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2,4-DIAMINO-5-CYANO-6-HALOPYRIDINES: A NEW CLASS OF CYCLIC AMP PHOSPHODIESTERASE INHIBITORS WITH THERAPEUTIC POTENTIAL

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Abstract—2,4-Diamino-5-cyano-6-halopyridines have been described previously as oral insulinotropic agents and have been found recently to have bronchodilatory properties. In the present report the synthesis of the iodo compound is newly described, and it is established that HI- or HBr-mediated condensation and cyclization of malononitrile in 1,2-dichloroethane yield selectively the 5-cyanopyridine derivatives. The pyridine derivatives were found to constitute a new class of potent cyclic AMP phosphodiesterase inhibitors. Inhibition of purified dog kidney cyclic AMP phosphodiesterase was of the mixed type. Since cyclic AMP phosphodiesterase inhibitors are known to enhance glucose-induced insulin secretion and to activate glucose production by the liver, the finding that the pyridine derivatives described here inhibited cyclic AMP phosphodiesterase opens new avenues of interpretation for their insulinotropic action as well as for the paradoxical lack of improvement of glucose disposal by elevation of insulin after oral drug administration. Cyclic AMP phosphodiesterase inhibition also has the potential of explaining the bronchodilatory effects of these drugs.

Hershfield and Richards [1] found 2,4-diamino-5-cyano-6-bromopyridine (compound I) to inhibit glucose efflux from erythrocytes. These authors noted steric similarities in hydrogen bonding possibilities and bulkiness of substituents between the pyridine derivative and glucose. It was demonstrated recently that this compound and a number of analogues augmented glucose-stimulated rat insulin secretion in the perfused pancreas or *in vivo* after oral administration [2], suggesting the possibility that these compounds mimicked glucose activity on a putative glucose receptor. However, the finding that enhancement by orally administered compound I of plasma insulin elevation in response to an intravenous glucose bolus was not accompanied by an acceleration of glucose disposal [2] made us search for possible molecular mechanisms whereby insulin action may be counteracted. The present report shows that compound I and its 6-iodo analogue are potent cyclic AMP phosphodiesterase inhibitors. This opens new possibilities of explanation of both its insulinotropic action and its insulin antagonizing effects and provides new tools for the study of cyclic AMP phosphodiesterases in general. It also bears on the mechanism of the bronchodilatory properties of compound I [3]. Because of the low acute toxicity of this drug [2], it appears to have therapeutic potential.

MATERIALS AND METHODS

Materials. Malononitrile and 2-amino-1-propene-1,1,3-tricarbonitrile were products from the Aldrich Chemical Co., Milwaukee, WI. HI and HBr gases were obtained from Matheson Gas Products, Newark, CA, and streptozotocin, C^2HCl_3, $(C^2H_3)_2$-SO and tetramethylsilane (TMS) from the Sigma Chemical Co. St. Louis, MO. Other solvents and reagents were standard reagent grade. Male Wistar rats (315–330 g) were obtained from Simonsen Laboratories, Gilroy, CA, and fed a Purina rat chow *ad lib.* until the start of the experiment.

2,4-*Diamino-5-cyano-6-iodopyridine.* Malononitrile (33 g, 0.50 mole) was dissolved in 500 ml of 1,2-dichloroethane and filtered through a Whatman GF/C glass fiber filter. The clarified solution was stirred in an ice-cooled reaction vessel equipped with a reflux condenser, and HI gas was introduced through a non-submerging inlet. A yellow precipitate formed immediately in a slightly exothermic reaction. After the absorption of 1 mole of HI as measured by weight increase, the rate of absorption slowed, and no new precipitate formed. The yellow precipitate was collected by filtration and washed with 1,2-dichloroethane. Suspension in 300 ml of water and neutralization with NaOH resulted in an orange precipitate that was dissolved in 1.7 l of boiling 50% ethanol/water and treated with charcoal. On cooling, the product crystallized. Silica gel thin-layer chromatograms developed with ethyl acetate showed the main product with R_f = 0.51 and a contaminant with R_f = 0.69. Spots with the same R_f values were also observed during the synthesis of the bromo analogue (see below). After several cycles of charcoal treatment and crystallization, a colorless

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product giving a single spot on thin-layer chromatograms was obtained. Yield: 38.4 g (59% of theoretical). Melting point: 244–245°. Anal. Calc. for $C_6H_5IN_4$: C, 27.71; H, 1.94; I, 48.88; N, 21.55; mol. wt, 260.0. Found: C, 27.79; H, 1.67; I, 49.65; N, 21.37.

2,4-Diamino-5-cyano-6-bromopyridine. This compound was synthesized and purified exactly as described for the 6-iodo analogue. The final product was identical with that obtained by Carboni *et al.* [4] as judged by melting point, thin-layer chromatography, and elemental analysis. The crude precipitate formed in the initial reaction was identified as predominantly the monohydrobromide of the title compound, even if a large excess of HBr gas were used. Anal. Calc. for $C_6H_6Br_2N_4$: C, 24.51; H, 2.06; Br, 54.37; N, 19.06; mol. wt 294.0. Found: C, 23.57; H, 1.98; Br, 50.24; N, 17.15. During recrystallization of impure product, a by-product formed needle-like crystals that could be collected separately because of their faster sedimentation. A major spot with R_f = 0.69 was observed on silica gel thin-layer chromatograms developed with ethyl acetate. Mass spectrometry identified this side-product as aminodibromo-cyanopyridine.

Spectroscopy. Proton and natural abundance ^{13}C Fourier transform nuclear magnetic resonance (NMR) spectra were taken on a Varian spectrometer (model CFT-20) at 80 and 20 MHz respectively. Pyridine derivatives were dissolved in $(C^2H_3)_2SO$ while 2-amino-1-propene-1,1,3-tricarbonitrile, after recrystallization from cyclohexane, was dissolved in C^2HCl_3. IR spectra were taken in KBr pellets on a Perkin Elmer No. 727-B instrument. Mass spectra were performed on a VG Data Systems mass spectrometer (model VG 7070 H) in the electron bombardment mode using a direct insertion probe, a source temperature of 200°, and a filament emission of 100 μA.

Elemental analyses. These were performed by Chemalytics, Inc., Tempe, AZ, and Canadian Microanalytical Service Ltd., Vancouver, Canada.

Isolation of metabolite. Four normal male Wistar rats (315–330 g) were housed individually in metabolic cages for urine collection. They were offered only 30 g/day of powdered Purina rat chow containing 200 mg of 2,4-diamino-5-cyano-6-bromopyridine *ad lib.*, of which they consumed 15 g/day on day 1 and 24 g/day for the next 4 days. The urine of all animals contained a crystalline precipitate that was collected by filtration and recrystallization from hot water. Anal. Calc. for $C_6H_4BrN_4NaO_4S$: C, 21.77; H, 1.22; N, 16.92; S, 9.68; mol. wt 331.1. Found: C, 20.45; H, 1.28; N, 15.87; S, 8.04.

Preparation and assay of cyclic nucleotide phosphodiesterase. Cyano-2,4-diamino-6-iodopyridine and the 6-bromo analogue were tested as inhibitors of high affinity cyclic AMP phosphodiesterase (EC 3.1.4.17) from dog kidney. The high affinity form of the enzyme was purified to near homogeneity according to Thompson *et al.* [5]. Enzyme (0.5 mg/ml) was diluted in 40 mM Tris–HCl (pH 8.0), bovine serum albumin (0.1%), and used immediately for activity analysis.

Phosphodiesterase hydrolysis was determined by the radioisotopic procedure and reagents described previously [6]. Reaction mixtures (0.4 ml) contained 40 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 3.75 mM 2-mercaptoethanol, 30 μg bovine serum albumin, 0.025 to 0.75 μM cyclic [^{3}H]AMP (~100,000 cpm), and 10 ng enzyme. Incubations were at 30° for 10 min, and substrate depletion did not exceed 20%. Inhibitors were dissolved in saline and tested for a lack of inhibition of snake venom nucleotidase activity or anion-exchange resin displacement of cyclic AMP.

Kinetic analysis. Concentrations of half-maximal inhibition (IC_{50}) were obtained by non-linear least-squares fitting of a four parameter logit function developed by D. Rodbard, Y. Feldman, and M. Jaffee (National Institutes of Health, Bethesda, MD) to the data. For the inhibition of phosphodiesterase by 2,4-diamino-5-cyano-6-bromopyridine, a more complete kinetic analysis was performed. Various kinetic models were first explored using classical plots that linearize different types of enzyme inhibition data. For a more rigorous statistical estimation of kinetic parameters of the model that fit the data best, equation 1 (see Results and Discussion) was fit to the data by non-linear least-squares analysis using the Levenberg–Marquart modification of the Newton–Gauss iterative procedure [7]. The error estimates on fitted parameters are the standard errors obtained by the matrix inversion method [8].

RESULTS AND DISCUSSION

Synthesis and structures. Carboni *et al.* [4] first outlined the synthesis of cyano-2,4-diamino-6-bromo pyridine by the reaction of malononitrile with HBr in benzene or tetrahydrofuran. The cyano group was initially reported to be in the 3 position [9], but in a subsequent publication the problem was left open [4]. Later Boldt *et al.* [10] presented evidence that the 5-cyano derivative resulted when the reaction was performed in benzene. On the one hand, the low solubility of malononitrile in benzene limited the quantity of material that could be synthesized easily. On the other hand, the chemical reactivity of tetrahydrofuran precluded use of this solvent for the synthesis of the 6-iodoanalogue of compound I. In 1,2-dichloroethane, a reaction medium was found that not only made the synthesis easier, but also allowed access to the newly described 6-iodo analogue.

The synthesis of the pyridine compounds involves 2-amino-1-propene-1,1,3-tricarbonitrile as a reaction intermediate [4] (Fig. 1). Presumably, closure of the pyridine ring involves tautomerization of one nitrile group followed by addition of HBr and attack of the resulting enimine nitrogen at the other nitrile of the same molecule. Depending on whether the nitrogen from the cyano group in position 1 or 3 provides the ring nitrogen, the product would be a 5-cyano or a 3-cyano substituted pyridine respectively. If both mechanisms were to occur, a mixture of the two products would result. Since it was not certain that synthesis in 1,2-dichloroethane instead of benzene and with HI instead of HBr would yield the cyano group selectively in the 5 position, the structure of the products had to be ascertained.

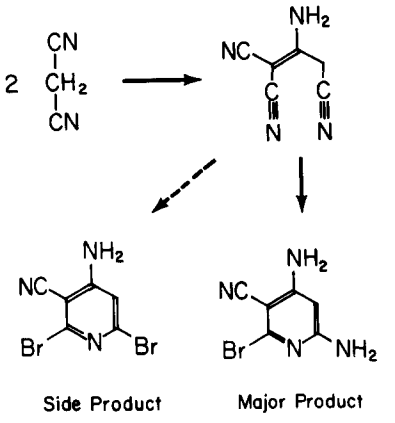

Fig. 1. Reactions of malononitrile with HBr. The same reactions also take place with HI, whereas with HCl the reaction stops after the first step.

The structure of the products obtained with either HBr or HI was established by elemental analysis, mass and NMR spectroscopy. Proton NMR spectra of both iodo and bromo compounds showed a sharp single signal from one aromatic proton. This demonstrated that the reaction did not produce a mixture of the 3- and 5-cyano compounds. The position of the cyano group in the major reaction products was investigated by natural abundance ^{13}C Fourier transform NMR spectroscopy. Chemical shifts were determined in proton decoupled spectra, and peaks were assigned by comparison to model compounds. Moreover, the single aromatic carbon bearing a proton showed the highest intensity, due to the nuclear Overhauser effect. Spectra with ^{1}H-^{13}C coupling are shown in Fig. 2. In the case of the 2,4-diamino-5-cyano-6-bromopyridine, the doublet ($\delta = 90.9$ ppm from TMS, J = 163.2 Hz) of pentets (J = 5.2 Hz) was interpreted as C-3 coupling strongly with a proton attached to it and weakly with all the amino protons in the 2 and 4 position. The quartet ($\delta = 89.9$ ppm from TMS, J = 6.1 Hz) was interpreted as C-5 coupling with the aromatic proton and with those of the 4-amino group, apparently with the same coupling constant.

In the case of 2,4-diamino-5-cyano-6-iodopyridine, the resolution of the doublet ($\delta = 91.2$ ppm from TMS, J = 164.8 Hz) stemming from the C-3 carbon into pentets was not discernable due to line-broadening. This was probably caused by the quadrapole moment of iodine. However, the quartet ($\delta = 96.1$ ppm from TMS, J = 6.0 Hz) did not show any sign of line-broadening. The interpretation of the coupling was the same as that for the bromo compound. Compounds synthesized in either benzene or 1,2-dichloroethane gave the same spectra. It was concluded, therefore, that the reaction of malononitrile with HI or HBr in inert solvents yields selectively the 5-cyanopyridine derivative.

Carboni *et al.* [4] showed that the reaction intermediate is 2-amino-1-propene-1,1,3-tricarbonitrile (Fig. 1). The selective formation of the 5-cyanopyridine derivative required an attack of the 1-nitrile nitrogen trans to the amino group at the 3-nitrile carbon, whereas the reverse would have yielded the

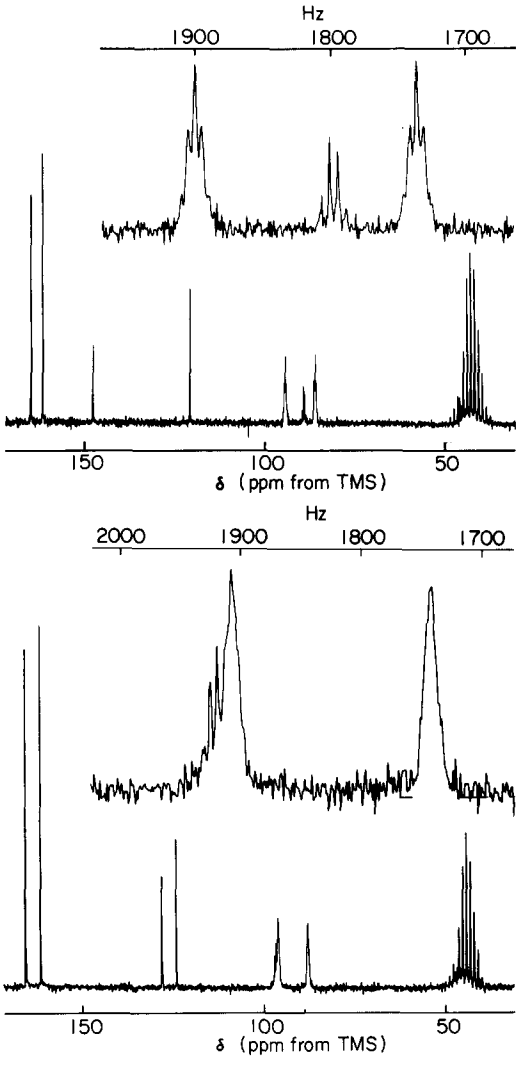

Fig. 2. Proton-coupled natural abundance ^{13}C NMR spectra. Upper panel: 2,4-diamino-5-cyano-6-bromopyridine. Lower panel: 2,4-diamino-5-cyano-6-iodopyridine. Inserts show areas of ^{1}H-^{13}C coupling on an expanded scale. Spectra were taken in $(C^2H_3)_2SO$ (signals $\delta < 50$ ppm) with tetramethylsilane as reference. For details see Materials and Methods.

3-cyanopyridine derivative. The amino group is not easily protonated since 2-amino-1-propene-1,1,3-tricarbonitrile could not be crystallized as an HCl salt even from concentrated HCl, and, in the apolar medium in which cyclization to the pyridine derivatives is performed, protonation would be even less likely. Space-filling molecular models allowed both mechanisms because of free rotation around the C2–C3 single bonds, an observation confirmed by the singlet proton NMR signal ($\delta = 7.26$ ppm from TMS in C^2HCl_3) from the methylene group. The electronegativity was the same for the two 1-nitrile carbons ($\delta = 119.3$ ppm and $\delta = 118.2$ ppm from TMS in C^2HCl_3) and the 3-nitrile carbon ($\delta = 118.5$ ppm from TMS in C^2HCl_3) according to their ^{13}C-NMR signals, which were identified by proton coupled spectra. Despite this apparent similarity, the non-

conjugated nitrile is the better electrophile than the conjugated nitrile, resulting in the observed selective cyclization to the 5-bromo derivative.

Purification of the pyridine derivatives required extensive recrystallization. A contaminant particularly difficult to eliminate was isolated, and its mass spectrum identified it as an amino-dibromo-cyanopyridine, most likely 4-amino-2,6-dibromo-5-cyanopyridine. A compound with identical chromatographic behavior accompanied the iodopyridine analogue during crystallization.

As mentioned by Carboni *et al.* [4], 2,4-diamino-5-cyano-6-chloropyridine could not be synthesized by the route used to obtain the bromo- and iodopyridine derivatives. Because in aprotic dipolar solvents the order of nucleophilicity of halide ions toward carbon and the order of acidity are inverted [11], the reaction with HCl was attempted in dimethylformamide both in the cold and at elevated temperatures, but without success. Attempts to replace the 6-bromo group in 2,4-diamino-5-cyano-6-bromopyridine by fluoride according to Finger and Starr [12] met with only partial success, as judged by a mass spectrum.

Metabolism of 2,4-diamino-5-cyano-6-bromopyridine. It had been demonstrated previously that 2,4-diamino-5-cyano-6-halopyridines are oral insulinotropic agents of low toxicity [2]. It was thus of interest whether, and how, they may be metabolized. The metabolism of the bromo compound was studied in rats that received drug mixed into their chow at the rate of 160 mg/day. The urine collected in metabolic cages contained a crystalline precipitate which was recrystallized from hot water. The strong similarity of the IR spectrum of the original drug with that of the crystalline metabolite was consistent with the continued presence of a pyridine ring substituted with amino, cyano and bromo groups. The most striking difference between the drug and the metabolite was the loss of the strong absorption band at 850 cm^{-1}. One explanation is the loss of the out-of-plane deformation mode of the hydrogen in position 3 accompanied by the appearance of two new absorption bands at 1440 cm^{-1} and in a cluster of bands between 1220 and 1280 cm^{-1}. The new bands in the metabolite were consistent with the presence of a sulfuric acid ester. An elemental analysis of the crudely purified material (see Materials and Methods) showed that the material had not been obtained in a completely homogeneous form. Nonetheless, this evidence together with the spectroscopic data strongly indicated that the metabolite was mostly sulfuric acid mono(2,4-diamino-5-cyano-6-bromo-3-pyridyl) ester Na-salt. The proton NMR spectrum was consistent with this structure although it suggested the presence of a small fraction of the corresponding free alcohol. These data show that 2,4,-diamino-5-cyano-6-bromopyridine was metabolized in the rat by hydroxylation of the 3 position, followed by formation of the sulfuric acid ester, a common sequence of drug biotransformation [13].

Inhibition of cyclic AMP phosphodiesterase. The insulinotropic action of 2,4-diamino-5-cyano-6-halopyridines [2] raised the possibility that these drugs might influence cyclic AMP metabolism. Tests on the purified low K_m cyclic AMP phosphodiesterase from dog kidney showed an IC_{50} of 36 ± 3 μM using a 0.25 μM cyclic AMP substrate concentration. Virtually identical IC_{50} values were obtained with the iodo and bromo analogues. Inhibition by the bromo compound was characterized more fully using cyclic AMP concentrations near the apparent K_m of the enzymes (Fig. 3). Non-linear least-squares analysis fitting various models of inhibition revealed that the best model was one in which the inhibitor interacted both with the enzyme alone (K_i) and the enzyme–substrate complex (K_i') associated with inhibition of enzymatic activity of the latter, i.e. so-called mixed inhibition (Fig. 4). This situation is described by the equation:

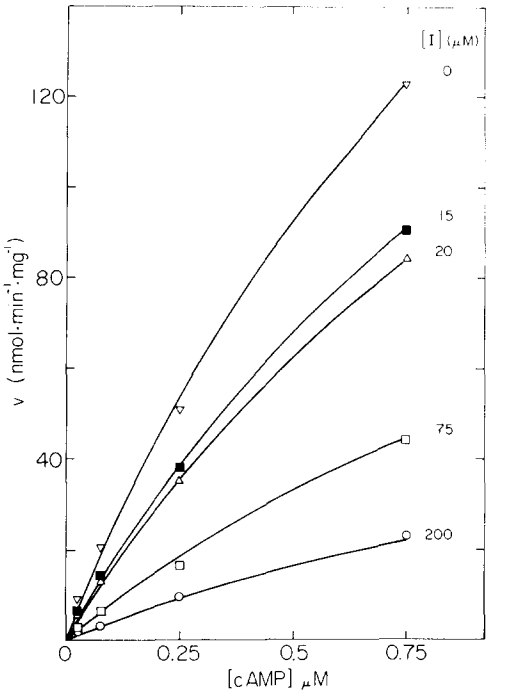

Fig. 3. Inhibition of purified low K_m cyclic AMP phosphodiesterase from dog kidney by 2,4-diamino-5-cyano-6-bromopyridine. Values are means of duplicate determinations. Solid lines are the non-linear least-squares fit of equation 1 to the data.

$$v = \frac{V_{max}}{1 + K_m[S] + [I](1/K_i + K_m/K_i'[S])}. \quad (1)$$

Non-linear least-squares estimates for the kinetic parameters were $K_m = 1.35 \pm 0.20$ μM, $K_i = 36.7 \pm 6.1$ μM, $K_i' = 64 \pm 35$ μM and $V_{max} = 342 \pm 34$ nmoles·min^{-1}·mg^{-1}. Essentially identical data were obtained with the iodo compound. While the present analysis is compatible with the data, the present data are not ultimately proof of the kinetic model proposed. However, they clearly indicate that inhibition by the pyridine derivatives differs from the strictly competitive inhibition observed with several other classes of phosphodiesterase inhibitors, including xanthines, isoquinolines, imidazolidinones, quinazolines, phenothiazines, and pyrazolopyridines, tested on this enzyme [14]. Preliminary studies indi-

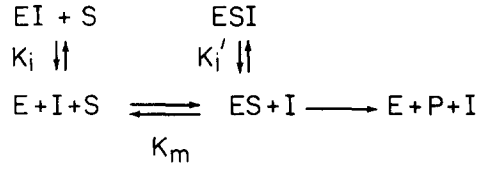

Fig. 4. Scheme of mixed inhibition described by equation 1. Key: E: enzyme; S: substrate (cyclic AMP); I: inhibitor; and P: product (AMP). K_m, K_i and K_i' are kinetic constants.

cated that inhibition of Ca^{2+}-calmodulin-activated high K_m cyclic AMP phosphodiesterase from bovine heart was inhibited by the bromo compound, but in a purely competitive manner. Consistent with the action at the enzymatic site of phosphodiesterase rather than at calmodulin, the bromo compound up to 0.4 mM had no effect on Ca^{2+}–calmodulin activation of human erythrocytes ($Ca^{2+} + Mg^{2+}$) ATPase (Dr. Beat U. Raess, personal communication). Further studies are in progress to define the actions of 2,4-diamino-5-cyano-6-bromopyridine on other forms of cyclic nucleotide phosphodiesterase and the enzyme systems from other tissues, including those from pancreatic islets of Langerhans.*

Phosphodiesterase inhibitors of the methylxanthine class are also inhibitors of alkaline phosphatase [15]. In contrast, 2,4-diamino-5-cyano-6-bromopyridine up to 0.4 mM was without effect on partially purified human bone, kidney and liver isozymes of alkaline phosphatase (Dr. John R. Farley, personal communication). It had been suggested previously that 2,4-diamino-5-cyano-6-bromopyridine inhibits glucose transport and stimulates insulin secretion because the geometry of its hydrogen-bonding capabilities is so similar to that of glucose [1,2]. Glucose is a known allosteric inhibitor of phosphorylases *a* and *b*, and caffeine (a phosphodiesterase inhibitor) acts synergistically with glucose [16], although not through the same allosteric site [17]. Glucose also accelerates phosphorylase *a* to *b* conversion by phosphoprotein phosphatase [18]. Thus, the ability of our compound (0.4 mM) to mimic glucose or caffeine in their effects on rabbit muscle phosphorylases *a* and *b* and on the dephosphorylation by phosphorylase phosphatase was tested. In concentrations up to 0.4 mM the bromo compound was inactive (Dr. Edmond H. Fischer, personal communication).

Finally, it is worth noting that the class of compounds described has added a further example to the list of agents with widely differing chemical structures which are simultaneously inhibitors of phosphodiesterases and glucose transport i.e. papaverine, dipyridamole, caffeine and now, 2,4-diamino-5-cyano-6-halopyridines. This situation is intriguing considering the fact that current ideas about glucose transport assign no role to phosphodiesterase action, and glucose was found not to inhibit phosphodiesterase.

* D. J. Johnson, J. M. McCreary, W. J. Thompson and C. de Haën, Abstr., *First Symposium on Cyclic Nucleotide Phosphodiesterases*, The University of Texas, Houston, TX, April 15–17, 1982.

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