Br. J. Pharmac. (1970), **39**, 639–646.

Studies on the receptors involved in the action of the various agents in the phenylbenzoquinone analgesic assay in mice

S. E. SMITS* AND A. E. TAKEMORI

Department of Pharmacology, College of Medical Sciences, University of Minnesota, Minneapolis, Minnesota 55455, U.S.A.

Summary

1. Tolerance to the activity of several narcotic analgesics (morphine, levorphanol, and methadone) and several narcotic-antagonist analgesics (pentazocine, cyclazocine, and nalorphine) was studied in the mouse phenylbenzoquinone stretching test. Virtually complete tolerance was induced by chronic treatment with each of the narcotic agents, while no apparent tolerance was induced by the narcotic antagonists.

2. In morphine-tolerant mice there was a high degree of cross-tolerance to the effects of not only the other narcotic drugs but also to those of the narcotic antagonists, acetylsalicylic acid, and physostigmine.

3. The effects of morphine and pentazocine were antagonized by naloxone but not by atropine, while the effects of physostigmine were antagonized by atropine but not by naloxone. Neither atropine nor naloxone antagonized the effect of acetylsalicylic acid.

4. The results of the tolerance study suggest that there is a fundamental difference in the consequences of receptor interaction for the narcotic and the narcotic-antagonist analgesics. Morphine-tolerant mice exhibit cross-tolerance non-specifically. The selectivity of naloxone and atropine differentiates the narcotic and narcotic-analgesics from the other two agents used in this analgesic test.

Introduction

The question of whether narcotic drugs and the narcotic antagonists produce their analgesic effects at the same or different receptor sites has not been resolved. Martin (1967) has advanced the theory of competitive dualism to explain the actions of these drugs. His model is characterized by two stereochemically similar but not identical analgesic receptors. Nalorphine is assumed to act at one receptor and morphine at the other; in addition nalorphine acts as a competitive antagonist at the morphine receptor. Studies using the quantitative antagonism of several narcotic and narcotic-antagonist analgesics by naloxone have suggested that these two types

* Present address: The Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46206, U.S.A.

of agents interacted with either two different receptors or with the same receptor in a different manner (Smits & Takemori, 1970). Taber, Greenhouse, Rendell & Irwin (1969) concluded from their results in a study of cross-tolerance and of combinations of morphine and nalorphine that these two drugs act at the same receptor site, morphine as a full agonist and nalorphine as a partial agonist.

The present investigation was undertaken to further elucidate the relationship between the receptors for the narcotic and the narcotic-antagonist analgesics. This was done by performing studies on the development of tolerance and cross-tolerance and on the specificity of certain antagonists, using the mouse phenylbenzoquinone (PBQ) analgesic assay.

A preliminary report was presented at the meeting of the American Society for Pharmacology and Experimental Therapeutics in August 1969.

Methods

Animals. Male Swiss-Webster albino mice weighing 15 to 22 g (Simonson Laboratories, White Bear, Minnesota) were used in all experiments. They were housed for at least 1 day after arrival before starting the experiment. Each mouse was used only once.

Chemicals and drugs. The narcotic analgesics used in these experiments were levorphanal tartrate (Hoffman-La Roche, Inc.), methadone hydrochloride (Mallinckrodt), and morphine sulphate (Mallinckrodt) while the narcotic antagonists were cyclazocine (Win 20,740, Sterling Winthrop Research Institute), nalorphine hydrochloride (Merck and Co., Inc.), pentazocine (Win 20,228, Sterling Winthrop Research Institute) and naloxone hydrochloride (Endo Laboratories, Inc.). Other agents used were acetylsalicylic acid (aspirin, Sigma Chemical Co.), atropine sulphate (K & K Laboratories, Inc.) and physostigmine sulphate (Calbiochem). Dosages are given in the form in which the drug was obtained—as the salt, free acid or free base.

PBQ analgesic assay. The assay procedure was the same as that described in the preceding paper (Smits & Takemori, 1970).

Conditioning schedule. From previously determined dose-response curves (Smits & Takemori, 1970) the dose needed just to produce 100% inhibition of stretching was estimated. This ED100 dose was administered on the first day three times, at 4 to 5 h intervals. For the next 3 consecutive days the injection dose was double that of the previous day. An additional injection was given on the night preceding the day on which the PBQ assay was to be performed. The conditioning schedule is presented in more detail in Table 1.

Results

Effect of chronic treatment with narcotic or narcotic-antagonist analgesic on the development of tolerance

The details of the chronic injection schedule are presented in Table 1. Three narcotic and three narcotic-antagonist agents were included in this experiment. After 4 days of treatment, the mice were tested on the fifth day with the ED100 dose of the drug which had been chronically administered. The results of this

TABLE 1. *Schedule of the chronic injections based on the dose of analgesic required to produce 100% inhibition of stretching (ED100 dose)*

Analgesic	ED100 dose* (mg/kg)	Dose per injection† (mg/kg) Day of conditioning treatment 1	2	3	4
Morphine sulphate	1·4	1·4	2·8	5·6	11·2
Levorphanol tartrate	0·25	0·25	0·5	1·0	2·0
Methadone hydrochloride	1·2	1·2	2·4	4·8	9·6
Pentazocine (free base)	14·0	14·0	28·0	56·0	56·0‡
Cyclazocine (free base)	0·11	0·11	0·22	0·44	0·88
Nalorphine hydrochloride	1·5	1·5	3·0	6·0	12·0

* This dose was estimated from previously determined dose-response curves (Smits & Takemori, 1970) and tested to establish that a 100% inhibition of stretching does occur.
† Three injections were given each day at 4 to 5 h intervals. On the day before the determination of tolerance or cross-tolerance an additional injection was given 10 to 14 h before performing the PBQ analgesic assay.
‡ The dose was not increased further due to toxicity.

TABLE 2. *Tolerance and cross-tolerance to narcotic and narcotic-antagonist analgesics*

Group	Drug* Conditioning	Test	Total number of stretches/ten mice Mean±S.E.	(*N*)†
A	None (control)	Water	91±11	(49)
B	None	Morphine	0±1	(7) [0–2]¶
		Methadone	0	(4)
		Levorphanol	1±1	(5) [0–2]¶
C	Water	Morphine	0	(1)
		Methadone	0	(1)
		Levorphanol	0	(1)
D	Morphine	Water	103	(1)‡
	Methadone		97	(1)‡
	Levorphanol		112	(1)‡
E	Morphine	Morphine	91±4	(4)‡
	Methadone	Methadone	75	(1)‡
	Levorphanol	Levorphanol	112	(1)‡
F	None	Pentazocine	0	(5)
		Nalorphine	6±2	(4) [0–8]¶
		Cyclazocine	5±2	(4) [0–9]¶
G	Water	Pentazocine	0	(1)
		Nalorphine	6±2	(3)
		Cyclazocine	6	(1
H	Pentazocine	Water	91	(1)‡
	Nalorphine		92	(1)‡
	Cyclazocine		113	(1)‡
I	Pentazocine	Pentazocine	4	(1)§
	Nalorphine	Nalorphine	13	(1)§
	Cyclazocine	Cyclazocine	28	(1)§
J	Morphine	Methadone	86	(2)‡
		Levorphanol	80	(2)‡
		Pentazocine	89	(2)‡
		Nalorphine	100	(2)‡
		Cyclazocine	81	(2)‡

* See Table 1 for the conditioning schedule and the ED100 (test) doses of the drugs. The ED100 doses of each drug were tested on the fifth day, 10 to 14 h after the last injection of the conditioning treatment.
† (*N*)=number of groups of ten mice observed.
‡ Values not significantly different from control (group A) ($P>0.05$).
§ Values significantly smaller than control (group A) ($P<0.01$).
¶ Range of stretches observed.

experiment are shown in Table 2. The ED100 doses of the drugs were, as expected, fully effective (groups B and F) and the injection of water, as a control for the conditioning drugs, did not alter these responses (groups C and G). The conditioning injections only slightly altered the stretching response of the mice (groups D and H). The effects of morphine, methadone and levorphanol were absent, indicating complete tolerance to the test doses (group E). Unlike the narcotics, none of the narcotic antagonists induced any substantial tolerance on this injection schedule (group I). Cyclazocine was the only agent in this group to which even a slight degree of tolerance appeared to develop, but the degree of tolerance was much less than those observed with the narcotic agents.

Cross-tolerance to other narcotic and narcotic-antagonist analgesics in morphine-tolerant mice

Mice were treated with morphine according to the schedule presented in Table 1. On the fifth day the mice were given a test ED100 dose of either one of the three narcotic agents or one of the three narcotic antagonists. The results are shown in Table 2. Marked cross-tolerance to the effects of the other narcotics was observed (group J). Chronic administration of morphine can also induce cross-tolerance to the narcotic antagonists as well (group J), although the chronic administration of these agents alone failed to induce tolerance (group I).

Specificity of the cross-tolerance exhibited by morphine-tolerant mice

To determine whether the cross-tolerance induced by morphine showed any specificity, the effects of the non-narcotic analgesic, acetylsalicylic acid, and the cholinesterase inhibitor, physostigmine sulphate, were studied in morphine-tolerant mice. Both of these agents have been reported to be active in inhibiting the PBQ-induced stretching response in mice (Siegmund, Cadmus & Li, 1957 ; Hendershot & Forsaith, 1959). Dose-response curves were determined for these two drugs in order that an estimate of their ED100 doses might be obtained. These doses of acetylsalicylic acid and physostigmine were unexpectedly without effect in morphine-tolerant mice (Table 3). In other words there was complete cross-tolerance to these two miscellaneous agents which are quite different from morphine both chemically and pharmacologically.

TABLE 3. *Cross-tolerance to acetylsalicylic acid and to physostigmine sulphate in morphine-tolerant mice*

Drug*		Total number of stretches/ten mice Mean±S.E. (*N*)†	
Conditioning	Test		
None	Water (control)	91±11	(49)
None	Morphine	0±1	(7)
	Acetylsalicylic acid	2±1	(4)
	Physostigmine	5±2	(4)
Morphine	Morphine	91±4	(4)‡
	Acetylsalicylic acid	84	(1)‡
	Physostigmine	80	(1)‡

* Tolerance to morphine was induced according to the chronic injection schedule shown in Table 1. An ED100 dose of each drug was tested 10 to 14 h after the last injection of morphine. The ED100 dose of acetylsalicylic acid was 60 mg/kg; that of physostigmine sulphate was 0·15 mg/kg.
† (*N*)=number of groups of ten mice observed.
‡ Value not significantly different from control ($P>0·2$).

Since cross-tolerance to the effects of acetylsalicylic acid and physostigmine was found in morphine-tolerant mice, it was of interest to determine whether tolerance itself developed to the effects of these two agents upon chronic administration. Injections were performed similarly to the schedule presented in Table 1. Because of toxicity the injection dose could not be doubled each day. For acetylsalicylic acid the ED100 dose (60 mg/kg) was given three times on the first day, 120 mg/kg three times on the second day, 240 mg/kg three times on the third and fourth days. For physostigmine the initial injection dose (0·15 mg/kg) was increased to 0·3 mg/kg on the second day and subsequent days. The results for the test of tolerance are shown in Table 4. The mice which had been chronically treated with either acetylsalicylic acid or physostigmine and then acutely tested with water had a severely

TABLE 4. *Effect of acetylsalicylic acid and physostigmine sulphate as conditioning drugs*

Drug		Total number of stretching responses/ten mice		
		Control	Time after last injection	
Conditioning	Test	Mean±S.D.(*N*)†	10 to 14 h	34 to 38 h
None	Water	91±11 (49)		
	Acetylsalicylic acid	2±1 (4)		
	Physostigmine	5±2 (4)		
Acetylsalicylic acid	Water		1	8
	Acetylsalicylic acid		0	0
Physostigmine	Water		21	11
	Physostigmine		2	3

* The schedule of conditioning was based on an ED100 dose and followed the same time course as shown in Table 1 for the analgesics. Due to toxicity the maximum injection dose was 240 mg/kg with acetylsalicylic acid and 0·30 mg/kg for physostigmine sulphate. An ED100 dose of each drug was tested in the PBQ analgesic assay at various times after the last dose of the conditioning treatment. The ED100 dose of acetylsalicylic acid was 60 mg/kg, while that of physostigmine sulphate was 0·15 mg/kg.
† (*N*)=number of groups of ten mice observed.

TABLE 5. *Antagonist effect of naloxone hydrochloride and atropine sulphate on the activity of some drugs in the PBQ analgesic assay*

Treatment	Total number of stretches/ten mice Mean±S.E. (*N*)†
Water (control)	91±2 (49)
Atropine	75±4 (4)‡
Naloxone	92±3 (10)
Morphine	0±1 (7)
Morphine+atropine	0 (3)
Morphine+naloxone	57±5 (3)¶
Pentazocine	0 (5)
Pentazocine+atropine	0 (3)
Pentazocine+naloxone	38±4 (3)¶
Acetylsalicylic acid	2±1 (4)
Acetylsalicylic acid+atropine	6±2 (4)
Acetylsalicylic acid+naloxone	2±1 (4)
Physostigmine	5±1 (4)
Physostigmine+atropine	62±6 (4)§¶
Physostigmine+naloxone	5±2 (4)

* These drugs were tested at ED100 doses. The antagonists were given at the following doses: atropine sulphate: 10 mg/kg; naloxone hydrochloride: 0·4 mg/kg.
† (*N*)=number of groups of ten mice observed.
‡ Value is significantly lower than control ($P<0·05$).
§ This value does not significantly differ from that obtained with atropine alone ($P>0·1$).
¶Values represent significant antagonism ($P<0·05$).

decreased ability to exhibit the PBQ-induced stretches. In an attempt to reduce the possibility of residual drug being present at the time of the test, another group of chronically treated mice was tested 34 to 38 h after the last injection. There was still an impaired response to PBQ, so that tolerance could not be assessed.

Antagonism of the activity of some drugs in the PBQ assay

The ability of naloxone to antagonize the effects of both narcotic and narcotic-antagonist analgesics in the PBQ assay has been reported (Blumberg, Dayton, George & Rapaport, 1961 ; Blumberg, Dayton & Wolf, 1966) and has been used in previous experiments to determine apparent pA_2 values (Takemori, Kupferberg & Miller, 1969 ; Smits & Takemori, 1970). However, the effect of naloxone on the activity of non-narcotic agents capable of inhibiting stretching in this assay has not been studied. In addition to naloxone, the acetylcholine antagonist, atropine sulphate, was included in this experiment to determine if the specificity of these antagonists might be useful in classifying the receptors involved in this assay. The effects of these two agents on the activity of morphine, pentazocine, acetylsalicylic acid and physostigmine are shown in Table 5. Atropine was given at a dose of 10 mg/kg, which caused a slight inhibition of stretching. Atropine has been reported to be active in this assay at very high doses (Chernov, Wilson, Fowler & Plummer, 1967). This dose of atropine produced a reversal of the effects of the test dose of physostigmine. However, it had no effect on the activity of morphine, pentazocine or acetylsalicylic acid. Naloxone at a dose of 0·4 mg/kg had no effect on the activity of either acetylsalicylic acid or physostigmine but did antagonize the effects of morphine and pentazocine by expected amounts (Smits & Takemori, 1970).

Discussion

Since tolerance develops to the depressant effects of narcotics (Seevers & Deneau, 1963), the present results showing that tolerance developed to the activity of morphine, methadone and levorphanol in the PBQ analgesic assay are not surprising. On a similar injection schedule, however, very little if any tolerance developed to the effects of the narcotic antagonists, pentazocine, nalorphine and cyclazocine. This indicates a fundamental difference between the agonistic actions of these two groups of analgesics. Previous experiments (Smits & Takemori, 1970) utilizing apparent pA_2 value determinations suggested that these two groups of drugs inhibit stretching by interacting with either two different receptors or two different sites on the same receptor. Since the mice in the present study were given equivalent doses the results suggest not only a basic difference in the mechanisms of action but also in the consequences of their interactions with the receptors. The narcotic antagonists apparently do not effectively initiate with the sequence of events which results in the formation of tolerance. However, the cross-tolerance to the effects of both the narcotic and the narcotic-antagonist analgesics exhibited by morphine-tolerant mice suggests that their mechanisms of action are not completely independent. The unexpected finding that there was also cross-tolerance to the effects of acetylsalicylic acid and physostigmine shows that this cross-tolerance lacks specificity.

At the completion of these tolerance and cross-tolerance studies it was learned that Taber *et al.* (1969) also found cross-tolerance to nalorphine in morphine-tolerant mice using acetic acid-induced stretching responses for the analgesic assay. They considered this to be evidence that these two agents act at the same receptors with

morphine and nalorphine acting as full and partial agonists, respectively. As further evidence for this proposal they mentioned similar structures of the two drugs, different slopes of the dose-response curves with a ceiling effect for nalorphine, antagonism of both drugs by naloxone and previous reports that nalorphine acted as partial agonists on guinea-pig ileum (Gyang & Kosterlitz, 1966) and on respiration (Bellville & Fleischli, 1968). The lack of tolerance with the narcotic antagonists could then conceivably be explained by the thesis that, as partial agonists, the antagonist analgesics are unable to exert a sufficient cellular effect to result in tolerance.

Our data and those of others do not completely support the concept that nalorphine acts as a partial agonist. First, the present study showed that the cross-tolerance observed between nalorphine and morphine in the stretching assay is not specific and cannot be regarded as strong evidence that the agents act at similar receptors as agonists. Second, although the slopes of the log dose-response curves for narcotic and narcotic-antagonist analgesics have been reported to be different (Taber, Greenhouse & Irwin, 1964 ; Chernov *et al.*, 1967), our findings (Smits & Takemori, 1970 ; Hayashi & Takemori, unpublished observations) show that the slope of the log dose-response curve for morphine does not differ significantly from those for pentazocine, cyclazocine or nalorphine. Blane (1967) also found similar slopes for the log dose-response curves of morphine and nalorphine. Pearl & Harris (1966), on the other hand, reported that the slope of the log dose-response curve for morphine differs from that of nalorphine but not from those of other antagonist analgesics such as pentazocine, cyclazocine and cyclorphan. Third, a maximal analgesic effect of less than 100% is not observed with any of the narcotic antagonist analgesics in this study (Table 2). Blumberg *et al.* (1966) also used doses of several narcotic-antagonist analgesics which produced near 100% (87 to 93%) analgesia. The above observations are not wholly harmonious with the hypothesis that narcotic-antagonist analgesics act as partial agonists at the same receptor site as that of narcotic analgesics.

Since morphine-tolerant mice exhibited cross-tolerance to the effects of acetylsalicylic acid and physostigmine, the effect of naloxone on the activities of these two miscellaneous agents was studied. Naloxone antagonized the effects of the narcotic and the narcotic-antagonist analgesics but not those of acetylsalicylic acid or physostigmine. The cholinergic antagonist, atropine, had no effect on the activity of morphine, pentazocine or acetylsalicylic acid but completely reversed the effect of physostigmine. Similar results were observed by Ireson (1969), who showed that effects of morphine or nalorphine are antagonized by naloxone but not by atropine and the effect of physostigmine are antagonized by atropine but not by naloxone. These results show that although cross-tolerance to acetylsalicylic acid and physostigmine is observed in morphine-tolerant mice, antagonists can be used to differentiate between the receptors for the two miscellaneous agents and those for the narcotic and narcotic-antagonist analgesics.

The receptor mechanisms and their inter-relationships in the PBQ analgesic assay require further investigation. From apparent pA_2 determinations (Smits & Takemori, 1970) and the ability to induce tolerance, it is probable that there are different receptors for the narcotic agents and the narcotic antagonists. If there is only a single receptor population for both types of analgesic then the binding function of the two types of drug with the receptor must be sufficiently different to result in

different apparent pA_2s. From the selectivity of naloxone and atropine, it appears that the receptors for the narcotic and the narcotic-antagonist analgesics are stereochemically similar but not identical and also are different from those for acetylsalicylic acid and physostigmine. However, the lack of specificity of the cross tolerance indicates that even the latter diverse agents do not act through completely independent mechanisms.

We wish to thank Mrs. S. E. Smits and Dr. Goro Hayashi for their assistance. The analytical and programming assistance of the staff and use of the computing facilities of the Biomedical Data Processing Unit (supported under NIH grant FR 00267) is gratefully acknowledged. We are also grateful for receiving generous supplies of naloxone from Dr. Ralph Jacobsen, pentazocine from Dr. S. Archer and cyclazocine from Dr. J. G. Bird. This investigation was supported by U.S. Public Health Service Grant GM 15477.

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(*Received December* 15, 1969)