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PARALLEL CENTRAL EFFECTS OF 5-ADENYLIC ACID AND SUBSTANCE P PREPARATIONS OF VARIOUS POTENCIES

Powders obtained in isolation of SP show widely different activities when tested on different organs from different animal species. Such behaviour indicates the presence of substances interfering with the stimulating effects of the active principle. v. Euler and Gaddum (1931) in their first paper on SP suspected ATP-like admixtures to be responsible for the depressive effects of some preparations. In unpublished experiments carried out in order to concentrate SP we found that some fractions exerted a marked depressive effect on the guinea pig ileum; we also observed that certain preparations containing SP, on addition to the bath fluid, first caused a dilatation of the organ, and afterwards only the expected contraction. Laszlo (1960) suggested 5-adenylic acid (5-AA) to be one of the ATP-type impurities present in raw SP. By the action of adenosine monophosphatase (AMP-ase) this acid is converted to 5-inosinic acid, a product completely devoid of any action on guinea pig ileum. Two of us (Stern and Huković, 1960) noticed that strongly active preparations (270 units SP per mg) lack some of the central effects described by Zetler (1956), who worked with weak preparations of 8 U./mg potency. The concentrated preparation did not inhibit strychnine convulsions, nor did it lengthen hexobarbital narcosis, but it antagonized the analgetic action of morphine in mice even in very small doses (1 unit per gram body weight). This antagonism is fully in agreement with Zetler's (1956) findings. Lembeck (1953) forwarded the hypothesis that SP represents the transmitter substance of the sensory neurons. It is entirely acceptable that a substance concerned with sensory transmission should antagonize inhibitors of sensitivity.

Since 5-AA goes along with SP in organ extracts it was necessary to establish the central effects of the former in order to hold them apart from those of the latter.

Method

In these experiments we used mice of both sexes, weighing 18 g on the average. Controls were set up in all experiments by injecting a number of animals with the same volume of saline or other solvent

used for preparing the solutions of SP injected to experimental animals. The injections in both experimental and control animals were applied on identical places at the same time intervals. Injections of strychnine were made according to Orlow, Williams and Pfeiffer (1949): strychnine nitrate (36 μg/ml) in saline was injected into the dorsal vein of the tail at a rate of 0.05 ml/0.1 min. till the appearance of tonic convulsions. Hexobarbital (sodium salt) (2.20 μg/ml) was applied in the same manner, at the same rate, in a total dose of 55 μg per g body weight. The duration of narcosis was measured from the end of the injection until the first signs of wake-up (righting reflex). Solutions of the sodium salt of 5-AA (10 mg/ml) were freshly prepared and injected i. p. in doses of 0.20 mg per g body weight 15 min. prior to strychnine, hexobarbital or to the production of pain by application of heat. The degree of pain was rated according to the method by Woolf and McDonald (1944). Morphine hydrochloride solution (0.24 mg/ml) in a dose of 6 μg/g was injected s. c. 30 min. before 5-AA and its analgetic effect estimated 45 minutes later. AMP-ase was prepared from rabbit muscle by the procedure of Schmidt (1928). The enzyme solution was neutralized with 10 per cent acetic acid, filtered, and stored under toluene. Enzymatic activity was determined by measuring the volume of ammonia evolved from 5-AA after incubation for 2 hours at 30°C and pH 5.9 (phosphate buffer) in a van Slyke—Cullen volumetric apparatus. The enzyme after neutralization contained an extremely small amount of ammonia. It liberated from an SP preparation of 25 U./mg potency practically the same amount of ammonia as from the same weight of 5-AA.

Statistical evaluation was carried out according to Fisher (1948) and the statistical significance between mean values judged by Student's t-test. Standard error of the mean is denoted by SE in the Tables.

Results

The first series of experiments was intended to show the influence of 5-AA on the action of strychnine, hexobarbital and morphine (Table I).

The dose of 662.50 ± 29.22 μg/g strychnine nitrate was absolutely lethal, after heavy convulsions, in the controls. Administration of 5-AA 15 min. prior to the strychnine salt protected the animals from convulsions and raised the lethal dose to 914.70 ± 47.31 μg/g. The difference is significant, $p<0.01$. At a longer time-interval between 5-AA and strychnine, e. g. more than 30 min., there was no more protection against convulsions.

Controls receiving hexobarbital alone sleep 10.2 min. in the average (SE ± 2.63 min.). Sleeping time is doubled when 5-AA is given prior to hexobarbital (20.76 ± 3.155 min.). The difference is significant, $p < 0.01$.

Controls react to pain 0.20 ± 0.131 min. after application of heat. Animals previously injected with 5-AA react only after 0.38 ± 0.0268

min. The difference is significant, $p < 0.01$, thus, 5-AA acts analgetically. When morphine is administered 45 min. prior to application of heat the animals react after 0.27 ± 0.0155 min. With 5-AA the corresponding time lag is 0.74 ± 0.0393. The difference is significant, $p < 0.05$, 5-AA potentiates the effect of morphine.

TABLE I

THE PROTECTIVE EFFECT OF 5-ADENYLIC ACID TOWARD CONVULSIONS PRODUCED BY STRYCHNINE

Previous administration	Strychnine i. v. µg/kg	S E	n	t	p
0.90% NaCl, i. p.	662.50	29.22	12	—	—
5-Adenylic Acid, 0.20 mg/g i. p.	914.70	47.31	12	3.552	< 0.01

THE PROLONGATION OF EVIPAN NARCOSIS BY 5-ADENYLIC ACID. EVIPAN SODIUM 55 µg/g i. v. IN MICE

Previous administration	Duration of narcosis in minutes	S E	n	t	p
0.90% NaCl, i. p.	10.02	2.625	10	—	—
5-Adenylic Acid, 0.20 mg/g i. p.	20.76	3.155	10	3.153	< 0.01

THE ANALGETIC OF 5-ADENYLIC ACID AND POTENTIATION OF ANALGETIC EFFECT OF MORPHINE

Previous administration	Time of reaction in min.	S E	n	t	p
0.90% NaCl, i. p.	0.20	0.0131	12	—	—
5-Adenylic Acid, 0.20 mg/g i. p.	0.38	0.0268	12	5.789	< 0,01
5-Adenylic Acid and Morphine, 6 µg/g	0.74	0.0393	12	2.448	< 0.05

The next series of experiments was carried out with SP. The preparations had 25 U./mg potency and were previously incubated with AMP-ase. The same drugs were tested as before. Controls were injected with buffered enzyme solution alone. The results are shown in Table II.

The controls exhibited strychnine convulsions and died of 542.90 ± ± 20.92 µg/g strychnine salt. With incubated SP solutions the animals were protected against convulsions and died only after 888.30 ± 80.91 µg/g strychnine salt. The difference is significant, $p < 0.01$. Sleeping time after hexobarbital in controls was 14.14±4.466 min. With incubated SP solution the sleeping time was increased to 23.14±4.734, but the difference is not significant at $p < 0.30$. After morphine alone animals reacted to applied heat after 0.46 ± 0.0477 min. With incubated SP solution injected 30 min. after morphine this interval was reduced to 0.27 ± 0.0277, which is almost the same as in controls after saline and buffered enzyme solution alone (0.30±0.0216 min.). The difference in reaction interval between controls and experimental animals is significant at $p < 0.01$.

TABLE II

THE PROTECTIVE EFFECT OF SUBSTANCE P TOWARD CONVULSIONS PRODUCED BY STRYCHNINE NITRATE i. v. ON MICE

Previous administration	Strychnine i. v. μg/kg	S E	n	t	p
0.90% NaCl, i. p.	542.90	20.92	10	—	—
Incubated SP, 10 μ/g i. p.	888.30	84.91	10	3.590	< 0.01

THE PROLONGATION OF EVIPAN NARCOSIS BY SUBSTANCE P. EVIPAN SODIUM 55 μ/g i. v. IN MICE

Previous administration	Duration of narcosis in minutes	S E	n	t	p
0.90% NaCl, i. p.	14.14	4.466	12	—	—
Incubated SP, 10 μ/g i. p.	23.04	4.734	12	1.358	< 0.30

THE INHIBITION OF ANALGETIC EFFECT OF MORPHINE BY SUBSTANCE P. MORPHINE HYDROCHLORIDE s. c. 6 μg/g OF MICE

Previous administration	Time of reaction in min.	S E	n	t	p
0.90% NaCl, i. p.	0.30	0.0216	12	—	—
Morphine, s. c.	0.46	0.0447	12	5.678	< 0.01
Morphine and Incubated SP, i. p.	0.27	0.0274	12	5.987	< 0.01

Injection of a large dose (0.0125 ml/g i. p.) of SP resulted in hypnotic, toxic and lethal effects. Unincubated SP powder of 25 U./mg potency was lethal in a dose of 40 U./g body weight. The same dose of incubated SP of identical potency killed 11 out of 12 animals. After incubation of the same powder with chymotrypsin the same dose, again, killed 7 out of 8 animals. Both unincubated and incubated powder still had a lethal effect if given in a dose of 30 U./g. With 20 U./mg only toxic effects in form of narcosis were observable. With further reduction of the dose other effects became manifest. With 14 U./g one can well see muscular pareses and weakness of the skeletal muscles. The toxic effects of SP start with paresis of the hind legs, difficulties in respiration, and, in some animals, rolling round their longitudinal axis. Cessation of symptoms and awakening occurs suddenly.

Discussion

Two of us (Stern and Huković, 1960) have already reported earlier that an SP powder of 16.3 U./mg potency inhibited strychnine convulsion, lengthened hexobarbital sleeping time, and antagonized morphine analgesia. In the present work we obtained the same effects with a more concentrated preparation of 25 U./mg, in which polluting 5-AA was destroyed by incubation with AMP-ase. 5-AA, itself, proved in our experiments to inhibit strychnine convulsions and lengthen hexobarbital narcosis. This substance, however, potentiated the analgetic action of morphine, and exhibited moreover some analgetic action of its own. The

difference in action between both incubated and unincubated SP preparations on the one hand, and 5-AA on the other lies in the effect on the central action of morphine. In trying a high-potency SP preparation (270 U./mg) we found that it did not protect the animals from strychnine convulsions, nor lengthen hexobarbital narcosis, but it antagonized morphine analgesia, in small doses*). Thus all SP preparations examined by us had one feature in common, namely the antagonism to morphine analgesia (Table III). The depression of strychnine convulsions and the lengthening of hexobarbital narcosis observed with raw SP preparations are probably to be attributed to the action of 5-AA. The antagonism to morphine, however, is a characteristic feature of SP, and 5-AA acts here in the opposite sense. These results show that in addition to previously established inhibition of SP-effects on the guinea pig ileum 5-AA also interferes with central effects of SP. It will, therefore be interesting to study, by the same methods, a high-potency SP preparation (30,000 U./mg) obtained recently by Franz, Boissonnas and Stürmer (1961) which appears to be the pure polypeptide, in order to establish definitely the true central effects of SP.

TABLE III

COMPARISON OF THE CENTRAL EFFECTS OF SP AND 5-ADENYLIC ACID AFTER STRYCHNINE, HEXOBARBITAL AND MORPHINE ON MICE

Previous administration	Strychnine convulsions	Hexobarbital narcosis	Morphine analgesia
Powders of SP 25 μ/mg Incubated with AMP-ase 10 μ/g i. p.	Inhibition $p < 0.01$	Prolongation $p < 0.01$	Inhibition $p < 0.05$
Powders of SP 16.2 μ/mg Nonincubated. 10 μ/g i. p.	Inhibition $p < 0.01$	Prolongation $p < 0.30$	Inhibition $p < 0.05$
Powders of SP 270 μ/mg Nonincubated 10 μ/g	Stimulation $p < 0.20$	0	0
Powders of SP 270 μ/mg Nonincubated 1 μ/g	0	0	Inhibition $p < 0.05$
5-Adenylic Acid 0.20 mg/g i. p.	Inhibition $p < 0.01$	Prolongation $p < 0.01$	Stimulation $p < 0.01$

Our results confirm the transmitter rôle of SP in sensory neurons proposed by Lembeck (1956). Antagonism to analgetic action in a factor supposed to facilitate the transmission of sensory impulses is an obvious property. This idea is also suggested by findings of Holton (1960), Serafimov (1958) and Stern and Kocić-Mitrović (1958). The two latter authors supplied some evidence to the above statement by showing the influence of light and dark on the amount of SP in the retina.

The lethal effect, toxicity, and hypnotic action of the 25-U./mg SP-powder, unincubated as well as incubated with AMP-ase and chy-

*) It must, however, be pointed out that morphine antagonism could be observed only with small doses, 1 U. per g body weight. Doses of 10 U./g failed to antagonize morphine and even potentiated strychnine convulsions.

motrypsin, point to the possible existence of further impurities. In this respect it will be particularly interesting to examine the type of muscular pareses by which the toxic symptoms start. One may ask first whether they are a consequence of peripheral or central SP effects.

Finally, to turn back to 5-AA, it is important to mention that the action of this substance is strongest within the first 15 min., and diminishes during the next 30 min. Such time-action relationships have also been found for SP by v. Euler and Pernow (1954) and Zetler (1959), and this coincidence affords additional support to the inference that the central effects of SP are in part due to 5-AA. So once more one can say that the central effects peculiar to SP shall only be known when large amounts of pure SP, free from interfering admixtures, will be available for further study.

Summary

5-Adenylic acid inhibits strychnine convulsions, lenghthens hexobarbital sleeping time, and stimulates the analgetic action of morphine.

SP-preparations, up to 25 U./mg potency, act in the same way on strychnine convulsions and hexobarbital narcosis, but antagonize morphine analgesia. These effects remain unchanged after incubation with AMP-ase carried out in order to destroy polluting 5-Adenylic acid.

The difference in action between SP-preparations containing 5-Adenylic acid and authentic 5-Adenylic acid lies in the effect on morphine analgesia alone.

Toxic effects observed with SP-preparations up to 25 U./mg potency are probably due to further impurities.

In order to study the true central effects of SP in detail very pure preparations with high SP potency are needed.

PARALELNI CENTRALNI EFEKTI 5-ADENILNE KISELINE I SP-PREPARATA RAZLIČITE JAKOSTI

5-adenilna kiselina djeluje inhibitivno na strihninske grčeve, produžuje heksobarbitalsku narkozu i stimulira analgetsko djelovanje morfina.

Preparati SP do jakosti od 25 jed./mg djeluju jednako na strihninske grčeve i heksobarbitalsku narkozu, ali na analgetsko djelovanje morfina reaguju antagonistički. Ovi efekti ostaju bez promjene poslije inkubacije s adenozin-monofosfatazom u cilju inaktivacije primiješane 5-adenilne kiseline.

Različito djelovanje preparata SP s primjesom 5-adenilne kiseline i same adenilne kiseline očituje se jedino u odnosu na analgetsko djelovanje morfina.

Toksički efekti zapaženi kod preparata SP do jakosti od 25 jed./mg su, vjerojatno, posljedice daljih onečišćenja.

Za proučavanje pravih centralnih efekata SP u pojedinostima potrebni su veoma čisti preparati s velikom jakošću.

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DISCUSSION

ZETLER: Did you try to destroy the strychnine-antagonistic and the hexobarbital-synergistic activity of SP-powder by proteolytic enzymes?

HUKOVIĆ: We only destroyed the 5-adenylic acid by monoaminophosphatase, using the method described by Laszlo. We did not perform experiments with powders of SP previously incubated with proteolytic enzymes.

PERNOW: If Lembeck is right when he thinks that SP is a transmitter of the first sensory neurons, and I think that is an attractive hypothesis, then it is convincing to hear, that with the more purified SP preparations there is no prolongation of the Evipan narcosis, which has been shown to be obtained with the crude preparations. If the SP is supposed to facilitate the transmission of sensory nerves, then rather a shortening of the narcosis time would be expected.

Have you tried to separate the factor in the crude SP preparation responsible for the prolongation on the narcosis from the real SP factor?

HUKOVIĆ: In these experiments we have used crude SP. As regards separating of different factors from crude SP, we started separating them, but we still did not inject them.