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FACTORS INFLUENCING THE ACTION OF MORPHINE ON ACETYLCHOLINE RELEASE IN THE GUINEA-PIG INTESTINE

BY

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Morphine depresses the resting output of acetylcholine (Ach) in the guinea-pig ileum (Schaumann, 1957) and also the raised output following electrical stimulation (Paton, 1957). Since the latter effect is consistent with a site of action of morphine on post-ganglionic cholinergic nerves in the intestine (Paton, 1957, 1963), the question arises whether effects of the drug on Ach release in the resting intestine may also be attributed to inhibition of nervous structures. This possibility is supported by Johnson's (1963) finding that the resting output of Ach in the guinea-pig intestine is reduced by as much as 85% by procedures which are likely to depress nervous activity, including incubation with cocaine (5 μg/ml.), procaine (10 μg/ml.), hemicholinium, lowered calcium, raised magnesium, and cooling to 25° C. In the present study we have sought further information of the site of action of morphine in the resting intestine by examining its inhibitory effect under a variety of conditions which modify nerve transmission and/or neurotransmitter release.

METHODS

The resting output of acetylcholine in the guinea-pig intestine has been measured under two experimental conditions:

The first procedure (a) was that of Schaumann, in which small segments of intestine, closed by ligatures at both ends, were randomly distributed among a number of incubating flasks containing magnesium-free Tyrode solution gassed with 95% oxygen, 5% carbon dioxide.

The magnesium-free Tyrode solution was of the following composition: NaCl 138 mM, KCl 2.7 mM, $CaCl_2$ 1.8 mM, $NaHCO_3$ 12.0 mM, NaH_2PO_4 3.5 mM, glucose 5.5 mM.

In experiments where sodium ion concentration was reduced the osmotic pressure of the solution was adjusted by adding the appropriate amounts of sucrose. Incubation was carried out in a shaking bath at 37° C and the incubation fluid was removed periodically for bioassay; physostigmine (10^{-5}M) was present throughout.

The second procedure (b) was designed to allow removal of intraluminal contents, which may accumulate over long periods of incubation and possibly interfere with the generation of acetylcholine. A 10-cm portion of the intestine was drawn over a perforated polythene tube (diameter 3 mm) and ligated at each end as shown in Fig. 1. The preparation was placed in a vertical position in an organ-bath containing Mg-free Tyrode solution. The centre tube was connected to a reservoir of

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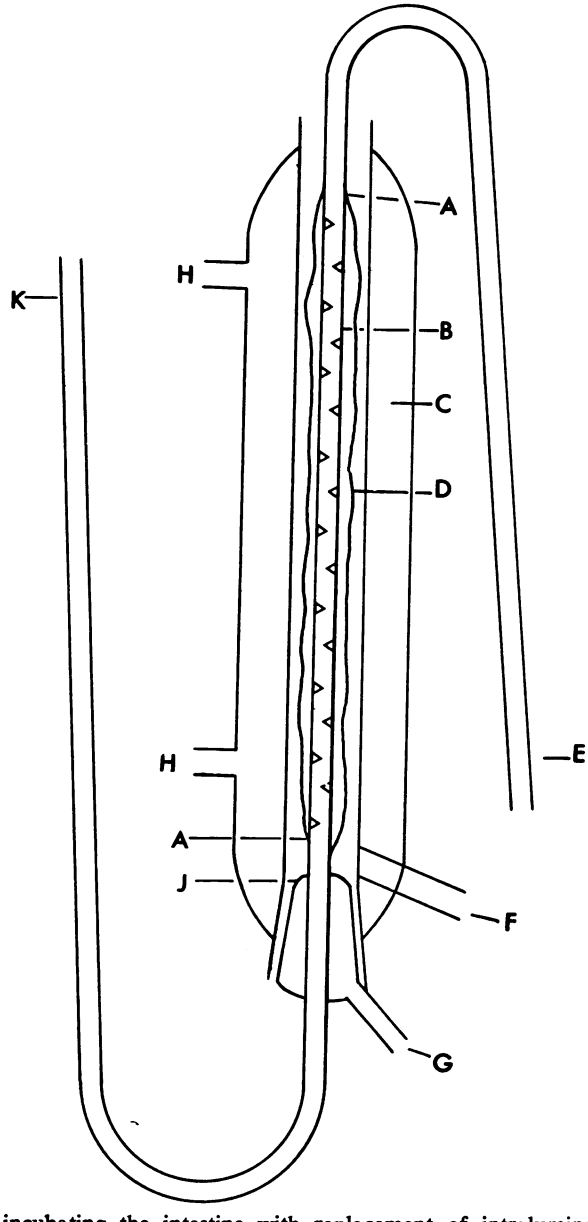

Fig. 1. Method of incubating the intestine with replacement of intraluminal fluid. A piece of intestine D was threaded over a length of perforated polythene tube B attached to a ground class cone at the bottom of the organ-bath. The intestine was tied on to the polythene tube at the top and bottom (marked A) to prevent intraluminal contents exuding into the bath. The bath was filled with incubation solution from the top and drained through the outlet F. The contents were gassed through small holes in the cone at J from a lead at G. The lumen could be flushed by means of the tube KBE, attached to a reservoir of fluid at K; intraluminal pressure was raised by lifting the reservoir and closing the clip at E. Temperature was maintained by circulating water at constant temperature through the outer jacket C *via* the nozzles at H.

Tyrode solution so that, when required, the intraluminal surface of the gut could be brought into contact with the Tyrode solution *via* the perforation in the tube. Hence the intraluminal surface could be flushed by gravity downflow, to permit continuous removal of intraluminal secretion. The precaution was routinely adopted of ensuring that the intestine was not under longitudinal tension as a result of its fixed position on the centre tube.

Bioassay

Solutions were applied directly to the toad rectus abdominus muscle sensitized with tacrine (80 μg/l.) as described previously (De la Lande & Porter, 1963 ; Porter, 1965).

In experiments where incubations were of short duration—for example, 2-min intervals—assays were carried out on the guinea-pig ileum as described by Paton (1957).

RESULTS

In exploratory experiments it was determined that acetylcholine accumulates in the physostigmine-containing solution, bathing intestinal segments at a steady rate for at least 1 hr (Fig. 2), and that segments in which the bathing solution was replaced at 20-min intervals generated acetylcholine for many hours. The rate of generation was initially low but rose rapidly until, after 1–2 hr, the output was sufficiently steady to permit the effect of morphine to be assessed by comparing the output during a brief exposure to the drugs with the outputs immediately preceding or following the drug (see, for example. Figs. 4*a* and *b*).

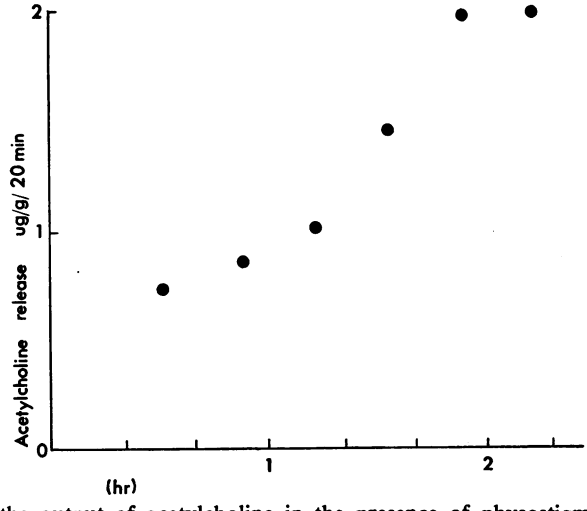

Fig. 2. Increase in the output of acetylcholine in the presence of physostigmine (10^{-5}M) added at zero time. No acetylcholine could be detected in the absence of physostigmine.

The effect of morphine was also studied by comparing the amounts of acetylcholine present in the bathing fluids of control and drug-treated segments under otherwise identical conditions. When tested by either procedure, morphine 10^{-5}M reduced the rate of accumulation. In accordance with the observation of Schaumann (1957) the effect of morphine was maximal or near maximal in this concentration, since a ten-fold increase in concentration produced little further inhibition (Fig. 3). The effect of morphine was readily reversible and could be reproduced over periods of 6–8 hr.

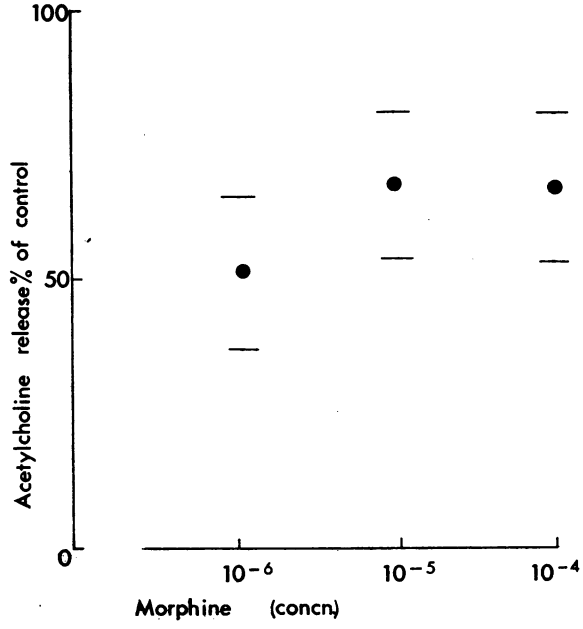

Fig. 3. Effect of the concentration of morphine on the release of acetylcholine. The output of acetylcholine in the presence of morphine is expressed as % of that in the absence of morphine. Horizontal bars indicate standard deviation (based on 8, 8, 10, experiments of morphine 10^{-6}, 10^{-5}, 10^{-4}, respectively).

The action of morphine on Ach output was examined on preparations subjected to subdivision, pre-cooling, anoxia, procaine, and changes in the electrolyte and glucose composition of the incubating medium.

Subdivision of the intestine

The effect of chopping the gut into small rings approximately 2 mm in length was examined in order to assess the possible contribution of long nerve pathways to acetylcholine (Ach) generation and its suppression by morphine. Subdivision caused a decline in output of Ach compared with control segments (incubation procedure (a)); the average output was 45% of that prevailing in undivided loops. The undivided segments in these experiments were not closed by ligatures, so that the drug was in contact also with the mucosal surface, as in the chopped preparations. The inhibitory effect of morphine was greatly reduced by subdivision (Table 1).

TABLE 1

EFFECT OF SUB-DIVISION OF INTESTINE ON THE OUTPUT OF ACH AND ON THE ACTION OF MORPHINE (SEVEN EXPERIMENTS)

	Average output expressed as % of unchopped segments	% inhibition by morphine
Unchopped preparation	—	42± 8
Chopped preparation	45 ± 13	5 ± 17

Cooling

Harry (1962) showed that after cooling ileum at 13° C for 30 min the output of Ach produced by transmural electrical stimulation on subsequent incubation at 37° C was slightly depressed.

In our experiments, segments of intestine which were stored at 4° C for 24 hr showed both a reduced rate of Ach generation when subsequently reincubated at 37° C, and a greatly reduced effect of morphine (six experiments, Table 2). The comparison in this case is based on determination of resting output of Ach and of its inhibition by morphine in the previously cooled segments, as compared with the output from a control segment from the same animal measured on the preceding day (incubation procedure (a)).

TABLE 2

EFFECT OF PREVIOUS COOLING OF THE INTESTINE ON THE OUTPUT OF ACETYLCHOLINE AND ON THE ACTION OF MORPHINE

	No. of experiments	Rate of output of Ach	% inhibition by morphine
Cooled preparation	6	7·5 ± 2·4 μg/g/hr	9 ± 16
Control segment	6	9·6 ± 2·1 μg/g/hr	49 ± 9

Procaine

As found by Johnson (1963), procaine caused the output of Ach to decline to a low level. In the presence of procaine, morphine had no apparent effect.

In 12 experiments the output of Ach measured 1 hr after the addition of procaine (2.7 mM), by procedure (a), was 32 ± 5% of control segments and was not significantly different from that of segments incubated with a mixture of procaine (2.7 mM) and morphine (10^{-5}M).

Anoxia

Gassing with 95% nitrogen to 5% carbon dioxide (procedure (b)) led to a decline in the resting output of Ach so that after 35–45 min in seven experiments the average output was 37 ± 10% of that of oxygenated control segments. The inhibitory effect of morphine was not apparent in the anoxic preparation, the output of Ach in such preparations amounting to 100 ± 35% of that of the corresponding drug-free controls (seven experiments).

Changes in the composition of the incubating medium

Glucose lack, sodium ion lack, and potassium ion excess each caused a decline in the resting output of Ach, and reduced or abolished the inhibitory action of morphine.

Glucose lack. In 18 experiments, using both procedure a and b, the output of Ach was reduced by subtraction of glucose to 49 ± 15% of the control outputs. Under these conditions, morphine no longer exerted its inhibitory action. A typical experiment illustrating the reversible depression of output by glucose lack and the effect of this procedure on morphine's action is shown in Figs. 4*a* and *b*.

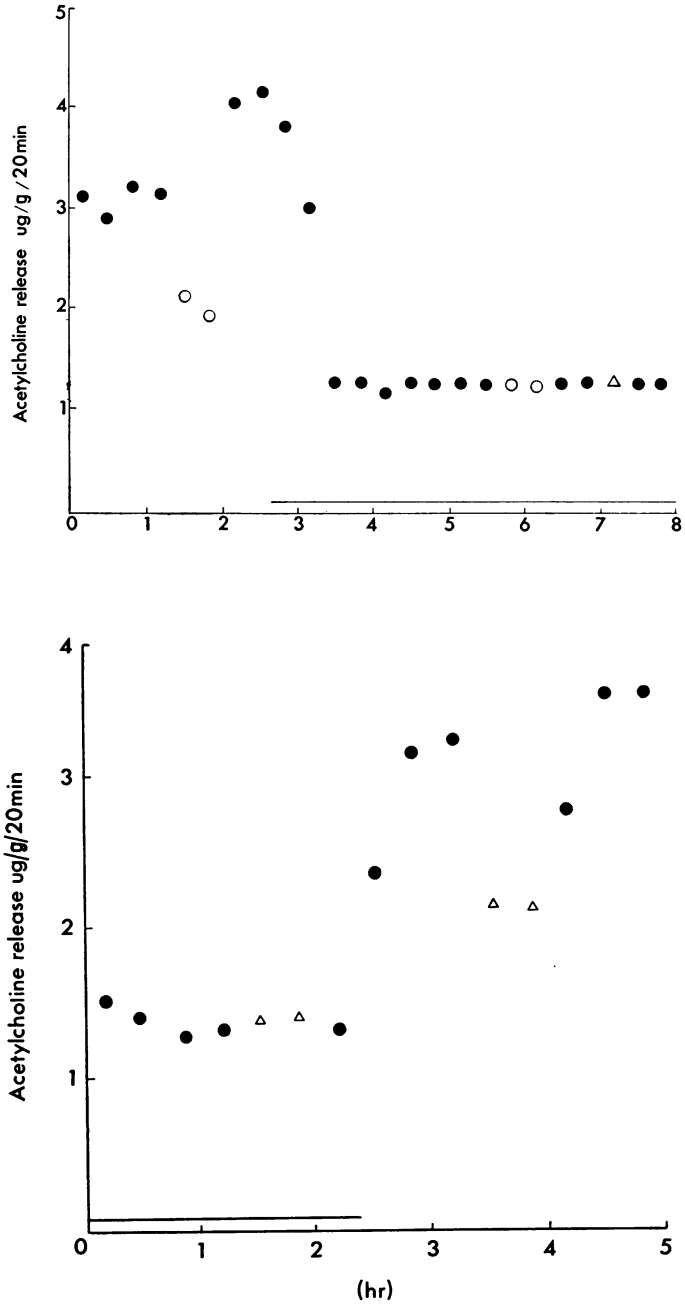

Fig. 4*a* and 4*b*. The effect of glucose lack on the output of acetylcholine and on the action of morphine. (*a*) Glucose to glucose-free conditions and (*b*) glucose-free to glucose conditions. The figures show the output of acetylcholine in μg/g/20 min. ○=the presence of morphine in a concentration of 10^{-6}M. △=a concentration of 10^{-5}M morphine. The abscissal bar indicates periods during which glucose was absent from the incubation fluid.

Sodium lack. Gerhards, Röttcher & Straub (1964) reported that liberation of Ach from guinea-pig intestine was markedly reduced by incubation in low sodium solutions. We found the reduction in Ach output by reducing the sodium ion concentration to approximately one tenth of normal (15 m-equiv/l.) amounted to $17 \pm 12\%$ of the controls (six experiments). The output was not further reduced by morphine.

A less drastic reduction in sodium ion concentration to one-half normal reduced the output to $34 \pm 14\%$ of the control (six experiments), but under these conditions the inhibitory effect of morphine was present and amounted to $50 \pm 22\%$ of the output in the absence of the drug.

The latter finding is of interest as it indicates that a reduction in output of Ach *per se* is not associated with a loss of the inhibitory action of morphine.

Potassium ion excess. An increase in the potassium ion concentration from 2.7 m-equiv/l. to 27 m-equiv/l. caused the output of Ach to decline to $43 \pm 14\%$ (six experiments, procedures (a) and (b)). The output was not further decreased in the presence of morphine, indicating that the inhibitory effect of the drug did not occur in the presence of an excess of potassium ions. 27 m-equiv/l. of K^+ was selected on the basis that it depolarized and abolished conduction in neurones ; a test of this assumption was provided by the finding that electrical stimulation under conditions which normally stimulate cholinergic neurones in gut (Paton, 1957 ; 1963) failed to increase the output of Ach in the presence of 27 m-equiv/l. of K^+, although causing considerable increases when the K^+ concentration was reduced to 2.7 m-equiv/l. (Fig. 5).

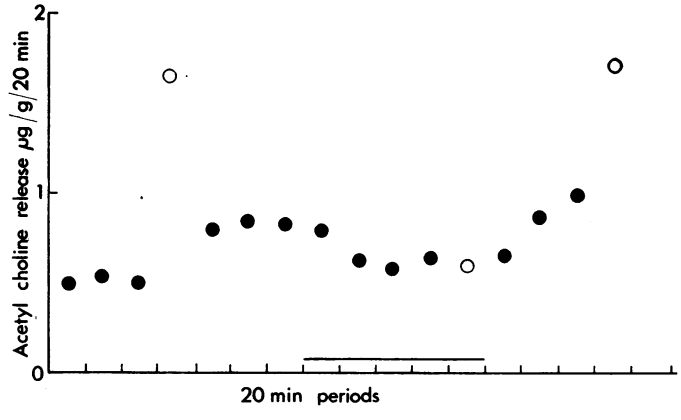

Fig. 5. Effect of increasing the potassium ion concentration to 27 m-equiv/l. (indicated by abscissal bar) on the output of acetylcholine from isolated guinea-pig intestine at rest (●) and upon electrical field stimulation (○).

In contrast to its effect on intestinal segments incubated in ordinary Tyrode solution, potassium ion excess caused a small but definite increase in Ach output in preparations incubated in sodium deficient (Table 3) fluid ; again there was no inhibition of Ach output by morphine.

TABLE 3

EFFECT OF MORPHINE ON THE RELEASE OF ACETYLCHOLINE FROM GUINEA-PIG INTESTINE INCUBATED IN LOW SODIUM (15 M-EQUIV/l.) AND IN LOW SODIUM, HIGH POTASSIUM (27 M-EQUIV/l.) MEDIA.

1st hr of incubation	2nd hr of incubation		
Control (μg/g/hr)	Control	+ High K^+	+ High K^+ + Morphine
Expt. 1			
0·37	—	0·73 (200)	—
0·33	—	—	0·6 (180)
0·30	0·33 (110)	—	—
0·30	—	0·73 (250)	—
0·33	—	—	0·84 (255)
Expt. 2			
0·20	0·23 (115)	—	—
0·21	0·26 (125)	—	—
0·22	—	0·45 (200)	—
0·25	—	0·56 (225)	—
0·25	—	—	0·65 (260)
0·26	—	—	0·58 (225)
Expt. 3			
0·60	0·55 (92)	—	—
0·60	0·60 (100)	—	—
0·50	—	1·5 (300)	—
0·47	—	1·3 (280)	—
0·45	—	—	1·4 (310)
0·45	—	—	1·3 (290)

* Numbers in brackets indicate the release expressed as a percentage of the control output.

DISCUSSION

Each of the conditions employed, namely chopping of the gut into small rings, pre-cooling, procaine, anoxia, and glucose lack, cause a reduction in output of Ach and a marked reduction or abolition of the inhibitory effect of morphine. This finding indicates that morphine inhibition is highly dependent on both the anatomical and metabolic integrity of the tissue. Each of the above conditions was selected on the assumption that it would impair the contribution of nervous structures to the resting output of Ach.

A basis for this assumption lies in the studies of Ambache (1946, 1949); Harry (1962); Johnson (1963); Day & Vane (1963), which indicate that cooling, procaine, and anoxia have little effect on smooth muscle function as judged by sensitivity to direct stimulation to, for example, Ach, but produce a marked loss of sensitivity to indirect stimulation—for example, nicotine, electrical field stimulation. Some of these procedures—namely, cooling and procaine—were shown earlier by Johnson to depress resting Ach output, and he has proposed that the Ach generated by the resting intestine is derived largely from nervous structures. Our data confirm Johnson's results and indicate further that the same structures include the morphine-sensitive component of the Ach-releasing system. By analogy with well-studied cholinergic neurones, it may be assumed that at least

two mechanisms are available for Ach generation from nerves—namely, spontaneous release of transmitter at nerve endings, and augmentation of this release by conducted impulses in nerve axons (intrinsic activity). Although intrinsic activity has not been demonstrated in cholinergic neurones in the resting intestine by direct techniques, there is no doubt that isolated segments of guinea-pig ileum undergo some spontaneous contractions when incubated in eserinized Tyrode solution, and it has been pointed out by Gerhards *et al.* (1964b) that in sodium deficient media, spontaneous activity is greatly reduced and is associated with a marked decrease in Ach output. They also showed that, provided changes in the rate of synthesis were taken into account, the residual output of Ach in sodium deficient solutions responded to various electrolyte changes in a similar way to cholinergic synapses. An assumption that a component of resting output results from conducted axonal activity in intramural nerves accords well with our findings that subdivision of the gut, and also excess potassium ions, depress Ach output, the former effect being explained by the severance of nerve axons, and the latter effect by the depolarizing block of conduction in such axons. Since in other tissues—for example, brain autonomic and skeletal neuromuscular junction—excess potassium ions accelerate spontaneous neurotransmitter release or the frequency of miniature end plate potentials (Brown & Feldberg, 1936 ; Mann, Tennenbaum & Quastel, 1939 ; Liley, 1956), it might be assumed that the effects of depolarizing block in intestine outweigh a stimulant effect of K^+ on spontaneous resting release. This assumption is supported by the finding that potassium ion excess does not depress Ach output in the chopped or sodium deficient preparation, where the contribution of intrinsic nerve activity would be small ; instead, an increase in output occurs. In general our findings with respect to resting Ach output confirm and extend those of Johnson (1963) and Gerhards *et al.* (1964a and b). However, the significant feature is that the morphine effect disappears under all conditions in which nerve activity is depressed, and particularly when intrinsic activity—that is, conduction in neurones—is likely to be abolished. Hence the neuronal sites of spontaneous or resting release of Ach in the gut do not appear to be implicated in the action of morphine. Instead, the hypothesis may be advanced that morphine acts proximally to such sites of release, presumably on neurones, by a process akin to that of local anaesthetics on nerve conduction. This hypothesis implies that nerve activity is continuously present in the isolated guinea-pig ileum. Since Paton has pointed out that an increase in Ach output can be observed to follow a single stimulus applied by transmural stimulation to the intestine, it does not appear necessary to invoke a high order of intrinsic activity in the intestine to account for the observed rates of resting release. The nature of such morphine-sensitive neurones is uncertain. However, the possibility that they involve relatively long pathways is indicated by the observation that subdivision of the gut abolished the depressant action of morphine.

SUMMARY

1. The output of Ach from eserinized guinea-pig intestine was reduced by chopping into small rings, previous cooling for 24 hr at 4° C, procaine, anoxia, lack of glucose or raised potassium ion concentration. Under these conditions morphine failed to depress Ach output further.

2. High concentrations of potassium ions increased the output when either the sodium ion concentration was reduced to one-tenth of normal, or the intestine was subdivided into small rings ; in both conditions morphine was again ineffective.

3. By analogy with other neuromuscular preparations it is proposed that the output of Ach from resting guinea-pig intestine may be the result of at least two factors: one involving conduction in long nerve pathways which are depressed by morphine ; the other a spontaneous release from nerve endings, which is not affected by morphine.

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