Inhibition of kallikrein (HoUK) using histones as substrates

Incubations	Half times of hydrolysis* (min)	Per cent Inhibition**
HoUK	80	–
HoUK + SBTI	81	0
HoUK + 0.1 M benzamidine	155	48
HoUK + 0.05 M $NaHSO_3$	No hydrolysis	100

* Hydrolysis of 50% of the histones in the conditions described in the text. ** The inhibition was compared by the half times of hydrolysis.

which is a competitive inhibitor for both enzymes[3], did inhibit the histones hydrolysis. In the table the effectiveness of inhibition of HoUK hydrolysis of chicken erythrocyte histones by both inhibitors is indicated. The hydrolysis of histones by trypsin was inhibited by both soybean-trypsin-inhibitor (SBTI) and benzamidine as would be expected. The table shows that hyposulfite is a potent inhibitor of kallikrein; this inhibition is similar to that promoted by this agent towards proteases contaminating crude preparations of histones[19].

Calf thymus histones were also cleaved by horse plasma kallikrein (HoPK). It is known that this preparation of histone can be fractionated, at least, into 5 fractions by electrophoresis in polyacrylamide gel[20]; those fractions were prepared and all of them were hydrolyzed by HoPK, forming initially large fragments stained with amidoblack and finally soluble peptide chains.

The proteolysis of proteins other than kininogen by kallikreins is unusual, because these enzymes have a very strict specificity; arginine-rich substrates, however, were expected to be cleaved unless the susceptible bonds were not accessible to the protease. The primary structures of some histones are known[21], and this group of proteins may be used as model compounds to study the specificity of kallikreins in the same way as the the B-chain of insulin is used for several proteolytic enzymes.

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The effect of bovine myelin basic protein on uptake and release of H^3-labelled 5-hydroxytryptamine, L-noradrenalin and γ-aminobutyric acid in rat cortex slices

C. G. Honegger, L. M. Krepelka, M. Steiner and H. P. von Hahn[1]

Abteilung Neurochemie, Neurologische Universitätsklinik, Socinstrasse 55, CH–4051 Basel (Switzerland),
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Summary. At concentrations above 10^{-5} M myelin basic protein (MBP) induced a small inhibition of the uptake of H^3-5HT and H^3-NA into rat cortex slices. Release of 5HT, NA and Gaba was not affected by 10^{-5} M MBP.

The first indication that myelin basic protein (MBP) could have electrophysiological activity came from Jankovic et al.[2], who reported that MBP, injected intraventricularly into the rabbit, caused major bioelectric and behavioural changes. Interference of MBP with the action of 5-hydroxytryptamine (5HT) in particular has been suggested by Carnegie[3,4]; he proposed that the encephalitogenic region of MBP has a structure similar to that of the natural 5HT receptor, and that interaction of MBP with 5HT could be the chemical basis of its electrophysiological action. Honegger et al.[5] have provided evidence that MBP has an inhibitory effect on the bioelectric activity of mouse cerebellum cultures and of rat spinal cord in situ, but the biochemical correlate of this electrophysiological activity is not yet known. Direct activation of L-noradrenalin (NA), L-dopamine (DA) or γ-aminobutyrate (Gaba) receptors does not seem to be responsible[5]. We have therefore investigated the presynaptic mechanisms, by testing the action of MBP on the uptake and release of 3 H^3-labelled neurotransmitters in rat cortex slices. Some of these results have been published as an abstract[6].

Materials and methods. Myelin basic protein (MBP) was prepared from fresh bovine spinal cord by a slight modification of the standard methods[7,8]. The solutions of purified MBP were not older than $^1/_2$ h when utilized for the experiments.

Transmitter uptake and subcellular distribution. Male adult SIV 50 rats (150–270 g) were used. 100 mg cerebral cortex slices were pre-incubated (5 min/25 °C) in Krebs-Henseleit medium under oxycarbon. H^3-labelled 5HT or NA (20 µl, 10^{-5} M) and 20–50 µl MBP solution were added to give final concentrations of 10^{-7} M for 5HT and NA, and 10^{-4} to 10^{-7} M for MBP, in 2 ml incubation volume. After 10 min incubation at 25 °C, the reaction was stopped by chilling, and the slices rapidly washed with cold medium. 0.9 ml 0.32 M sucrose was added to 100 mg slices for homogenization. The cell nuclei were removed by

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Table 1. Tissue/medium ratios for the uptake of 1×10^{-7}M H^3-5-hydroxy-tryptamine and H^3-noradrenalin into rat cortex slices in the presence of 1×10^{-7}M to 1×10^{-4}M bovine myelin basic protein (MBP)

Added MBP	H^3-5-Hydroxytryptamine N	T/M ratio	Per cent inhibition	H^3-Noradrenalin N	T/M ratio	Per cent inhibition
None	20	7.81±0.45	–	20	5.24±0.39	–
1×10^{-4}M	4	6.44±0.34	17.5%*	–	–	–
5×10^{-5}M	4	6.94±0.42	11.1%**	4	4.15±0.39	20.8%*
1×10^{-5}M	4	7.35±0.92	5.9%	4	4.59±0.40	12.4%***
5×10^{-6}M	–	–	–	4	4.83±0.35	7.8%
1×10^{-6}M	4	6.91±0.45	11.5%**	4	4.84±0.32	7.8%
5×10^{-7}M	–	–	–	4	5.18±0.41	1.1%
1×10^{-7}M	4	7.26±0.30	7.0%***	–	–	–

10-min-incubation at 25 °C. Means ± S.D.
* Difference to controls (no MBP) significant at $p < 0.001$. ** Difference to controls significant at $p < 0.005$. *** Difference to controls significant at $p < 0.01$.

centrifugation, 0.5 ml of the nuclei-free homogenate was layered on a 12.2 ml sucrose step gradient (see[9] for details). After centrifugation (60,000×g, 90 min) 34 fractions were collected and prepared for liquid scintillation counting. The results of the fractionations are given as cpm per mg original tissue weight, in each of the 4 major density regions: mitochondria, synaptosomes, myelin and supernatant. Transmitter release: 100 mg rat cortex slices were preincubated (10 min, 25 °C) in 10 ml of the high-Ca^{++}, low-K^+ medium of Raiteri et al.[10]. Then 100 µl of 10^{-5} M H^3-5HT, -NA, or -Gaba were added (final concentrations 10^{-7} M) and incubation was continued for 10 min. After rapid chilling, the slices were washed free of radioactivity, and 4.9 ml releasing medium (high-Ca^{++}, high-K^+ medium of Raiteri et al.[10]) were added. Then 50 µl of 10^{-3} M MBP were rapidly added

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Table 2. Subcellular fractionation of rat cortex slices after uptake of 1×10^{-7}M H^3-5-hydroxytryptamine (5HT) and H^3-noradrenalin (NA) in the presence of 1×10^{-7}M to 1×10^{-4}M bovine myelin basic protein (MBP). The distribution of the label is given in cpm per mg tissue fresh weight.

	MBP	N	Total Fr. 1–33	Mitochondria Fr. 1–8	Synaptosomes Fr. 9–25	Myelin Fr. 26–29	Supernatant Fr. 30–33
5HT	None	20	1144 ± 87	30.8 ± 3.3	588 ± 37	93.1 ± 9.9	432 ± 57
	1×10^{-4}M	4	1026 ± 85	33.1 ± 2.4	*393 ± 30	80.0 ± 6.0	*520 ± 54
	5×10^{-5}M	4	1047 ± 73	40.2 ± 10.1	*466 ± 30	77.4 ± 9.4	463 ± 63
	1×10^{-5}M	4	1075 ± 102	38.3 ± 5.9	541 ± 70	93.0 ± 16.7	403 ± 22
	1×10^{-6}M	4	967 ± 42	26.7 ± 4.0	536 ± 22	83.9 ± 8.9	321 ± 18
	1×10^{-7}M	4	1022 ± 116	32.5 ± 3.3	532 ± 47	87.9 ± 8.0	369 ± 60
NA	None	20	668 ± 58	15.0 ± 2.6	378 ± 30	37.9 ± 6.0	237 ± 27
	5×10^{-5}M	4	*478 ± 47	15.9 ± 1.8	*237 ± 31	28.9 ± 5.1	*196 ± 23
	1×10^{-5}M	4	*574 ± 27	14.3 ± 1.4	*308 ± 22	33.0 ± 3.6	**219 ± 3
	5×10^{-6}M	4	**570 ± 67	11.9 ± 2.9	**335 ± 25	30.9 ± 6.4	*193 ± 39
	1×10^{-6}M	4	603 ± 46	12.7 ± 1.2	**331 ± 19	31.8 ± 3.2	228 ± 28
	5×10^{-7}M	4	646 ± 42	14.8 ± 1.0	358 ± 11	36.0 ± 0.1	237 ± 31

* Difference to controls (no MBP added) significant at $p < 0.001$. ** Difference to controls significant at $p < 0.01$.

Table 3. Effect of 1×10^{-5}M myelin basic protein (MBP) on the release of H^3-labelled 5-hydroxytryptamine, L-noradrenalin, and γ-aminobutyrate from rat cortex slices by the high-K^+, high-Ca^{++} medium of Raiteri et al.[10].

	5-Hydroxytryptamine	Noradrenalin	γ-Aminobutyrate
Tissue/medium ratio after uptake (5mM K^+, 2.7mM Ca^{++})	6.58 ± 0.42 (8)	7.33 ± 0.63 (12)	44.3 ± 5.9 (14)
Per cent release, spontaneous (5mM K^+, 2.7 mM Ca^{++})	–	17.0%; 17.6% (2)	3.6%; 3.8% (2)
Per cent release, K-Ca-activated (56 mM K^+, 2.7 mM Ca^{++})	57.4% ± 1.6 (4)	61.3% ± 0.5 (4)	40.7% ± 1.9 (6)
Per cent release, K-Ca-activated, with MBP (56 mM K^+, 2.7 mM Ca^{++})	58.7% ± 0.7 (4)	60.2% ± 1.1 (4)	43.5% ± 0.7 (4)

Slices were incubated 10 min/25 °C with 1×10^{-7}M H^3-neurotransmitters in uptake medium (5 mM K^+, 2.7 mM Ca^{++}). Percentage released label was determined after 10 min/25 °C incubation in release medium (56 mM K^+, 2.7 mM Ca^{++}). Number of experiments in brackets. Means ± S.D.

(final concentration 10^{-5} M), and the reaction mixture was incubated for 10 min at 25 °C. After stopping the reaction by chilling, the slices were collected, washed and prepared for scintillation counting; 100 µl aliquots of the uptake medium, and of the release medium after the release incubation were also counted. From these data, the tissue/medium ratios after uptake and the percentage of label released in 10 min into the medium were calculated.

Results and discussion. As the tissue/medium ratios (table 1) show, there is a small but significant inhibition of the uptake of both 5HT and NA into rat cortex slices by MBP at concentrations above 10^{-5} M. This inhibition is about twice as strong for NA as for 5HT. At lower MBP concentrations, no significant effect on the uptake of either neurotransmitter was observed. The apparently significant value of 11.5% inhibition of 5HT uptake by 10^{-6} MBP is probably an artefact due to the small number of experiments. When the subcellular distributions of 5HT and NA after uptake in the presence of MBP are compared (table 2) 2 different patterns of inhibition are found. For 5HT, 10^{-4} M and 5×10^{-5} M MBP inhibit transmitter uptake into the synaptosomes and induce a corresponding increase in the supernatant, which contains the glial as well as the neuronal cytoplasmic material. In the total tissue, the decrease in uptake is very small. Thus, MBP produces a shift in the uptake of 5HT from the synaptosomes to the cytoplasmic material. This is particularly evident when the cpm are calculated as percentage of the total cpm recovered from the gradient. With 10^{-4} MBP the reduction from 51.5% (controls) to 38.3% in the synaptosomes can be compared with an increase from 37.7% (controls) to 50.6% in the supernatant.

The pattern for NA is clearly different. 5×10^{-5} M and 1×10^{-5} M MBP both induce a larger and significant decrease in cpm per mg in the whole tissue. This loss of accumulated labelled NA is found not only in the synaptosomes, as for 5HT, but also in the supernatant. Since this reduction in uptake is larger in the synaptosomes than in the supernatant, there is an (apparent) small increase in the percentage of labelling in the supernatant But as with 5HT, MBP concentrations below 10^{-5} M have no effect on NA uptake.

Raising the K^+-concentration to 56 mM in the presence of 2.7 mM Ca^{++} induces a rapid and intensive release of all 3 neurotransmitters tested from rat cortex slices (table 3). Addition of 10^{-5} M MBP to the release medium had no effect on the release of any of the 3 substances. These results lead to the conclusion that the site of action correlated with the bioelectric blocking activity of MBP[4] is not presynaptic, i.e. that there is no interference with uptake or release of these neurotransmitters at synaptic terminals by the electrophysiologically active concentrations of MBP. This is evident from the finding that a concentration of 5×10^{-6} M MBP, which totally abolished the spontaneous firing rate of mouse Purkinje cells in culture[5], was ineffective in our experiments, where interference with pre-synaptic biochemical mechanisms was tested. Only high MBP concentrations showed slight activity. Other mechanisms of action of MBP must be considered. For instance, it is possible that MBP acts on biochemical processes in the mitochondria, or by blocking the Na/K pump[5,11]. Since the experiments with various selective antagonists of NA, DA and Gaba have led to the conclusion that the postsynaptic receptors for these neurotransmitters are not involved in MBP action[5], and if we exclude presynaptic action, then the suggestion of Carnegie[3,4] that MBP interacts with 5HT at its postsynaptic receptor site is still a possible explanation.

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Quantification of melanin in hepatic and cardiac lipofuscin[1]

L. M. Ambani, J. W. Jhung, L. M. Edelstein and M. H. Van Woert[2]

Departments of Internal Medicine and Pharmacology, Yale University School of Medicine, New Haven, Connectitut 06510 (USA), and Department of Pathology and Medicine, St. Vincent Hospital, Worcester, Massachusetts 01610 (USA), and University of Massachusetts Medical School, Worcester, Massachusetts 01610 (USA), 27 July 1976

Summary. Melanin pigment in liver and heart tissue, obtained at autopsy from patients, was isolated and quantified. The quantity of melanin extracted was directly proportional to lipofuscin granule counts. Infrared and electron spin resonance spectrographs of the isolated pigments from liver and heart showed absorption characteristics identical to those of known melanins. The pigment was absent in fetal and neonatal life, increased in brown atrophy of the heart and liver, and diminished in livers with fatty metamorphosis.

Previous spectroscopic analyses have demonstrated a melanin component in cardiac lipofuscin granules in addition to its larger lipid fraction[3-6]. In this report, we describe the isolation, identification and quantification of melanin in heart and liver tissues obtained at post mortem from patients of different ages, dying of various causes. The amount of pigment obtained using this method was compared with the number of lipofuscin granules determined by microscopic examination.

Materials and methods. Heart and liver tissues were obtained at autopsy from patients ranging in age from 5 months (fetus) to 90 years. 5 g (wet weight) of tissue was cut into small pieces; 30 ml of concentrated hydrochloric acid was added and left at room temperature. After 1 week, the mixture was centrifuged at $1000 \times g$ in an International centrifuge for 6 h. The supernatant was removed and the acid digestion of the pellet was repeated. 25–30 ml boiling chloroformmethanol (v/v $^1/_2$) mixture was added to the pellet and incubated

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2 Reprint requests should be addressed to M. H. V.W., Department of Neurology, Mount Sinai School of Medicine, Fifth Ave. and 100th Street, New York, N. Y. 10029, USA.

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