Unilateral intrapallidal injection of EKC caused ipsiversive circling behaviour [1]. So this may result from increased dopamine release in the globus pallidus leading either directly or indirectly to a reduction in pallidal outflow. Conversely, neither DADLE nor FK 33-824 had any behavioural effect when injected unilaterally into the globus pallidus and similarly they had no effect on ^{3}H-dopamine release from pallidal slices. However, DADLE (50 μM only) decreased the rate of KCl-evoked release of ^{3}H-GABA from prelabelled pallidal slices. This may explain the behavioural consequence of bilateral intrapallidal injections of DADLE, which is to increase locomotor activity [1].

However, it remains puzzling as to why the higher dose of DADLE was without effect on the KCl-evoked release of ^{3}H-GABA. For some reason perhaps high doses of opioid agonist drugs have no effect in this type of release experiment. This may explain the ineffectiveness of FK 33-824 in this work. This drug is 100 times more potent than DADLE in increasing locomotor activity following bilateral intrapallidal injection. Perhaps much lower concentrations of FK 33-824 might have an effect. DADLE also has some μ-receptor actions and so the possibility that these receptors, as well as δ-receptors, are involved in the modulation of GABA release cannot be ruled out. The failure of any of the opioid agonist drugs to alter the release of ^{3}H-5HT from prelabelled pallidal slices suggests that opioid receptors are not located on 5HT terminals in the globus pallidus.

These preliminary findings suggest that different opioid agonist drugs can modulate neurotransmitter release within the globus pallidus. It remains to be established if these effects on pallidal release are due to an action on opioid receptors by investigating the effects of the opioid antagonists.

Acknowledgements—This study was supported by the Parkinson's Disease Society, the Medical Research Council and the Research Funds of the Bethlem Royal and Maudsley Hospitals, and King's College Hospital. We are grateful to Sandoz for gifts of FK 33-824, the Wellcome Research Laboratories for BW 180C, and Sterling Winthrop for ethylketocyclazocine.

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Biochemical Pharmacology, Vol. 36, No. 10, pp. 1741–1747, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

Arachidonic acid monooxygenase and lipoxygenase activities in polymorphonuclear leukocytes

(*Received* 5 *June* 1986; *accepted* 7 *November* 1986)

Polymorphonuclear leukocytes (PMNs) metabolize arachidonic acid (AA) via several distinct enzymatic pathways. The 5-lipoxygenase enzyme is responsible for the production of 5-hydroxyeicosatetraenoic acid (5-HETE) and 5,12-diHETE (LTB_4) [1]. The formation of 12- and 15-HETE by 12- and 15-lipoxygenases, respectively, has also been described [2, 3]. AA metabolism in PMNs of some species also proceeds via the cyclooxygenase pathway to produce thromboxane A_2 [4, 5], although this activity is not detectable in human cells. The profile of metabolites formed could be stimulus dependent since the calcium ionophore, A23187, appears to selectively stimulate the 5-lipoxygenase [1], whereas bradykinin activates only the 15-lipoxygenase [6]. More recently, we have demonstrated that canine PMNs metabolize AA by a mechanism independent of either the cyclooxygenase or lipoxygenase pathways [7]. The products, called peak 1 (P1) and peak 2 (P2) pending structural analysis, are formed by a cytochrome

P-450-dependent mixed-function oxidase (MFO) [7]. This third route of AA metabolism is referred to as the epoxygenase pathway since it can lead to the formation of unstable epoxides [8].

The object of the present study was to determine the importance of this cytochrome P-450-dependent pathway in metabolism of AA by comparing AA metabolism in human, canine and rat PMNs, together with the activity of aryl hydrocarbon hydroxylase (AHH), an enzyme used as an index of cytochrome P-450-dependent activity. A previous study on the time–course of formation of P1 and P2 suggested that P2 may be derived from P1 [7]. This was addressed by incubating P1 with canine neutrophils and determining the time-dependent conversion to P2. In addition, the enzyme involved in the conversion of P1 to P2 was studied using inhibitors of the cyclooxygenase, lipoxygenase, and MFO enzymes.

Materials and methods

(1) *Neutrophil harvesting*

Canine neutrophils. Canine neutrophils were harvested 3 hr after the intraperitoneal instillation (via 14 gauge angiocath, Critikon) of 400 ml of 0.1% glycogen (Sigma), dissolved in sterile normal saline and containing 2.5 units of heparin/ml (Sigma) [9]. Contaminant red blood cells in the recovered peritoneal fluid were destroyed by hypotonic lysis. The cell suspension was then washed in normal saline before being resuspended in Hanks' balanced salt solution (HBSS), pH 7.4 [composition in g/l = 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 7.15; NaCl 6.67; KCl, 0.33; Na_2HPO_4, 0.08; KH_2PO_4, 0.08; $CaCl_2$, 0.17; $MgSO_4$ 0.17; glucose 0.92; and $NaHCO_3$, 1.17; all reagents obtained from Sigma]. Wright–Giemsa staining revealed 85–95% neutrophils with monocytes (esterase positive) accounting for 3–6% of the remaining leukocytes. Neutrophil viability, as assessed by Trypan blue exclusion was typically 95–98%. Platelet contamination was low and never exceeded 1 platelet:2 neutrophils. The neutrophils remained at room temperature until used, always within 3 hr of the completion of neutrophil harvesting.

Human neutrophils. Human buffy coat leukocytes from peripheral blood were obtained from the Hudson Valley Blood Bank (Valhalla, NY) on the day of experimentation. Following dextran sedimentation the suspension was centrifuged at 400 *g* for 5 min, the supernatant fraction was discarded, and the pellet was subjected to hypotonic lysis to remove any residual erythrocytes. The remaining leukocytes were then washed with 0.9% saline and resuspended in HBSS. PMNs constituted 68 ± 5% of the leukocyte population with a viability of 97–98%, as assessed by Trypan blue exclusion. Monocytes constituted approximately 15–20% of the leukocyte population, with the remainder mainly lymphocytes and some eosinophils.

Rat neutrophils. Rat peritoneal neutrophils were harvested following the instillation of 12 ml of 8% casein (Sigma; prepared in sterile normal saline and containing 2.5 units of heparin per ml) into the peritoneal cavity for 12 hr [10]. Following recovery of the neutrophil-rich peritoneal fluid, isolation of the neutrophils proceeded as described for canine neutrophils, with PMN viability exceeding 95%.

(2) *Cytochrome P-450 activity*

Preparation of neutrophil microsomes. Aliquots (8.0 ml) of PMN suspensions (5×10^8/ml) were centrifuged at 900 *g* for 5 min. The supernatant fraction was discarded and the cells were lysed by resuspension in 2 vol. of ice-cold water. The suspensions were then homogenized with eight strokes in a glass Teflon homogenizer. Phosphate buffer (0.2 M), pH 7.6, containing 0.4 ml phenylmethylsulfonyl-fluoride was then added to the homogenates to yield a final buffer concentration of 0.1 M phosphate buffer as previously described [11]. The homogenate was then centrifuged at 600 *g* for 10 min to remove cellular debris and unbroken cells, and the supernatant fraction was respun at 27,000 *g* for 10 min to remove the mitochondrial fraction. The resulting supernatant fraction was spun at 105,000 *g* for 90 min yielding a microsomal pellet, which was resuspended in 0.1 M potassium phosphate buffer, pH 7.8. Protein was determined by the Biorad method (BioRad Protein Assay Kit, BioRad Laboratories).

Aryl hydrocarbon hydroxylase activity. PMNs were assayed for AHH activity by the fluorometric procedure first described by Nebert and Gelboin [12] and modified by Abraham *et al.* [13]. Briefly, neutrophil microsomes were incubated in a potassium phosphate buffer, 0.1 M, pH 7.8, containing $MgCl_2$, 3 mM; NADPH, 1 mM; and NADH, 1 mM (Sigma) in a total volume of 1 ml. An NADPH-generating system was subsequently used in which the content of the incubation mixture was as follows: 0.1 M potassium phosphate buffer, pH 7.8, containing $MgCl_2$, 3 mM; NADP, 1 mM; glucose-6-phosphate, 1 mM; glucose-6-phosphate dehydrogenase, (GGPD, Boehringer Mannheim Biochemicals) 0.5 units; and NADH, 1 mM in a total volume of 1 ml. All reagents except GGPD were obtained from Sigma. The reaction was initiated by the addition of 0.1 mM benzo[*a*]pyrene (BaP) in 20 μl acetone to the incubation and allowed to progress for 10 min ulmess otherwise indicated. Fluorescence of the 3-OH metabolite of benzo[*a*]pyrene proportional to cytochrome P-450 activity was determined at excitation and emission wavelengths of 396 and 522 nm respectively.

Cytochrome P-450 content. Dog PMNs were homogenized by freeze–thawing using liquid nitrogen. PMNs were then suspended in ice-cold 10 mM Tris, pH 7.5, containing 0.15 M KCl and 0.25 M sucrose. The broken cells were centrifuged twice at 10,000 *g* for 20 min and the pellet was discarded. The supernatant fraction was centrifuged at 105,000 *g* for 90 min. The microsomal pellet was resuspended in 0.15 M KCl, centrifuged at the same force for 90 min, and resuspended finally in 0.1 M potassium phosphate buffer, pH 7.5. The microsomal suspension was assayed for cytochrome P-450 content according to the procedure of Omura and Sato [14]. Measurements were made using a dual beam Cary 14 Spectrophotometer at an extinction coefficient of 91 mM^{-1} cm^{-1} between 450 and 510 nm. Protein was determined by the method of Lowry *et al.* [15].

(3) *Arachidonic acid metabolism by canine, human and rat neutrophils*

Neutrophils (10^7/ml) were preincubated with one of the following inhibitors of AA metabolism or their vehicle controls for 10 min at 37°: BW755c (Wellcome Research Laboratories, 47–94 μM), prepared in distilled water; nafazatrom (Miles Laboratories, 37 μM) prepared in 0.9% saline at pH 10.0 with NaOH; SKF-525A (β-diethylamine-ethyl-2,2 diphenyl-pentanoate, Proadifen, 100 μM; Smith, Kline & French), prepared with distilled water; indomethacin (Sigma, 5 μM) prepared as a stock solution with 4.2% $NaHCO_3$ and diluted with Krebs buffer prior to use, or A23187 (Sigma, 2 μM), prepared as a stock solution with dimethyl sulfoxide (DMSO) and diluted with Krebs buffer prior to use. 1-[^{14}C]AA (0.25 to 1.0 μCi, specific activity 60.1 mCi/mmole, Amersham, final concn of 5–20 μM) was then added to the incubates or their boiled controls, and the reaction was terminated after 10 min by high speed centrifugation in an Eppendorf fixed-speed microcentrifuge. The supernatant fraction was acidified to pH 3.5 with 1 M citric acid, and the products were extracted twice with 3 vol. of ethyl acetate.

(4) *Separation of AA metabolites*

Thin-layer chromatography. The ethyl acetate extracts were evaporated under nitrogen, and the samples were reconstituted in 100 μl ethyl acetate and applied to Brink-

mann silica gel TLC plates under a continuous stream of nitrogen. AA metabolites were separated using the A-9 solvent system (organic phase of ethyl acetate/iso-octane/acetic acid/water, 55/25/10/50, by vol.), visualized by autoradiography on Kodak XAR-5 film, and compared to known standards. Zones appropriate to the standards were cut, and radioactivity was quantified using standard liquid scintillation counting techniques. Product formation is expressed as the percentage of total radioactivity recovered.

High pressure liquid chromatography. The ethyl acetate extracts were evaporated under nitrogen, and reconstituted in 100 μl of acetonitrile for separation of the AA metabolites by HPLC. Reverse-phase HPLC was performed on a C_{18} μBondapak column (4.6 mm × 20 mm, Waters Associates) using a linear gradient of 1.25%/min from acetonitrile–water (1:1, v/v) plus 0.1% acetic acid to acetonitrile plus 0.1% acetic acid at a flow-rate of 1 ml/min [16]. Radioactivity was monitored "on-line" by a radioactive flow detector (Radiometric Instrument & Chemical Co., Inc.).

(5) *Formation of* [^{14}C]*-peak* 1 *by intact canine neutrophils*

Canine PMNs (10^7/ml) were preincubated with BW755c (47–94 μM) for 5 min at 37°. 1-[^{14}C]AA (10–20 μM, containing 1–2 × 10^6 cpm, specific activity 60.1 mCi/mmole, Amersham) was then added to the incubates, and the reaction was terminated after 5 min by centrifugation at 10,000 *g* for 30 sec in an Eppendorf fixed-speed microcentrifuge. The supernatant fraction was acidified to pH 3.5–4.0 with 1 M citric acid and extracted for acidic lipids with 3 vol. of ethyl acetate. 1-[^{14}C]-P1 was purified from the ethyl acetate extract by reverse-phase HPLC as described above.

(6) *Metabolism of peak* 1 *by canine neutrophils*

Canine neutrophils (10^7) in 1 ml of buffer were incubated with 50,000 cpm of HPLC-purified [^{14}C]-P1 (prepared in 50 μl Tris buffer, pH 7.4; Sigma) at 37° in a shaking water bath for 2–30 min. Then the reaction was terminated by centrifugation at 10,000 *g* for 30 sec in an Eppendorf fixed-speed microcentrifuge. Additional samples were preincubated with 100 μM SKF-525A, 94 μM BW755c or 5 μM indomethacin, prior to incubation with [^{14}C]-P1 for 30 min. Following centrifugation, the supernatant fractions were acidified to pH 3.5–4.0 with 1 M citric acid and extracted for acidic lipids twice with 3 vol. of ethyl acetate. Samples were stored in ethyl acetate under nitrogen at −70° prior to the separation of products by TLC.

Results and discussion

Spectrofluorometric determinations of BaP metabolism by microsomes of canine PMNs revealed appreciable AHH activity (Fig. 1), resulting in the formation of 6.4 ± 0.5 pmol 3-OH BaP/mg protein/min (mean ± SEM, N = 3). In comparison, AHH activity in canine liver microsomes was 169 ± 30 pmol 3-OH BaP/mg protein/min (mean ± SEM, N = 5). Analysis of AHH activity in human neutrophils gave a value of 7.3 ± 1.4 pmol 3-OH BaP/mg protein/min (mean ± SEM, N = 4; Fig. 1), with the value for human liver being 110 ± 21 pmol/mg protein/min. In contrast, rat PMNs failed to demonstrate measurable AHH activity, despite similar amounts of protein used in the assay.

The cytochrome P-450 content of human PMNs was determined spectrally by the difference in absorbance at a wavelength of 450 nm after bubbling with CO and reduction by sodium dithionite. A value of 0.08 pmol/mg protein (mean of two experiments) was obtained which is similar to the value of 0.12 ± 0.05 pmol/mg protein (N = 3) for canine PMNs which we reported previously [7].

Incubation of [^{14}C]AA (5 μM) with human PMNs (10^7/ml) for 10 min resulted in the formation of a number of products, including 5-HETE (7 ± 3%), 12/15-HETE (16 ± 10%), LTB_4 (5,12-diHETE, 14 ± 5%) and unmetabolized AA (21 ± 5%). The identity of LTB_4 and 12-HETE is suggested by their mobility on TLC and characteristic u.v. absorbance profiles, similar to those obtained with authentic standards. Two other products with R_f values of 0.57 and 0.62 by TLC utilizing the A-9 solvent system were recovered from intact human neutrophils, which co-migrated with the cytochrome P-450-derived P1 and P2 purified from canine PMNs and accounted for 8 ± 2 and 7 ± 1% of the radiolabel recovered respectively. Preincubation of human PMNs with BW755c [17], a dual inhibitor of the cyclooxygenase and lipoxygenase pathways, enhanced the formation of these products 2- to 3-fold (Fig. 2) probably by diversion of non-utilized substrate, although we cannot rule out other interactions between the lipoxygenase and monooxygenase pathways. Similar results were obtained with another dual inhibitor, nafazatrom (results not shown, [18]). However, SKF-525A, an inhibitor

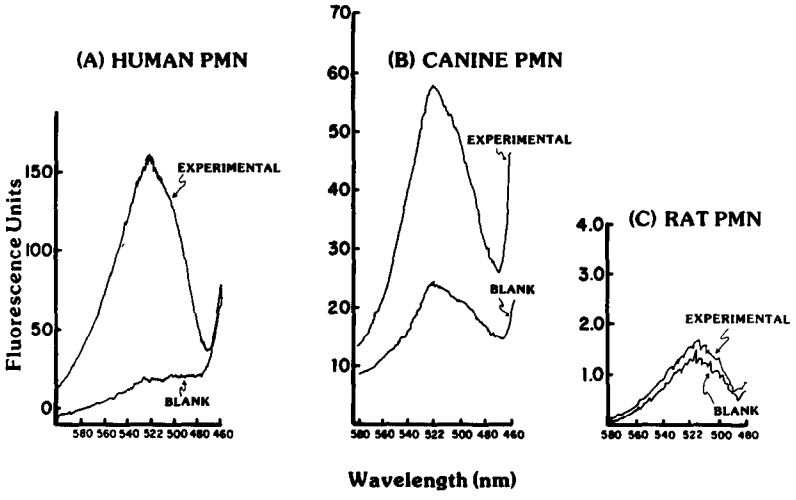

Fig. 1. Aryl hydrocarbon hydroxylase activity in human (A), canine (B) and rat (C) PMN microsomes. The abscissa is the emission wavelength, and the ordinate denotes aryl hydrocarbon hydroxylase activity, measured in fluorescence units. Similar amounts of protein were used in each incubate.

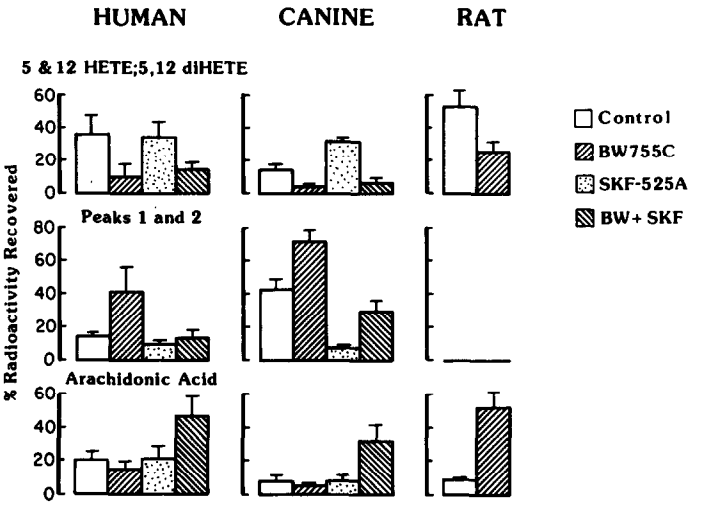

Fig. 2. Inhibition of arachidonic acid metabolism in polymorphonuclear leukocytes. The recovery of AA metabolites was measured following incubation of [^{14}C]AA with human, canine and rat PMNs. The amount of product formed is expressed as the percentage of radioactivity recovered. The top panels show the combined recovery of the lipoxygenase products 5-HETE, 12-HETE and 5,12-diHETE (LTB_4); the middle panels depict the recovery of P1 and P2 and the lower panels show the recovery of unmetabolized AA. Values shown are the mean ± SEM (human, N = 3; canine, N = 6; rat, N = 3). In the human cells, BW755c significantly ($P < 0.05$) reduced lipoxygenase activity, whereas the augmented production of P1 and P2 was reduced ($P < 0.05$) when SKF-525A was given in combination with BW755c. In dog leukocytes, the production of P1 and P2 was significantly augmented ($P < 0.01$) by BW755c and inhibited by SKF-525A ($P < 0.01$) when compared to control. In contrast, BW755c inhibited ($P < 0.005$) and SKF-525A enhanced ($P < 0.01$) lipoxygenase activity. The addition of SKF-525A plus BW755c reduced the formation of these products ($P < 0.005$) when compared to BW755c alone, and resulted in a significant increase ($P < 0.01$) in the recovery of unmetabolized AA. BW755c reduced lipoxygenase activity in the rat cells ($P < 0.05$) associated with a concomitant increase ($P < 0.05$) in unmetabolized AA.

of cytochrome P-450-mediated reactions via type I binding [19], inhibited the formation of both products (Fig. 2).

Intact rat PMNs metabolized AA to produce predominantly the lipoxygenase products LTB_4 (25 ± 3%) and 5-HETE (15 ± 7%) (Fig. 2). Cytochrome P-450-mediated metabolism of AA in rat PMNs was not apparent even in the presence of BW755c, which prevented the formation of all AA metabolites with a resultant increase in the amount of unmetabolized AA and neutral lipids recovered (Fig. 2). Thus, the failure to observe AA monooxygenase metabolites in rat PMNs is consistent with the lack of cytochrome P-450-dependent AHH activity.

In contrast to human and rat PMNs, the predominant pathway of AA metabolism in unstimulated canine PMNs occurred via a cytochrome P-450-dependent mechanism forming P1 and P2 which together accounted for 53 ± 4% of the recovered radiolabeled material (Fig. 3). Basal lipoxygenase activity in these cells was low, accounting for 16 ± 3% of radiolabel recovered.

A comparison of the AA–MFO-derived products purified from canine or human PMN products revealed similar mobilities on TLC, retention times on reverse-phase HPLC (Fig. 3), and the absence of u.v. absorbance in the 230–280 nm range, characteristic of lipoxygenase metabolites. In addition, the formation of these products in cells from both species was augmented by inhibition of alternative pathways of AA metabolism, whereas their production was diminished by SKF-525A (50–100 μM). Taken together, these results suggest that the P1 and P2 are formed via a cytochrome P-450-dependent MFO oxidase system in both canine and human PMNs, and that the products are identical from the two sources.

Despite a similar profile of AA metabolism by the human and canine PMNs, there were quantitative differences. Less P1 and P2 were formed by human cells when compared to the dog, despite similar levels of AHH activity and cytochrome P-450 content. The difference in AA metabolism may relate to different isozymic forms of cytochrome P-450 present in the human and canine PMNs. A specific isozyme may be involved in AA metabolism which would not be apparent when measuring AHH activity or overall cytochrome P-450 content. The relative numbers of PMN in the two preparations (68 ± 5 and 95 ± 5% for the human and canine leukocytes, respectively) could account for some of the quantitative differences. Indeed, we cannot discount the 15–20% mononuclear cells as a possible source of P1 and P2, but this appears unlikely in view of their formation by the PMN-rich canine leukocyte preparations.

Incubation of [^{14}C]-P1 with intact canine PMNs resulted in a time-dependent conversion to peak 2 (Fig. 4), with P2 accounting for 73 ± 2% of the radiolabel recovered at 30 min. The half-life of P1 was calculated at approximately 3.9 min. The reaction was enzymatic since approximately 85% of the radiolabel recovered from the boiled cell preparation remained as P1. Moreover, it is likely that a small percentage of the radiolabel within the starting [^{14}C]-P1 material can be ascribed to P2 contamination, since P2 elutes at a retention time of only 1–2 min before P1 on the HPLC system used, probably giving rise to some overlap of these metabolites in the 1-ml fractions collected.

The metabolism of P1 to P2 is mediated by a cytochrome P-450-dependent mixed-function oxidase since SKF-525A (100 μM) attenuated P2 conversion to 22 ± 12% ($P < 0.05$) at 30 min, whereas preincubation of the cells with BW755c (50 μM) did not alter the conversion of P1 to P2. Although P2 was the major product observed upon incubation of

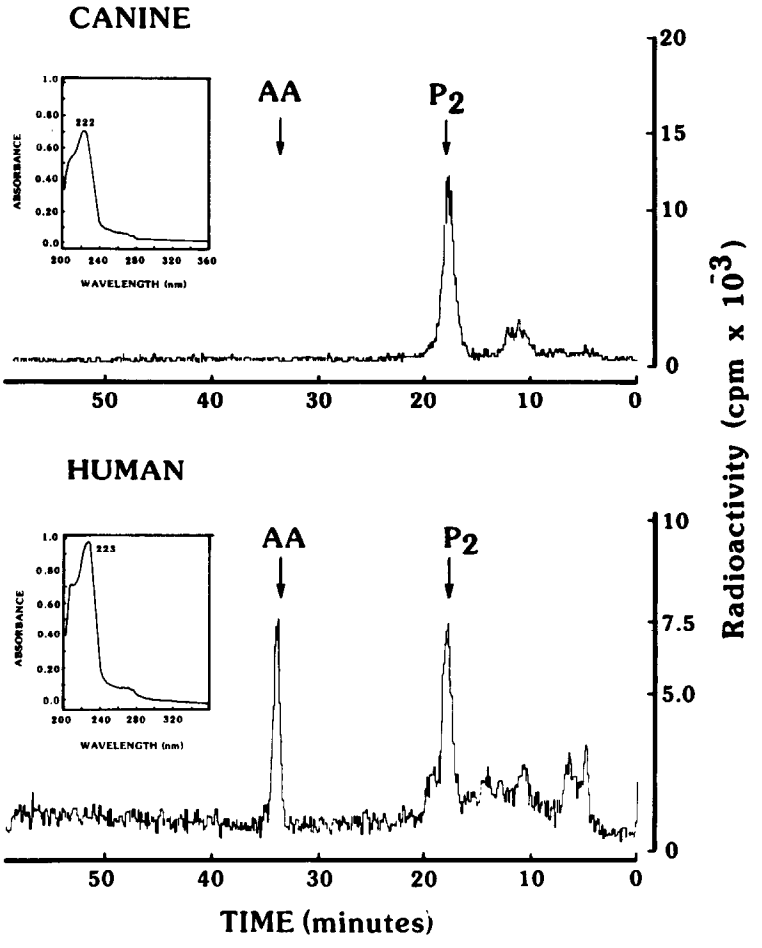

Fig. 3. Reverse-phase high pressure liquid chromatography of AA metabolites formed by canine and human PMNs following pretreatment with BW755c (94 μM). In this system, LTB_4 has a retention time of ~11 min; P1, 19 min; 12-HETE, 20 min; and 5-HETE, 21 min. Arrows depict the retention times of P2 and unmetabolized AA. The inset of each panel demonstrates the u.v. spectra obtained with P2 after its collection following separation by HPLC.

[^{14}C]-P1 with PMNs, two other metabolites were also observed (Fig. 4, inset). Following a 30-min incubation with P1, products with R_f values by TLC of 0.35 (P3) and 0.42 (P4) were observed, which accounted for 5 ± 2 and 3 ± 1% of the radioactivity recovered respectively (N = 3). In cells pretreated with SKF-525A, formation of the more lipophilic metabolite (P4) increased to 13 ± 4% of the radioactivity recovered, whereas the more polar metabolite (P3) remained unchanged at 6 ± 2%. Thus, the formation of these products does not appear to be cytochrome P-450 mediated. However, their formation was prevented by BW755c, but not indomethacin, suggesting that P1 can also be transformed by a lipoxygenase enzyme, but at a slower rate than its conversion to P2. These products were not characterized further.

Cytochrome P-450-dependent mixed-function oxidases can convert AA to a number of different products which include the mono-HETEs, ω and ω-1 hydroxylation metabolites and a novel series of epoxides and their corresponding diols [20–23]. Peak 1 and P2 are not mono-HETEs, since these latter products are identical to those formed via the lipoxygenase enzymes and exhibit peaks of u.v. absorbance in the 233–237 nm range. The structures of P1 and P2 are currently being analyzed. A cytochrome P-450-dependent ω-hydroxylase which metabolizes LTB_4 has been described recently in human PMNs [24]. Similarly, 12-HETE can undergo ω-hydroxylation to form 12,20-diHETE in human PMNs [25, 26], which could also be mediated via the cytochrome P-450 system. Interestingly, LTB_4 hydroxylase activity in rat PMN is only one-sixth of that in the human cells [27], supporting the relative lack of cytochrome P-450-dependent enzyme activity in PMNs of this species.

These results indicate that PMNs contain a number of enzymes capable of metabolizing arachidonic acid. The lipoxygenase and cytochrome P-450-dependent monooxygenase enzymes may be differentially regulated. We previously demonstrated that canine PMNs can produce P1 and P2 in the absence of extracellular calcium [7]. Moreover, human PMNs stimulated with the calcium ionophore, A23187, showed an increased conversion of [^{14}C]AA to 5-HETE and LTB_4, with no change in P1 or P2 formation (results not shown). These changes in AA metabolism may underlay functional changes of the PMNs since LTB_4 is a chemotactic agent which activates the cells and induces aggregation and cell adhesion, whereas the monooxygenase product P2 exerts opposing effects to inhibit aggregation and adhesion responses [28]. This possibility of differential regulation is under further investigation.

In summary, PMNs of human, canine or rat origin were examined for cytochrome P-450-dependent hydroxylase activity and metabolism of exogenous [^{14}C]AA by a cyto-

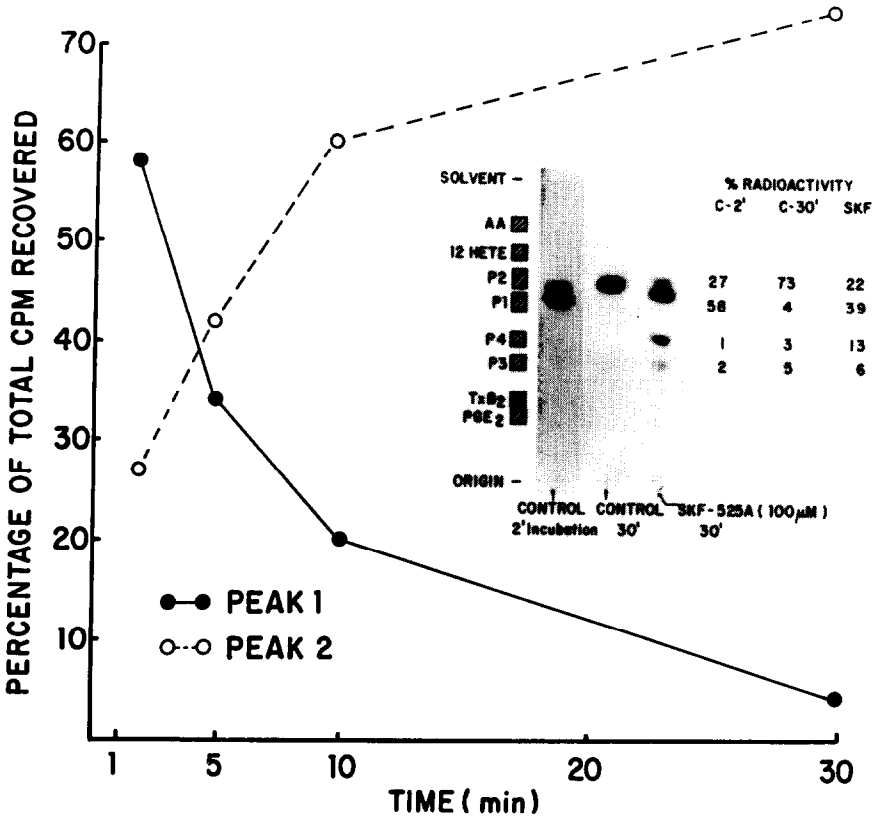

Fig. 4. Time–course of P1 conversion to P2 in canine PMNs. PMNs were incubated with 50,000 cpm [^{14}C]-P1 for 2–30 min. Each point represents the mean of three experiments. The inset depicts a representative autoradiographic plate showing the profile of products obtained upon incubation of [^{14}C]-P1 with canine PMNs. Products were separated by TLC in the A-9 solvent system. On the right side of the inset is the percentage of radioactivity recovered within the zones labeled P1, P2, P3 and P4. Values represent the mean of three experiments.

chrome P-450-dependent monooxygenase enzyme system. Human and canine leukocytes, in contrast to those of the rat, exhibited detectable hydroxylase activity and the formation of two SKF-525A inhibitable metabolites of AA designated P1 and P2. P2 is a metabolite of P1 and both products can be readily differentiated from the lipoxygenase metabolites of AA.

Acknowledgements—The authors would like to thank Pam Blank for typing this manuscript. This study was supported in part by U.S. Public Health Service Grants HL-31591 and HL-34300 from the National Heart, Lung and Blood Institute and by NIH Grant AM-29742. Dr. Mullane is the recipient of a Pharmaceutical Manufacturer's Association Faculty Development Award, Dr. Abraham of a Research Career Development Award (AM 00781) from the National Institute of Arthritis, Metabolism and Digestive Diseases, and Rosemary Kraemer of a Pharmaceutical Manufacturer's Association Pre-doctoral Fellowship.

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Biochemical Pharmacology, Vol. 36, No. 10, pp. 1747–1749, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

Effect of pentobarbital on cyanide-induced tremors in mice and calcium accumulation in PC12 cells

(*Received* 18 *August* 1986; *accepted* 1 *December* 1986)

Tremors, seizures and central lesions are common manifestations of acute cyanide poisonings [1]. Our laboratory recently reported that cyanide-induced tremors may be related to alterations in neuronal regulation of intracellular calcium [2, 3]. Cyanide produces accumulation of cytosolic Ca^{2+} as a result of decreased availability of ATP which is necessary for maintenance of energy-dependent calcium homeostatic processes. Elevated cytosolic Ca^{2+} can induce the release of neurotransmitters, leading to generalized CNS excitation manifested as convulsions or tremors.

The inhibitory actions of barbiturates on neurotransmitter release [4], as well as other calcium-dependent neuronal processes, have been established [5, 6]. The ability of pentobarbital to decrease the incidence of cyanide-induced convulsions has been reported [7], but a more detailed study has not been conducted. The objective of this study was to characterize the effect of pentobarbital on cyanide-induced tremors in mice and to correlate these efforts with cyanide accumulation of cytosolic Ca^{2+} in PC12 cells.

Materials and methods

Male, Swiss-Webster mice (Laboratory Supply, Indianapolis, IN), weighing 27 ± 1 g, were used for the tremor studies. The effect of pentobarbital on KCN-induced tremors was studied using the following treatment regimens: (1) saline was administered, i.p., 15 min before KCN (12 mg/kg, s.c.); (2) pentobarbital (60 mg/kg) was administered, i.p., 15 min before saline, s.c.; and (3) pentobarbital (60 mg/kg) was administered, i.p., 15 min before KCN (12 mg/kg, s.c.). Tremors were measured as previously described [8, 9]. Briefly, motor activity produced mechanical displacement of a free-floating platform which caused resistance changes across a wheatstone bridge. The fluctuations in voltage over time were converted to an intensity–frequency profile by Fourier transformation equations. A useful parameter for characterizing animal activity is the peak frequency which is defined as that frequency with the greatest intensity.

PC12 cells were obtained from Dr. William Tank (University of Colorado, Denver, CO). Cell maintenance has been described previously [10, 11]. Cytosolic Ca^{2+} levels were measured using Quin II/AM as previously reported [12].

All treatment groups were composed of four or more animals or, in the experiments with PC12 cells, four or more different cell samples. Analysis of variance was used to determine statistical differences between treatment groups at a significance level of 0.05. If multiple comparisons were not a factor, a Student's *t*-test (two-tail) was employed, and the means were ranked using a Neuman–Keuls multiple range test.

Results and discussion

The effect of KCN and/or pentobarbital on the intensity-frequency profile is illustrated in Fig. 1 and the change in peak frequency over time in Fig. 2. Mice pretreated with saline and 15 min later administered KCN (12 mg/kg, s.c.) developed severe whole body tremors. The onset of the tremors occurred 15 min after administration of KCN and were most severe at this time period with a peak frequency of 20.95 ± 0.95 Hz. The peak frequency steadily declined after 30 and 45 min. The intensity–frequency profile revealed that the tremors at 30 min were composed of high-frequency peaks ranging from 11 to 18 Hz (Fig. 2). Prominent frequencies were measured at 11, 15 and 18 Hz. After 2–3 hr, normal exploratory and grooming behavior was observed.

Administration of pentobarbital (60 mg/kg, i.p.) followed 15 min later with saline produced intermittent and sporadic shivers in the mice (Fig. 1). The shivers began after 15 min and lasted approximately 45 min. The peak frequency of pentobarbital-induced shivers varied considerably over a range of 3–16 Hz. The intensity–frequency profile of the most severe shivers at 30 min revealed multiple medium-ranged frequencies up to 16 Hz in which all exhibited similar degrees of intensity (Fig. 2). When mice were pretreated with pentobarbital, (60 mg/kg, i.p.), 15 min before KCN (12 mg/kg, s.c.), no movement or motor activity was observed. The combination of KCN and pentobarbital produced no motor activity, other than a small respirator peak at 3 Hz. Frequencies of 2–3 Hz reflect