Morphinomimetic activity in extracts from milk and body tissues[1]

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1. Introduction

The demonstration of opioid receptors in nervous tissue of vertebrates in 1973 (1–3) provoked intensive search for the endogenous ligands of these receptors. During following years, such endogenous opioids (endorphins) have, in fact, been isolated from brain, pituitary and other tissues and have been identified to be peptides in chemical nature (4, 5 and 6). More recently, a different type of opioid has been demonstrated in mammalian biofluids, e.g. in blood (7, 8). This type of opioids interacts like endorphins with opiate-receptors, but is resistant to pronase, questioning their peptidic nature. Pronase resistant compounds having opioid agonistic activity were also reported (9, 10, 11) to be present in bovine milk, milk products, baby foods and in enzymatic casein digests. One such opioid was later characterized to be a fragment of β-casein and named as β-casomorphin (10). Its N-terminal amino acid sequence differs from that of the other endogenous opioid peptides (e.g. enkephalins, β-endorphins and dynorphin).

In the present paper, the results obtained on an attempt to assess the opioid activities of the hydrophobic extracts of milk, milk proteins, body fluids and body tissues such as liver, mammary gland and brain, are reported.

2. Materials and methods

Milk samples: Cow milk was procured from the cattle yard of the Institute and skimmed with an Alfa-Laval Laboratory Cream Separator, lyophilized and the powder was stored in a desiccator until further use. Rabbit milk was collected from healthy, randomly selected rabbits under aseptic conditions. Milk was then skimmed off in a centrifuge, freeze-dried and stored in a desiccator until use.

Milk proteins: Casein was precipitated isoelectrically from cow skim milk at pH 4.6 by a modification of the procedure of McMEEKIN *et al.* (12). Whey obtained from the above procedure was slowly saturated with ammonium sulphate up to 80 % under cold and continuous stirring conditions. The precipitated protein was then spun at 1,00,000 x g for 30 min in a Beckman Model L-Ultracentrifuge. The precipitate obtained was dissolved in distilled water and dialyzed for 48 h to remove the salt. The dialyzed solution was finally lyophilized and stored in a desiccator until use.

Body fluids: To collect the blood samples, rabbits were bled from the marginal ear vein and blood was collected in ice-cold Petri dishes, lyophilized and the powder was stored in a desiccator until use. Blood from rats was also collected after sacrificing them and offered the same treatment as in the case of rabbit blood.

Urine from the normal rabbits was collected (20–30 ml), freeze-dried and then stored in a desiccator until use.

Body tissues: Adult female rats and rabbits (non-lactating and lactating) were obtained from the Small Animal House maintained at the Institute. For collecting the tissues, animals were anaesthesized with chloroform, liver as well as the entire thoracic, abdominal and inguinal mammary gland were excised into ice-cold containers. The brain tissue was removed after opening the skull as soon as possible. The tissues were then stored frozen at −20 °C until further use.

Preparation of chloroform-methanol extract (CMEX): The procedure used for the extraction of endogenous opioid peptides from milk, milk proteins and body fluids (blood and urine) was the modification of the procedure described by BRANTL *et al.* (9).

(i) *Milk and milk proteins:* One g each of the lyophilized samples from cow and rabbit skim milk, cow whole casein and whey protein was mixed thoroughly with 1.0 g of silicic acid. To this mixture 10 ml of chloroform-methanol (2:1 v/v) solution was added and the suspension was stirred for one hour and then filtered through Whatman 42 filter paper. The residue was then washed with 5.0 ml of chloroform-methanol mixture and the combined filtrate was evaporated to dryness under vacuum at low temperature (0–10 °C). The final residue was redissolved in a known volume of distilled water and stored at −20 °C until further use.

(ii) *Body fluids:* The lyophilized sample corresponding to 20–30 ml each of blood and urine, was mixed with 5.0 g of silicic acid and stirred thoroughly in 20–30 ml of chloroform-methanol (2:1 v/v) solution for 1 h. The suspension was filtered through Whatman 42 filter paper and the residue was washed with 5 - 10 ml of chloroform-methanol mixture. The combined filtrate was then evaporated under vacuum at low temperature. The final residue was redissolved in a known volume of distilled water and stored at −20 °C for further use.

Preparation of acetone-methanol extract (AMEX): The body tissues such as liver, mammary gland and brain, collected from adult female non-lactating as well as lactating rats and rabbits, were extracted for the endogenous opioid peptides, following the procedure of HUGHES (4) with certain modifications as described below:

Tissues collected from the anaesthetized animals were minced thoroughly under ice-cold conditions and homogenized in 5 ml/g of acetone containing 0.2 ml of 10 N HCl/l with a Potter-Elvehjem homogenizer for 1 min. The solution was then stirred continuously for 2 h and filtered through suction. The residue was re-extracted with 5.0 ml/g of acetone-water (80:20 v/v). The combined filtrate from the two extractions was evaporated down to the aqueous phase under reduced pressure at 28 °C. The aqueous extract was spun at 6,000 rpm for 30 min in a Sorvall centrifuge and the supernatant was lyophilized to dryness. The residue remaining after lyophilization was redissolved in methanol (10–20 vol.) and filtered to remove salt and residual proteins.

Finally, methanol was evaporated under vacuum at 34 °C and the resulting yellow waxy residue was suspended in a known volume of distilled water (10 g wet weight of tissue/ml distilled water) and stored at −20 °C until further use.

Thin layer chromatography of CMEX and AMEX: The crude extracts obtained from the above two extraction procedures were chromatographed (10 µl of each sample) on glass plates (20 x 20 cm) using silica gel G and butanol: acetic acid and water (4:1:1) as the solvent system. The spots were characterized for peptides using ninhydrin.

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Determination of analgesic activity of crude extracts: For this purpose, a modification of the rat tail-flick procedure of D'AMOUR and SMITH (13) was used. Mice (preferably male) were weighed in groups of five for each sample and injected intraperitonially (10 µl crude extract/g body wt. of mice). The reaction time (i.e. the time taken in sec to flic the tail) was recorded both before injecting the crude extracts (basal level) and after the intraperitonial injection (IPI) at 5, 15 and 30 min of administration. Saline was used as control and the standard narcotic drug used was pethidine with a dose of 10 mg/kg body weight to compare the potencies of the crude extracts. The analgesiometer used was a radiant heat type and in any case of the response which took more than 8 sec, the instrument was made off to avoid the burning of tail. For this purpose pethidine was used and its maximum potency exhibited at 5 min interval of administration was taken as the comparative parameter (see Fig. 1). The crude extracts were prepared by dissolving the tissue hydrophobic residues in distilled water in a ratio of 10 g starting material/ml distilled water and 10 ml of lyophilized blood and urine samples/ml distilled water. The samples which were found to have analgesic activity were tested on separate groups of mice after pre-treatment with naloxone (10 mg/kg body wt.) intraperitonitally, 20 min before the measurement of analgesia.

3. Results and discussion

3.1 Chemical nature of the crude extracts

Based on the available findings, there is a general opinion on the chemical characteristic of the tissue opioids to be peptidic and hydrophobic in nature. The characterization of the various extracts viz. CMEX and AMEX obtained from milk, milk proteins, body fluids and body tissues of rats and rabbits using thin layer chromatography revealed them to be ninhydrin positive and peptides in nature.

3.2 Analgesic potency of milk and body fluids

(i) *Evaluation of milk:* The maximum fold increase viz. 3.42-fold in response for cow skim milk was obtained at 5 min interval after IPI. With the same crude extract when administered after pre-treatment of the animal with naloxone (10 mg/kg body wt. IPI), the analgesic effect was found to be reversed (1.73-fold). This morphine like activity in the extract from milk was very close to the pethidine potency (Fig. 1) even in relation to the duration of the activity up to 30 min of administration in the mice. The potency of cow skim milk was maximum in comparison to all the other milk systems tested, namely whole casein, cow (1.48-fold), whey proteins, cow (1.65-fold) studied (Table 1). These observations again confirm the reports of BRANTL and TESCHEMACHER (9, 10, 11) on the presence of opioid activity in milk and milk products. In contrast, rabbit skim milk was not found to have any such activity which could be detected by the tail-flick method.

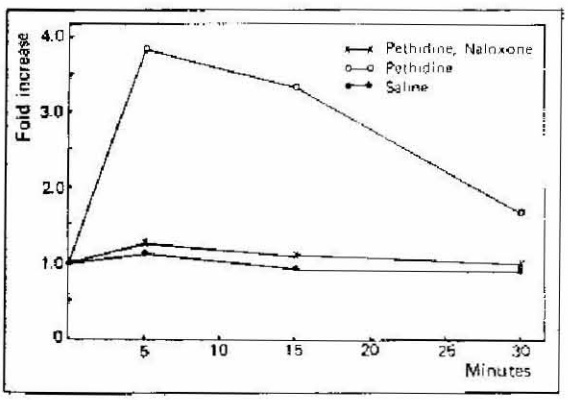

Fig. 1: Analgesic potency of pethidine and its reversal with naloxone as assessed by the mice-tail flick method. Saline was used as the control.
Pethidine was injected (intraperitonially) with a dose of 10 mg/kg body weight.
Naloxone was injected (intraperitonially) with a dose of 10 mg/kg body weight 20 min before the measurement of analgesia.

Table 1: Opioid potencies of hydrophobic extracts obtained from milk, milk proteins and other body fluids

Drug/extract administered	Mean basal level (s)	Fold increase[1] from basal level at 5 min interval of IPI
Drug used		
Saline (control)	1.85	1.13
Pethidine[2]	1.51	3.81
Pethidine + naloxone[3]	1.85	1.25
Extracts used		
Milk and milk proteins		
Cow skim milk	2.26	3.42
Cow skim milk + naloxone	2.01	1.22
Rabbit skim milk	2.12	1.33
Whole casein (cow)	2.56	1.48
Whey proteins (cow)	2.05	1.65
Body fluids		
Rat blood (NL)	2.20	1.45
Rat blood (L)	1.53	1.05
Rabbit blood (NL)	2.43	1.24
Rabbit blood (L)	2.22	1.54
Rabbit urine (NL)	1.90	1.34
Rabbit urine (L)	2.08	0.92

[1] Calculated for 10 g starting material per ml distilled water, and for body fluids 10 ml lyophilized samples per ml distilled water. See text.
[2] Used as standard narcotic drug (10 mg/kg body wt.) to bracket the responses of various treatments.
[3] Administered intraperitonially (10 mg/kg body wt.) 20 min prior to measurement of analgesia
(NL) = Non-lactating
(L) = Lactating

(ii) *Body fluids:* Body fluids like blood and urine from rabbits and rats were next examined for opioid potency. The effect of lactation was also examined. CMEX of blood samples from non-lactating rats and lactating rabbits showed their maximum responses after 5 min interval of administration (1.45 and 1.54-fold increase from their respective basal levels). However, similar extracts from blood samples of lactating rats and non-lactating rabbits exhibited maximum response (viz. 2.03 and 1.51-fold increase, respectively) after 15 min of administration.

CMEX of urine samples from non-lactating rabbits showed maximum response (1.34-fold) at 5 min, whereas, from lactating rabbits after 15 min and 30 min (1.10 and 1.12-fold increase, respectively).

Thus, in the biofluids tested namely blood and urine, no significant opioid activity could be detected. This was found irrespective of the species tested namely rat and rabbit and

independent of their physiological status regarding milk synthesis. However, the presence of opioids in blood cannot be ruled out in some cases, just on the basis of the assay system used (mice-tail-flick procedure) which is relatively less sensitive (13). As a matter of fact, a low molecular weight substance termed "anodynin" extracted from human blood plasma with opioid activity has already been reported (7). Blood systems deserve further investigation on these lines using more sensitive assay systems like guinea pig *ileum* and mouse *vas deferens* techniques (14, 15).

3.3 *Analgesic potency of body tissues*

AMEX of body tissues *viz.* liver, mammary gland and brain from rat and rabbits (non-lactating as well as lactating) were tested in a similar manner injecting the crude extracts (10 µl/g body wt.) intraperitonially. The responses were calculated (Table 2) on the basis of 10 g starting material dissolved in 1.0 ml of distilled water.

Table 2: Opioid potencies of crude extracts obtained from rat and rabbit body tissues

Extract administered	Mean basal level (s)		Fold increase from basal level at 5 min interval of IPI	
	Non-lactating	Lactating	Non-lactating	Lactating
Rat brain	2.80	3.90	3.28	2.14
Rat mammary	2.05	2.28	2.77	1.23
Rat liver	2.01	3.00	0.59	0.89
Rabbit brain	3.33	1.59	1.64	3.24
Rabbit mammary	1.85	1.86	0.80	1.58
Rabbit liver	3.36	1.95	0.70	0.69

It is clear that the mammary gland, the tissue responsible for milk synthesis, in one case of one sample from female rat (non-lactating) showed analgesic potency (2.77-fold increase at 5 min interval of adminfistration). When other tissues like brain and liver from either rat or rabbit were tested, brain tissue invariably and irrespective of the physiological status (non-lactating or lactating) showed significant analgesic activity.

Similar observations on brain opioids have been reported earlier by various workers (4, 16, 17). HUGHES (4) even proposed that the compound isolated from brain tissue forms a part of a central-pain-suppressive system and also have a wider neurochemical role in the brain. The liver tissue, however, does not appear to possess any such activity as revealed by the present assay technique. Similar findings were reported by other workers (18, 19).

4. References

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5. Summary

SINGH, S.K., HOSSAIN, M.A., GANGULI, N.C.: Morphinomimetic activity in extracts from milk and body tissues. Milchwissenschaft 40 (2) 74–77 (1985).

34 Morphines (milk proteins)

Cow skim milk was examined for its opioid potency. When extracted with chloroform-methanol, it was found to exhibit appreciable analgesic activity (3.42-fold) compared to the control as revealed by mice-tail flick procedure. This activity could be reversed with prior treatment of naloxone, a narcotic antagonist. Such activity could not be noticed either in similar extracts from skim milk samples obtained from healthy rabbits or in the extracts from cow milk proteins, namely casein and whey. Body fluids, such as blood from rats and rabbits, when extracted by similar procedure, showed less pronounced activity except in the case of lactating rats (2.03-fold compared to its basal level) when tested by the procedure used. No activity could be noticed in similar extracts from urine of rabbits (non-lactating and lactating).

Acetone-methanol extracts from brain tissue (rat and rabbit, both non-lactating and lactating) showed significant response in terms of latency to flick the tails of mice whereas such extracts from liver tissues of rats and rabbits (non-lactating and lactating) did not show any appreciable response. In general similar extracts obtained from the mammary gland tissue of these animals did not show comparable analgesic activity except in one sample from female non-lactating rat (2.77-fold).

SINGH, S.K., HOSSAIN, M.A., GANGULI, N.C.: Morphinomimetische Aktivitäten in Extrakten aus Milch und Körpergewebe. Milchwissenschaft 40 (2) 74–77 (1985).

34 Morphine (Milchproteine)

Kuhmagermilch wurde auf ihr opioides Potential untersucht. Bei Extraktion mit Chloroform-Methanol zeigte sich eine erhebliche analgetische Aktivität (3,42-fach) im Vergleich zur Kontrolle, wie dies durch die Mäuseschwanz-Reaktionstechnik festgestellt wurde. Diese Wirkung konnte durch vorherige Behandlung mit Naloxon, einem Betäubungsmittelantagonisten, aufgehoben werden. Eine entsprechende Aktivität konnte weder in Extrakten aus Magermilchproben gesunder Kaninchen noch in den Kuhmilchprotein-Extrakten (Casein und Molke) festgestellt werden. Körperflüssigkeiten wie Blut von Ratten und Kaninchen zeigten, wenn sie auf ähnliche Weise extrahiert wurden, eine weniger ausgeprägte Aktivität, außer bei laktierenden Ratten (2,03-fach verglichen mit dem Basiswert), wenn die Prüfung analog erfolgte. Bei Extrakten aus dem Kaninchenharn (laktierende und nichtlaktierende Tiere) konnte keine Aktivität ermittelt werden.

Aceton-Methanol-Extrakte aus Hirngewebe (Ratte und Kaninchen, laktierend und nichtlaktierend) zeigten eine signifikante Reaktion im Sinne einer Latenz bei der Mäuseschwanz-Reaktionstechnik, während Extrakte aus Lebergewebe von Ratten und Kaninchen (laktierend und nichtlaktierend) zu keiner deutlichen Reaktion führten. Im allgemeinen zeigten Extrakte aus dem Milchdrüsengewebe dieser Tiere keine vergleichbare analgetische Aktivität mit Ausnahme einer Probe einer nichtlaktierenden Ratte (2,77-fach).

SINGH, S.K., HOSSAIN, M.A., GANGULI, N.C.: Activité morphinomimétique dans des extraits de lait et des tissus de corps. Milchwissenschaft 40 (2) 74–77 (1985).

34 Morphines (protéines lactiques)

SINGH, S.K., HOSSAIN, M.A., GANGULI, N.C.: Actividad mofinomimética en extractos de leche y de tejido del cuerpo. Milchwissenschaft 40 (2) 74–77 (1985).

34 Morfinos (proteinas de leche)