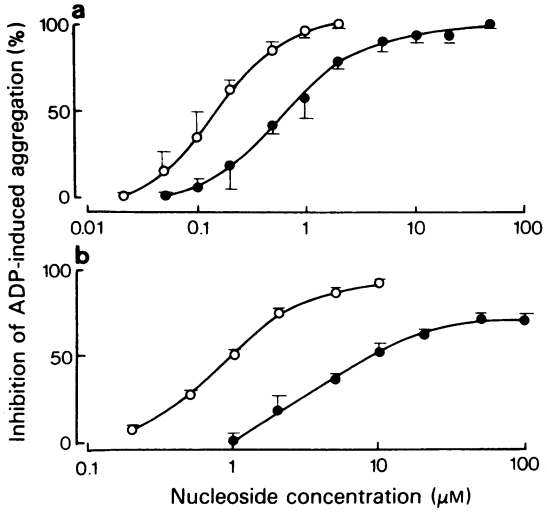

Figure 4 Inhibition by NECA and adenosine of ADP-induced aggregation. NECA (O) or adenosine (●) was incubated with platelet-rich plasma for 3 min at 37°C before addition of ADP (a) 5 μM; (b) 200 μM. Each point is the mean of at least 3 observations. Vertical bars show s.e. mean.

Effects of NECA on adenylate cyclase

NECA caused dose-dependent increases in concentrations of platelet cyclic AMP which were competitively inhibited by theophylline (100 μM) (Figure 5). Lineweaver-Burke analysis of this experiment gave a K_a value for NECA of 0.95 μM and a K_i value for theophylline of 8 μM (Figure 6). Adenosine had a K_a value of 1.2 μM (results not shown) so NECA was approximately 1.3 times more potent.

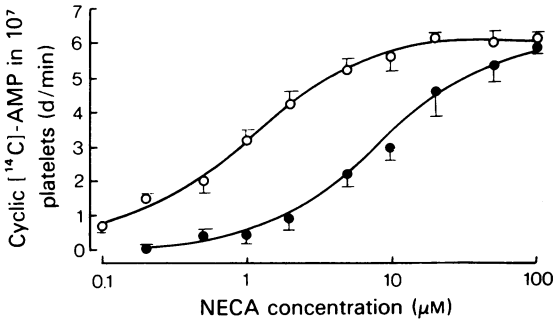

Figure 5 Effect of NECA on levels of platelet cyclic [^{14}C]-AMP. Platelet-rich plasma preincubated with [^{14}C]-adenine for 90 min at 37°C was treated with NECA for 30 s at 37°C in the absence (O) or presence (●) of theophylline (100 μM). All samples contained papaverine (2 mM). Each point is the mean of at least three observations. Vertical bars show s.e. mean.

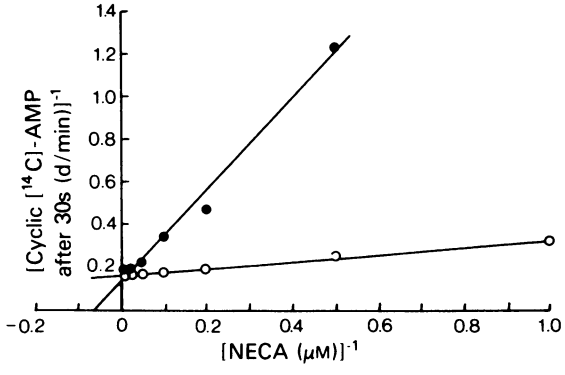

Figure 6 Double reciprocal plot of data taken from Figure 4. Lines were fitted by computer using least squares linear regression analysis. (O) NECA; (●) NECA and theophylline.

Effects of NECA on prostaglandin E_1-stimulated adenylate cyclase

Neither NECA (10 μM) nor adenosine at the same concentration prevented inhibition of PGE_1 (1 μM)-stimulated adenylate cyclase by ADP (5 μM), in the presence of papaverine (2 mM) and sufficient theophylline (100 μM) to block access of NECA or adenosine to the adenosine receptor (Figure 7).

Discussion

Of the many adenosine analogues tested so far as inhibitors of human platelet aggregation, significant activity is found only in those substituted at the C^2-position, such as 2-chloroadenosine and 2-azidoadenosine (Born, Haslam, Goldman & Lowe, 1965; Cusack & Born, 1977), or at the N^6-position, such as

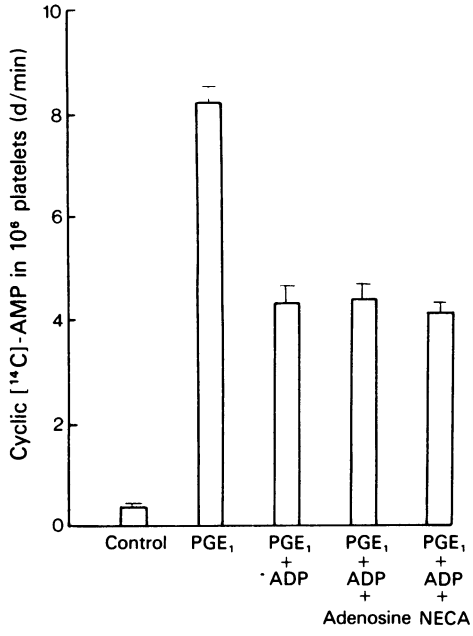

Figure 7 Effect of NECA (10 μM) and adenosine (10 μM) in the presence of papaverine (2 mM) and theophylline (100 μM) on the inhibition after 20 s at 37°C of prostaglandin E_1 (PGE_1 1 μM)-stimulated levels of platelet cyclic [^{14}C]-AMP by ADP (5 μM).

N^6-phenyladenosine (Kikugawa *et al.*, 1973a). None of these analogues is more than 3 times more potent than adenosine, and the most active inhibitors, 2-chloroadenosine and 2-azidoadenosine, were only more potent at high ($\geq$10 μM) concentrations (Haslam & Rosson, 1975; Cusack & Born, 1977). Our results show that NECA is 5 to 10 times more potent than adenosine even at micromolar concentrations, and would therefore appear to be the most potent adenosine analogue yet tested as an inhhibitor of human platelet aggregation.

Adenosine inhibits platelet aggregation by an action on an external receptor linked to adenylate cyclase (Haslam & Rosson, 1975), which has recently been shown to be stereospecific (Cusack, Hickman & Born, 1979). NECA also caused stereospecific increases in levels of platelet cyclic AMP, which are competitively inhibited by theophylline, a known (Haslam & Rosson, 1975) adenosine antagonist. NECA was, however, only about 1.3 times more potent than adenosine at raising levels of platelet cyclic AMP, compared to 5 to 10 times more potent as an inhibitor of platelet aggregation.

Some adenosine analogues, such as 2-azido-AMP and 2-ethylthioadenosine 5'-phosphorothioate, act by competing with ADP for an ADP receptor (Gough, Nobbs, Middleton, Penglis-Caredes & Maguire, 1978, Cusack *et al.*, 1979), and it was conceivable that the extra potency of NECA could be accounted for by such a supplementary action. Our results indicate that NECA does not act at an ADP receptor, as it was just as potent at inhibiting aggregation due to other agents such as adrenaline. Additionally, no direct antagonism by NECA of the action of ADP on PGE_1-stimulated adenylate cyclase was detected.

NECA is a departure from the known structure-activity relationships observed for inhibition of platelet aggregation by adenosine analogues, since such activity has so far only been retained by N^6 and C^2 substitutions, and all other 5' modifications have rendered adenosine inactive (for review see Haslam & Cusack, 1981). The ability of NECA to inhibit platelet aggregation and to activate adenylate cyclase must alter established concepts of the requirements of the platelet adenosine receptor.

We thank the Medical Research Council for financial support (G 978/38/SA), the British Heart Foundation for an equipment grant, the Vandervell Foundation for support for one of us (S.M.O.H.) and Professor G.V.R. Born, F.R.S. for encouragement. Blood was kindly donated by employees of Equity and Law Life Assurance Society Limited, and taken by Dr C. Fenn of this department. Correspondence to N.J.C. please.

References

ACTON, E.M., RYAN, K.J. & GOODMAN, L. (1964). Synthesis of L-ribofuranose and L-adenosine. *J. Am. Chem. Soc.*, **86**, 5352–5354.

BORN, G.V.R., HASLAM, R.J., GOLDMAN, M. & LOWE, R.D. (1965). Comparative effectiveness of adenosine analogues as inhibitors of blood-platelet aggregation and as vasodilators in man. *Nature*, **205**, 678–680.

COBBIN, L.B., EINSTEIN, R. & MAGUIRE, M.H. (1974). Studies on the coronary dilator actions of some adenosine analogues *Br. J. Pharmac.*, **50**, 25–33.

CUSACK, N.J. & BORN, G.V.R. (1977). Effects of photolysable 2-azido analogues of adenosine, AMP and ADP on human platelets. *Proc. R. Soc. Lond. B.*, **197**, 515–520.

CUSACK, N.J., HICKMAN, M.E. & BORN, G.V.R. (1979). Effects of D- and L- enantiomers of adenosine, AMP and ADP and their 2-chloro- and 2-azido- analogues on human platelets. *Proc. R. Soc. Lond. B.*, **206**, 139–144.

DIETMANN von, K., BIRKENHEIER, H. & SCHAUMANN, W. (1970). Hemmung der induzierten Thrombocyten-Aggregation durch Adenosin und Adenosin-Derivate. *Arzneim. Forsch. (Drug Res.)*, **20**, 1749–1751.

GOUGH, G., NOBBS, D.M., MIDDLETON, J.C., PENGLIS-CAREDES, F. & MAGUIRE, M.H. (1978). New inhibitors

of platelet aggregation. 5'-Phosphate, 5'-phosphorothioate, and 5'-O-sulphamoyl derivatives of 2-substituted adenosine analogues. *J. med. Chem.*, **21**, 520–529.

HARMAN, R.E., ZENEROSA, C.V. & GUPTA, S.K. (1969). Permanganate oxidation of purine nucleosides. *Chem. Ind. (Lond.)*, **1141**.

HASLAM, R.J. & CUSACK, N.J. (1981). Blood platelet receptors for ADP and adenosine. In *Purinergic Receptors: Receptors and Recognition*, Series B Volume 12, ed. Burnstock, G. London: Chapman and Hall.

HASLAM, R.J., DAVIDSON, M.M.L. & DESJARDINS, J.V. (1978). Inhibition of adenylate cyclase by adenosine analogues in preparations of broken and intact human platelets. *Biochem. J.*, **176**, 83–95.

HASLAM, R.J. & ROSSON, G.M. (1975). Effects of adenosine on levels of adenosine cyclic 3', 5'-monophosphate in human blood platelets in relation to adenosine incorporation and platelet aggregation. *Mol. Pharmac.*, **11**, 528–544.

KIKUGAWA, K., IIZUKA, K. & ICHINO, M. (1973a). Platelet aggregation inhibitors. 4. N^6-Substituted adenosines. *J. med. Chem.*, **16**, 358–364.

KIKUGAWA, K., SUEHIRO, H. & ICHINO, M. (1973b). Platelet aggregation inhibitors. 6. 2-Thioadenosine derivatives. *J. med. Chem.*, **16**, 1381–1388.

MICHAL, F. & BORN, G.V.R. (1971). Effect of the rapid shape change of platelets on the transmission and scattering of light through plasma. *Nature*, **231**, 220–222.

PRASAD, R.N. & TIETJE, K. (1978). N^1, N^6-ethenoadenosine 5'-(N-ethylcarboxamide). In *Nucleic Acid Chemistry: Improved and New Synthetic Procedures, Methods and Techniques.* ed. Townsend, L.B. & Tipson, R.S. pp. 701–707. New York, Chichester: Wiley.

RABERGER, G., SCHÜTZ, W. & KRAUPP, O. (1977). Coronary dilatory action of adenosine analogues: a comparative study. *Archs int. Pharmacodyn.*, **230**, 140–149.

(Received July 4, 1980, Revised August 18, 1980.)