Vol. 121, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1984 Pages 372-379

N-ACETYL-5-METHOXY KYNURENAMINE, A BRAIN METABOLITE OF MELATONIN, IS A POTENT INHIBITOR OF PROSTAGLANDIN BIOSYNTHESIS

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Received April 16, 1984

Although the hypothesis that melatonin may act by inhibiting prostaglandin synthesis arose in part from the similar structures of melatonin and indomethacin, melatonin does not share the in vitro efficacy of indomethacin in inhibiting prostaglandin synthesis. One possibility is that a metabolite of melatonin formed within the target cell might inhibit prostaglandin synthesis and in this study we have tested this by examining the action of the two oxidised metabolites, N-formyl-N-acetyl-5-methoxy kynurenamine formed by the action of indole 2,3-dioxygenase and N-acetyl-5-methoxy kynurenamine, formed in the brain by the action of formamidase. This latter compound which has a structure resembling the fenamate inhibitors of PG biosynthesis had a marked and time dependant inhibiting effect in synthesis using bovine and ovine seminal vesicle microsome preparations and measuring the products by using ($1-^{14}C$) arachidonic acid and by using a specific antiserum for PGE methyl oxime.

The striking similarity between the structure of indomethacin (Fig.1), a powerful inhibitor of prostaglandin (PG) synthesis, and melatonin, a pineal indole which regulates endocrine function (1), has led to the hypothesis that melatonin may act by inhibiting prostaglandin synthesis. This hypothesis has been supported in part by in vitro experiments in which melatonin (10^{-8} to 10^{-4} mol/l) inhibited PG production in platelets (2) and in tissue from the hypothalamus (3,4) and the uterus (4) moreover, when melatonin was injected into the cisterna magna of rabbits, shortly before cervical stimulation to induce ovulation, melatonin blocked the surge of PGE into the cerebrospinal fluid and the subsequent ovulation (5). This last action of melatonin is in accord with its antigonadotrophic effects in some species (6,7) and suggests that it acts on the brain by inhibiting hypothalamic production of PGE which is thought to mediate GnRH and gonadotrophin release (8-10). Despite this ability to inhibit PG production in intact tissue, melatonin is virtually

0006-291X/84 $1.50

inactive in inhibiting PG synthesis when isolated microsomal preparations of PG synthetase are used and an obvious possibility is that a metabolite of melatonin could be active in inhibiting prostaglandin synthesis.

Circulating melatonin is metabolised in the liver by 6-hydroxylation followed by sulphation to give the main urinary metabolite 6-sulphatoxy melatonin (11). However, there have been repeated observations of significant amounts of metabolites in urine which do not respond to Erlich's reagent and therefore do not possess an intact indole ring system (11,12,13). It is now clear that some at least of these metabolites could be kynurenamines (14) and that one likely site of production of these compounds is the brain, since Hirata et al. (14) have demonstrated that these metabolites are produced by incubation of labelled melatonin with brain tissue in vitro and by injection of the label intracisternally into rat brain. In neither the in vitro nor the in vivo case was 6-hydroxy melatonin detected in significant amounts. Kynurenamine production is thought to involve cleavage of the indole ring by indole 2,3-dioxygenase (also known as pyrrolase) to give N^{γ}-acetyl-N^{2}-formyl-5-methoxy kynurenamine (AFMK). This is acted upon by formamidase, which is also present in the brain, to give N-acetyl-5-methoxy kynurenamine (AMK). Indole 2,3-dioxygenase is a similar enzyme to PG synthetase in that both are heme containing dioxygenases and both have a peroxidase component (15). However, the indole dioxygenase is a cytosolic enzyme and the PG synthetase is membrane bound. In this study we have tested the effect of both kynurenamine metabolites for their ability to inhibit PG generation by seminal vesicle microsomes.

MATERIALS AND METHODS

($1-^{14}C$) arachidonic acid was obtained from Amersham Ltd. Unlabelled arachidonic acid N-acetyl-5-methoxy tryptamine (melatonin), 6-hydroxy melatonin, acetyl salicylic acid (aspirin) and adrenalin were purchased from Sigma. Bovine seminal vesicle microsomal powder was purchased from Miles laboratories (Stoke Poges). N-acetyl-5-methoxy kynurenamine and N-acetyl-N-formyl-5-methoxy kynurenamine were synthesized by ozonolysis of melatonin and subsequent acid hydrolysis as described by Hirata et al. and were characterized by infra red and NMR spectra. T.l.c analysis of the crystalline products showed single bands with Rf values identical to authentic standards kindly supplied by Dr. Hirata.

Thin layer chromatographic analysis of prostaglandins formed from (1-^{14}C) labelled arachidonic acid.

Inhibitors (200 μm) were evaporated in scintillation vials and bovine seminal vesicle microsomes were added in 0.9 ml HEPES buffer (50 mM pH 8.0) to give a concentration of 1 mg dry weight/ml in the final incubate. After 4 mins preincubation at 37°, 0.1 ml of a solution of (1-^{14}C) arachidonic acid (6.7 KBq), unlabelled sodium arachidonate and adrenalin bitartrate in HEPES buffer were added to give final concentrations of 8 um arachidonic acid and 100 um adrenalin. The tubes were incubated for 15 mins at 37°C in a shaking water bath and the reaction was stopped by the addition of 1 ml of 0.5 moles/l citric acid and 10 ml of ether/ethyl acetate 1:1. The prostaglandins were extracted, separated by t.l.c. and the zones corresponding to standards were counted using previously published techniques (16). The results are expressed as pg produced per mg BSV powder per 15 mins based on the counts in the bands, the known specific activity (55 mCi/mmol) of the label and the measured losses during extraction and transfer to the t.l.c plates.

Measurement of time dependent inhibition of prostaglandin synthetase by melatonin metabolite and aspirin

Sheep seminal vesicle microsomes were prepared by homogenisation of seminal vesicles (obtained from the local slaughter house) in tris HCl buffer pH 7.4, 50 mM, and centrifuging the homogenate at 3000 g and 11,000 g and discarding the pellets. The supernatant was centrifuged for 60 min at 105,000 g and the pellet resuspended in tris buffer (50 mM pH 7.4) and centrifuged again. The pellet was then resuspended in buffer and lyophilized. Microsomes were resuspended in HEPES buffer (pH 8.0) to give a final concentration of 1.0 mg dry weight per ml buffer. Tubes containing enzyme suspension, inhibitor and adrenalin (100 μM) were preincubated at 37°C in a water bath for appropriate times and the reaction was commenced by the addition of arachidonic acid to give a final concentration of 8 uM. The tubes were incubated in a water bath at 37° with an air atmosphere, with shaking for 2 mins and the reaction as terminated by cooling the tubes in an ice bath and by the immediate addition of methyl oximating solution (1.0 ml of a solution of methoxyamine hydrochloride, 20 mg/ml in sodium acetate buffer pH 5.1, 1.0 mol/l). Samples were left in oximating solution for 12 hours at room temperature then kept at 4°C. 5 μl aliquots of the methyloxime solutions were assayed by radioimmunoassay using a specific E antiserum raised in goats against the methyl oxime of PGE_2. This antiserum had a cross reactivity against A_2, D_2, and F_2 of $< 0.1\%$ and 100% against PGE_1. The cross reactivities against arachidonic acid and adrenalin were both $< 0.0001\%$. The radioimmunoassay had an intra-batch coefficient of variation of 9.8%, and an interassay coefficient of variation of 15.2%. The lines were fitted to points using linear regression analysis.

I_{50} values were determined by plotting reaction velocity (ng produced during reaction) against log molar concentration of inhibitor (20,40,80,160 and 320 μM/l for AMK and 80,160,320,640 and 1280 μM/l for aspirin, and determining the intercept corresponding to half uninhibited velocity. Values of I_{50} for aspirin could not be obtained from the data where the preincubation time was less than 4 minutes.

RESULTS

The thin layer chromatographic analysis of radiolabelled products obtained from incubating arachidonic acid with bovine seminal vesicles microsomes in the presence of melatonin metabolites showed that none of the inhibitors or aspirin essentially changed the distribution of products (Fig. 1). In the first

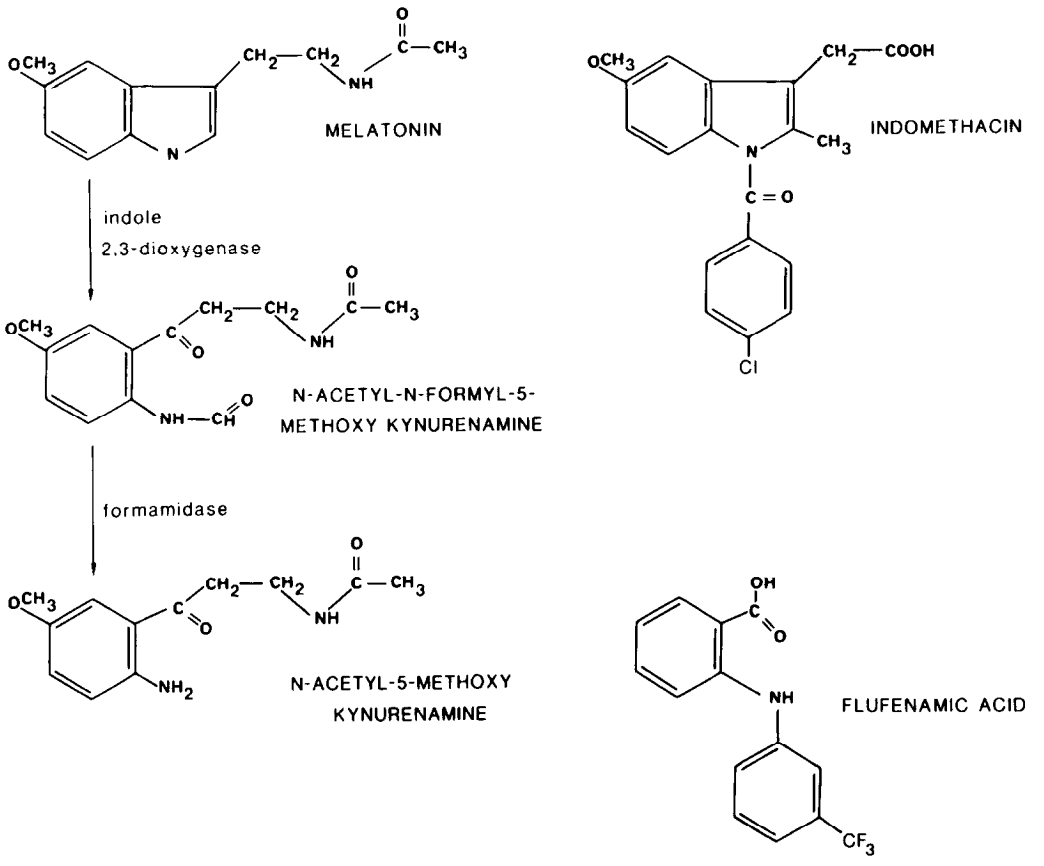

Figure 1. Structures of melatonin and its two kynurenamine metabolites compared with the structure of indomethacin and flufenamic acid.

experiment 6-hydroxy melatonin increased the production of all PGs (total counts in PG bands show a 110% increase over control). Melatonin showed a slight inhibitory effect on production of all PGs except 6-oxo $PGF_{1\alpha}$. In the second experiment neither N-acetyl-N-formyl methoxy kynurenamine nor aspirin had a significant effect on PG production with this preincubation time but AMK showed a highly significant ($P < .001$) reduction in PGE_2 and PGD_2 production. In both experiments using t.l.c analysis, variable amounts of PGA_2 were produced due to degradation of PGE_2 during extraction and chromatography.

Investigation of the effect of preincubation times on the inhibition by N-acetyl-5-methoxy kynurenamine and aspirin showed that Aspirin had a marked time dependant inhibitory effect (Fig.2) N-Acetyl-5-methoxy kynurenamine also showed a significantly greater inhibition after 8 minutes preincubation at all

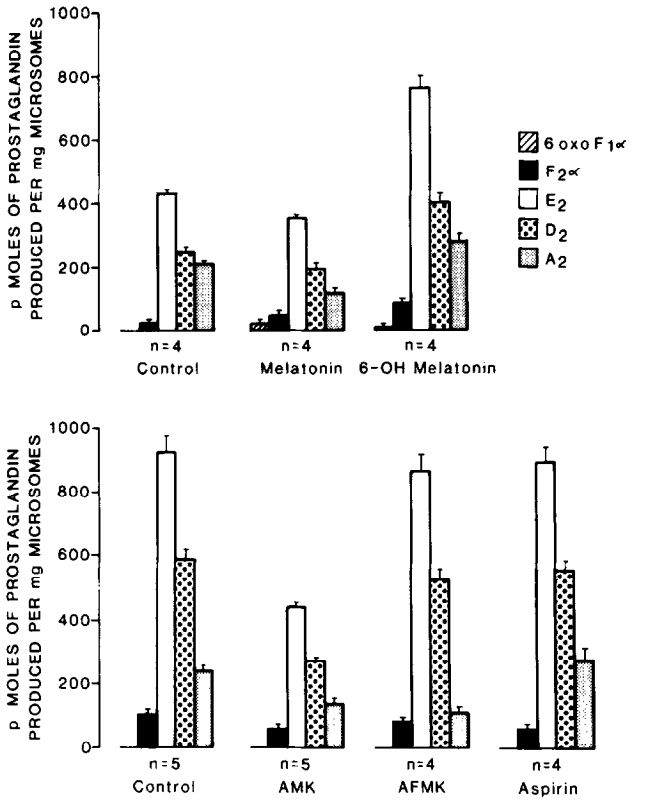

Figure 2. Product distribution from PG biosynthesis in the presence of melatonin, its metabolites and aspirin.

doses tested. Aspirin (80 μm) showed no significant inhibition at any preincubation time tested. I_{50} values for AMK and aspirin (Table 1) demonstrate a dependance of this parameter on preincubation time with a rapidly increasing inhibitory action with preincubation.

TABLE 1

I_{50} concentrations of N-acetyl-5-methoxy kynurenamine and aspirin (μM/l)

Preincubation time (min)	AMK	ASPIRIN
0	220	
1	160	
2	105	
4	56	440
8	14	160

DISCUSSION

Very few naturally occurring inhibitors of PG cyclooxygenase have been reported. Corticosteroids prevent production by phospholipase of the precursor arachidonic acid (17) and endogenous inhibitor or prostaglandins synthesis "(EIPS)" has not been identified though it is thought to act similarly to the corticosteroids (18). Certain fatty acids act as classical competitive inhibitors with arachidonic acid, particularly those with more unsaturated bonds than arachidonic acid (19) but as yet no endogenous compound with a structure and action similar to that of the non steroidal anti inflammatory drugs has been found. The finding that the main brain metabolite of melatonin, N-acetyl-5-methoxy kynurenamine inhibits PG synthesis suggests that this compound may play a key role in modulating the effects of melatonin and raises the possibility that similar metabolites of 5-hydroxy tryptamine may also inhibit prostaglandin synthesis.

Many inhibitors of prostaglandin synthesis exhibit a time dependent action which is competitive and irreversible in character (20,21). This has presented problems in the estimation of the potency of compounds such as aspirin although methods have been presented which deal with the kinetics of time dependent inhibition (20). Since aspirin is now thought to work by the acylation of a serine residue at the active site of the cyclooxygenase enzyme (22) it's time dependent irreversible nature can be appreciated. We have found that AMK behaves in a similar manner (Fig.3), showing increased efficacy following preincubation (Table 1) but although AMK could possibly participate in a transacylation reaction we have no evidence for such a mode of action. The *in vivo* situation obviously allows for such an induction time and thus AMK should be a powerful inhibitor of PG synthesis *in vivo*. Moreover, further studies on the ability of melatonin to inhibit PG synthesis in tissue has shown that this effect is also time-dependent (23). In the *in vivo* situation the increased lipophobic nature due to the amino group on AMK may preclude ready diffusion through membranes (or passage across the blood-brain barrier) and although melatonin may be free to enter the cells of the brain, conversion to AMK would

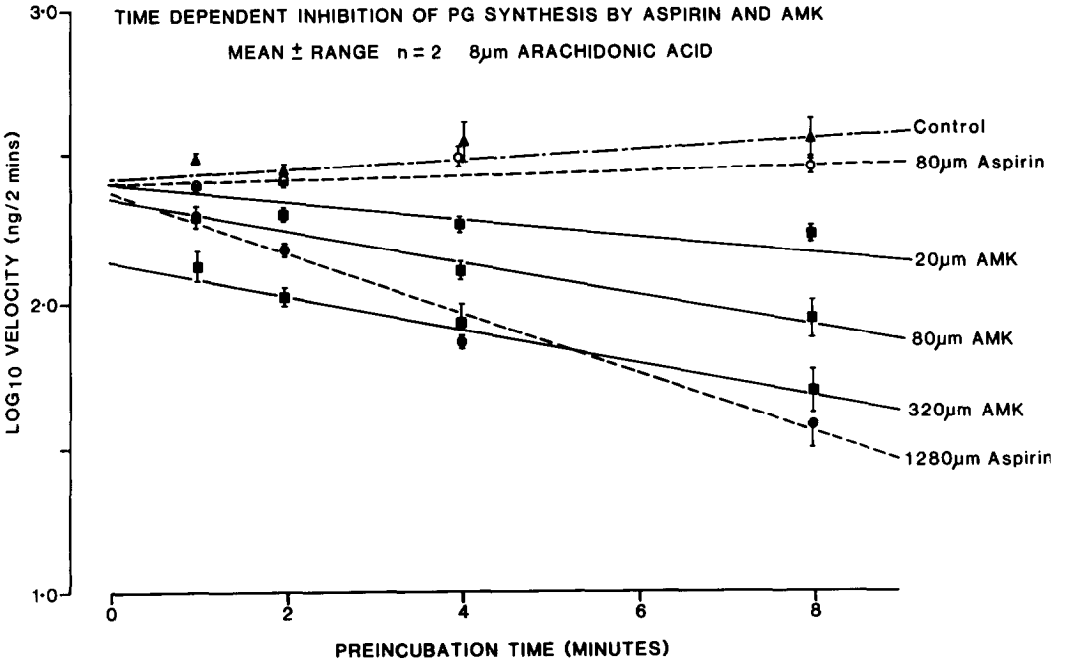

Figure 3. Time dependent effect of N-acetyl-5-methoxy kynurenamine and aspirin in prostaglandin biosynthesis.

tend to trap the compound within cells or organelles and thus further potentiate its effect. Furthermore the restricted ability of AMK to traverse membranes may have led to it's activity having been overlooked in cell or organ perfusates.

Several PG synthesis inhibitors are also chelating agents for transition metals; examples include the fenamates, based on a N-phenyl anthranilic acid structure, which form stable bidentate complexes with metal ions (24) and the salicylates which are known to complex ferric ions (25). AMK also shows a chelating ability exhibited as the appearance of a red colour in the presence of ferrous or ferric ions. Such an ability to chelate may be connected with its ability to inhibit PG synthesis in accord with several published theories of the structure-activity relationships of non-steroidal anti-inflammatory drugs (20,26). Whereas melatonin resembles indomethacin the structure of AMK shows a striking resemblance to that of the fenamates (Fig.1) and it is tempting to speculate that these widely used synthetic non steroidal anti inflammatory drugs may be analogues of compounds such as AMK.

These findings suggest that the metabolism of melatonin to the kynurenamine metabolites cannot be regarded as a detoxifiation mechanism and raise the possibility that melatonin may act via metabolites to govern PG synthesising enzymes of the hypothalamus and thereby regulate the role of PGs in neuroendocrine functions.

REFERENCES

1. Cardinali, D.P. (1981) Endocrin. Rev. 2, 3214-3220.
2. Leach, C.M. and Thorburn, G.D. (1980) Prostaglandins 20, 51-56.
3. Cardinali, D.P., Ritta, M.N., Fuentes, A.M., Gimeno, M.F. and Gimeno, A.L. (1980) European J. Pharmacology. 67, 151-153.
4. Gimeno, M.F., Lauda, A., Sterin-Speziale, N., Cardinali, D.P. and Gimeno, A.L. (1980) European J. Pharmacology. 62, 309-317.
5. Leach, C.M., Reynoldson, J.A. and Thorburn, G.D. (1982) Endocrinology. 110, 1320-1324.
6. Moguilevsky, J., Faigon, M.R., Scacchi, D. and Cardinali, D.P. (1979) Neuroendocrinology. 29, 163-168.
7. Kamberi, I.A., Mical, R.S., Porter, J.C. (1970) Endocrinology. 87, 1-12.
8. Ojeda, S.R., Harms, P.G. and McCann, S.M. (1975) Endocrinology. 97, 843-854.
9. Ojeda, S.R., Negro-vilar, A. and McCann, S.M. (1979) Endocrinology. 104, 617-624.
10. Behrman, H.R., Orczyk, G.P. and Greep, R.O. Prostaglandins. 1, 245-258.
11. Kopin, I.J., Pare, C.M.B., Axelrod, J. and Weissback, H. (1961) J. Biol. Chem. 236, 3072-3075.
12. Kveder, S. and McIsaac, W.M. (1961) J. Biol. Chem. 236, 3214-3220.
13. Reppert, S.M. and Klein, D.C. (1978) Endocrinology. 102, 582-588.
14. Hirata, F., Hayaishi, O., Tokuyama, T. and Senoh, S. (1974) J. Biol. Chem. 249, 1311-1313.
15. Groves, J.T. (1979) in: "Advances in Inorganic Biochemistry (1)". Eds. Eichorn, G.L. and Marzilli, L.G. Elsevier, Amsterdam, pp. 119-145.
16. Abel, M.H. and Kelly, R.W. (1979) Prostaglandins. 18, 16-23.
17. Flower, R. (1978) In: Advances in Prostaglandin and Thromboxine Research Vol. 3 Ed. C. Galli, Raven Press, N. York pp. 105-112.
18. Saeed, S.A., McDonald Gibson, W.J., Cuthbert, J., Copas, J.L., Schneider, C., Gardiner, P.J., Butt, N.M. and Collier, H.O.J. (1977) Nature. 270, 32-36.
19. Lands, W.E.M., Le Tellier, P.R., Rome, L.H. and Vanderkhok, J.Y. (1973) Adv. Biosci. 9, 15-28.
20. Lands, W.E.M. and Rome, L.H. (1976) In: Prostaglandins: Chemical and Biochemical Aspects. Ed. Karim, S.M.M. MTP Press, Lancaster pp. 87-137.
21. Ku, E. C. and Wasvary, J.M. ( ) Biochim. Biophys. Acta. 384, 360-369.
22. Roth, G.T., Stanford, N. and Majerus, P.W. (1975) Proc. Nat. Acad. Sci. U.S.A. 72, 3073-3076.
23. Gimeno, M.F., Ritta, M.N. Bonacossa, A., Lazzari, M., Gimeno, A.L. and Cardinali, D.P. (1981) In: Melatonin, current concepts and perspectives. Eds. Bivan, N. and Schloot, W. Pergamon Press, London pp. 147-150.
24. Decker, J.S. and Frye, H. (1966) Z. Naturforsch. B. 21, 527
25. Pitt, C.G. and Martell, A.E. (1980) In: Inorganic Chemistry in Biology and Medicine. Eds.
American Chemical Society pp. 279-312.
26. Egan, R.W., Humes, J.L. and Kuehl, F.A. (1978) Biochemistry. 17, 2230-2234.