J. Pharm. Pharmacol. 1984, 36: 326–330
Received August 3, 1983

The peripheral narcotic antagonist *N*-allyl levallorphan-bromide (CM 32191) selectively prevents morphine antipropulsive action and buprenorphine in-vivo binding in the rat intestine

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The assumed low ability of the the quaternary narcotic antagonist *N*-allyl levallorphan-bromide (CM 32191) to cross the blood-brain barrier and its selectivity in relieving the peripherally-elicited antipropulsive action of morphine while preserving analgesia has been tested. To ascertain the extent of penetration of CM 32191 into the cns, its relative potency in preventing the in-vivo binding of high specific activity [^{3}H]buprenorphine in the rat cns and small intestine was compared. Pretreatment was with CM 32191 at 16, 30 or 60 mg kg^{-1} s.c., 20, 60 or 120 min before buprenorphine, the concentrations of which in cerebrum and spinal cord were comparable with control values, but were consistently reduced in the intestine (longitudinal muscle with attached myenteric plexus). Pretreatment with naloxone (20 min, 0·5 or 1 mg kg^{-1} s.c.) lowered buprenorphine binding in intestine and cns. Neither narcotic antagonist produced significant changes in buprenorphine plasma concentrations. The peripheral selectivity of CM 32191, methyl naloxone and naloxone was examined by investigating in the same rats nociception in the hot plate (central opiate-sensitive mechanism) and the transit of a charcoal meal along the small intestine (local opiate-sensitive mechanism). Both effects were inhibited by morphine (5 mg kg^{-1} i.v.). Naloxone (10 min pretreatment, 0·5 or 1 mg kg^{-1} s.c.) did not selectively antagonize intestinal action of the morphine since the relief of charcoal transit inhibition was consistently associated with complete loss of analgesia. Pretreament (s.c. at 50 min) with the *N*-methyl quaternary analogue of naloxone, at low doses only, selectively prevented the slowing of transit by morphine without significantly impairing its antinociceptive action; higher doses (>16 mg kg^{-1}) suppressed the antinociceptive action. In contrast, CM 32191 (16, 30 or 60 mg kg^{-1}, 10, 50 or 110 min before morphine) always selectively antagonized constipation induced by the opiate and did not influence its action delaying hot-plate reaction.

Quaternary narcotic antagonists cross the blood-brain barrier poorly (Tavani et al 1979) and have therefore been regarded as useful tools for investigating the relative roles of central and peripheral opiate receptors in several conditions (Tavani et al 1979; Manara et al 1980; Ferretti et al 1981; Ramabadran et al 1982; Russel et al 1982; Carr & Simon 1983; Brown et al 1983). However, recent work pinpointing the shortcomings of currently available quaternary narcotic antagonists (Bianchi et al 1982) has prompted the search for compounds with better peripheral selectivity (Bianchetti et al 1982).

The present study with the quaternary narcotic antagonist *N*-allyl levallorphan-bromide (CM 32191) was designed to test its ability to penetrate the cns, as evidenced by inhibition of in-vivo binding therein of the powerful opiate buprenorphine radiolabelled at high specific activity, and to assess the peripheral selectivity of CM 32191 by comparing its ability to prevent local inhibition of gastrointestinal transit and centrally elicited analgesia in morphine-treated rats. Naloxone and its *N*-methyl quaternary analogue (MRZ 2593) were reference compounds.

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METHODS

Treatment of animals

Overnight fasted male CD-COBS rats (Charles River, Italy), 180–220 g, housed in standard conditions (60% relative humidity, 22 °C), were injected with tritium-labelled buprenorphine (5 μg kg^{-1} i.v.) at different times after s.c. injection of drugs or their vehicle. Thirty min after the labelled drug, rats were decapitated; cerebrum (brain without cerebellum), spinal cord, plasma and small intestine longitudinal muscle (with attached myenteric plexus; Rang 1964) were isolated, frozen on dry ice and stored at −25 °C for later assay of their buprenorphine content.

Other rats were tested for hot plate reaction time (55 °C, cut-off time 30 s) and for gastrointestinal transit as described by Bianchi et al (1982). Three training sessions with the hot plate were run the day before the experiment, then a baseline value (pre-drug jumping time) was obtained about 1–2 h before drug treatment (animals whose jumping time exceeded 10 s or was not consistent with that of the last training test were discarded). The hot plate results were scored as an analgesic index: (reaction time with drug–pre-drug reaction time)/(30-pre-drug reaction time), an index of '1·0' representing complete loss of nociception ('A' in Tables 2, 3). Analgesia was also expressed quantally ('B' in Tables 2, 3).

One min after the hot plate test, the animals were given by gavage a charcoal meal (2 ml/rat) consisting of 10% vegetable charcoal plus 10% gum arabic (F.U., Farmitalia-Carlo Erba, Milan, Italy) in water. Five minutes afterwards animals were decapitated and the small intestine was removed, its length measured (from the pyloric sphincter to the ileocecal junction) and the distance travelled by the test meal was recorded as a percentage of the total length (% transit), for validation of this procedure see Tavani et al (1980).

Under the test conditions, inhibition of charcoal transit by morphine is mainly due to impaired small intestinal propulsion and does not depend to any significant extent on direct action on the stomach, thereby delaying gastric emptying (Fiocchi et al 1982).

Drugs

Drugs were administered in 0·9% NaCl aqueous solution, 2 ml kg^{-1} s.c. (narcotic antagonists) or i.v. (labelled buprenorphine and morphine); controls received vehicle only. Doses were calculated for the following salts, gifts of which are gratefully acknowledged: CM 32191 (*N*-allyl levallorphan-bromide) and buprenorphine HCl, Midy S.p.A., Milan, Italy; naloxone HCl, Endo, Garden City, N.Y., USA; MRZ 2593 (*N*-methyl naloxone bromide), Dr H. Merz, Boehringer, Ingelheim, W. Germany. [^{3}H]Buprenorphine free base (tritiated in position 15, 16, specific activity 8 μCi $μg^{-1}$), custom synthesized, was purchased from the Radiochemical Centre, Amersham, U.K. and Morphine HCl from Farmitalia-Carlo Erba, Italy.

Buprenorphine assay

Tritium-labelled buprenorphine was assayed as described by Tavani et al (1979) by thin layer chromatography of all specimens, followed by liquid scintillation radioassay. No correction for recovery (about 90%) was made in the data presented, which were calculated as ng of free buprenorphine, based on the injected specific activity and amended according to the actual radiochemical purity found in our chromatography tests.

In naloxone-pretreated animals, intravenous administration of buprenorphine establishes lower than control whole-tissue levels in selected areas which are targets of well recognized actions of opiates; therefore measurements of buprenorphine in these areas are assumed to reflect in-vivo drug binding to functionally relevant sites (Manara et al 1978; Tavani et al 1979).

Experimental design and statistical analysis

The data presented in Table 1 were obtained in two separate experiments made on two different days but with the same drug solutions. All the experimental conditions were equally represented each day with the same number of replications, i.e. 2 and 3 rats for the 1st and 2nd experiment respectively. All tissues of the same type from the two experiments were processed simultaneously for assay of their [^{3}H]buprenorphine content. In the experiment shown in Table 2, all the animals were treated and tested on the same day. The results in Table 3 were obtained in several experiments performed on different days; one experiment for the 10 min, 2 for the 50 and 2 for the 110 min pretreatment interval.

For statistical evaluation based on analysis of variance and Duncan's test (Duncan 1955), results were processed by a specially programmed (Rocchetti & Recchia 1982) HP 85 minicomputer. Means and s.e. for gastrointestinal transit in Tables 2, 3 are presented as % of control for description purposes only, but all data were processed as % transit values.

RESULTS

Effect of naloxone and CM 32191 on buprenorphine in-vivo binding

The effects of pretreatment with naloxone (0·5 or 1 mg kg^{-1} s.c.) or with CM 32191 (16, 30 or 60 mg kg^{-1} s.c.) on [^{3}H]buprenorphine in rat tissues and plasma after 5 μg kg^{-1} i.v. are shown in Table 1. Naloxone injected 20 min before the labelled drug consistently reduced (from 40 to 69% of controls) the binding of buprenorphine in cerebrum, spinal cord and longitudinal muscle of the small intestine with attached myenteric plexus (MP). CM 32191 given 20, 60 or 120 min before buprenorphine reduced its binding in the MP to the same extent as naloxone but

Table 1. Effect of pretreatment with naloxone or CM 32191 on buprenorphine in rat tissues. Animals were all killed 30 min after buprenorphine (5 μg kg^{-1}) injected i.v. at different times after pretreatment as indicated. Data are expressed as the mean ± s.e.m. (n = 5). MP = small intestine longitudinal muscle with attached myenteric plexus.

Pretreatment		Buprenorphine ng g^{-1} or ml^{-1}			
Time min	Drug mg kg^{-1} s.c.	Cerebrum	Spinal cord	MP	Plasma
20	Saline	3·22 ± 0·23	2·14 ± 0·13	1·21 ± 0·09	0·37 ± 0·02
	Naloxone				
20	0·5	1·55 ± 0·05**	1·36 ± 0·03**	0·83 ± 0·02**	0·43 ± 0·04
	1·0	1·28 ± 0·12**	1·27 ± 0·12**	0·78 ± 0·06**	0·36 ± 0·04
	CM 32191				
20	16	3·46 ± 0·32	2·23 ± 0·09	0·81 ± 0·02**	0·46 ± 0·04
	30	2·89 ± 0·28	2·07 ± 0·20	0·97 ± 0·08*	0·44 ± 0·02
	60	3·02 ± 0·25	2·13 ± 0·17	0·86 ± 0·06*	0·38 ± 0·04
60	16	2·97 ± 0·29	2·24 ± 0·19	0·79 ± 0·15**	0·37 ± 0·03
	30	3·54 ± 0·32	2·11 ± 0·18	0·84 ± 0·08**	0·44 ± 0·04
	60	2·26 ± 0·29*	1·97 ± 0·17	0·75 ± 0·05**	0·35 ± 0·01
120	16	3·00 ± 0·15	2·14 ± 0·20	0·71 ± 0·08**	0·44 ± 0·03
	30	2·88 ± 0·17	2·10 ± 0·13	0·84 ± 0·07**	0·42 ± 0·04
	60	2·87 ± 0·27	2·06 ± 0·07	0·65 ± 0·02**	0·40 ± 0·03

* $P < 0·05$. ** $P < 0·01$ vs saline.

had no effect on buprenorphine binding in the cns (apart from a sporadic, slight effect of borderline significance in cerebrum by 60 mg kg^{-1} CM 32191 given 60 min before). Pretreatment with either narcotic antagonist caused no significant changes in plasma buprenorphine at any time.

Effect of naloxone, MRZ 2593, and CM 32191 on morphine-induced analgesia and gastrointestinal transit inhibition

Naloxone, its *N*-methyl quaternary analogue (MRZ 2593) and CM 32191 were tested in the same rats by investigating nociception in the hot plate test and the transit of a charcoal meal along the small intestine; both phenomena were strongly inhibited by 5 mg kg^{-1} i.v. morphine in the absence of antagonists (Table 2). Pretreatment with naloxone 0·5 or 1 mg kg^{-1} s.c. 10 min before morphine prevented its inhibitory action on gastrointestinal transit and nociception. In rats pretreated s.c. 50 min before with 4, 8 or 16 mg kg^{-1} MRZ 2593, morphine's intestinal effect was reduced without significant loss of analgesia. However, higher doses of MRZ 2593, i.e. 30 and 60 mg kg^{-1}, while relieving constipation induced by morphine, also definitely impaired its antinociceptive action. CM 32191, 16, 30 or 60 mg kg^{-1} s.c., given 50 min before morphine, did not significantly alter the hot plate score, but consistently reduced opiate-induced slowing of the test meal's progression.

Further similar but more extensive tests with CM 32191 included lower doses and different pretreatment intervals with morphine, ranging from 10 to 110 min (Table 3). Four and 8 mg kg^{-1} of CM 32191 did not significantly change morphine's effects on either of the investigated end-points regardless of the pretreatment interval. The doses of CM 32191, when

Table 2. Effect of pretreatment with naloxone, MRZ 2593 or CM 32191 on morphine analgesia and gastrointestinal transit inhibition in rats. Morphine 5 mg kg^{-1} was administered i.v. 10 min after saline or naloxone and 50 min after MRZ 2593 or CM 32191. Rats were tested with the hot plate, fed the test meal and killed respectively 4, 5 and 10 min after morphine. Hot plate score is given as an analgesic index ± s.e.m. (A) and number of rats jumping/number of rats tested (B). Scores for drug-free controls were: A, 0·16 ± 0·15; B, 5/5. G.I.T. = gastrointestinal transit ± s.e.m. as % of transit in drug-free controls, which was 46·2 ± 1·4 (% small intestine traversed ± s.e.m. in 5 min).

Pretreatment		Hot plate score		
Drug	mg kg^{-1} s.c.	A	B	G.I.T.
Saline	—	1 ± 0[2]	0/5	16 ± 4[2]
Naloxone	0·5	0·15 ± 0·05[4]	5/5	80 ± 10[4]
	1	0·13 ± 0·07[4]	5/5	86 ± 10[4]
MRZ 2593	2	1 ± 0[2]	0/5	35 ± 6[2]
	4	0·93 ± 0·07[2]	1/5	44 ± 9[2,3]
	8	0·80 ± 0·20[2]	1/5	54 ± 7[2,4]
	16	0·80 ± 0·20[2]	1/5	71 ± 4[1,4]
	30	0·49 ± 0·21[3]	4/5	68 ± 6[1,4]
	60	0·41 ± 0·19[4]	4/5	68 ± 3[2,4]
CM 32191	16	1 ± 0[2]	0/5	40 ± 4[2,3]
	30	1 ± 0[2]	0/5	52 ± 13[2,4]
	60	0·93 ± 0·07[2]	1/5	100 ± 11[4]

[1] $P < 0·05$. [2] $P < 0·01$ vs drug-free controls. [3] $P < 0·05$. [4] $P < 0·01$ vs saline.

administered 10 min before morphine, had no statistically significant influence on its actions, except for 30 mg kg^{-1} which prevented opiate-induced constipation.

The outcome of pretreatment with 16, 30 and 60 mg kg^{-1} CM 32191, 50 min before morphine, was consistent with the results in Table 2 obtained under the same conditions (i.e. significant antagonism of the intestinal action of morphine without any evident loss of analgesia). Findings were comparable when the interval between pretreatment with 32191 and morphine was prolonged from 50 to 110 min. Additional experiments, whose results are not reported in detail, showed no significant difference from drug-free control hot-plate and gastrointestinal transit scores in rats given CM 32191 alone, 16, 30 and 60 mg kg^{-1} s.c., 20, 60 or 120 min before death. Rats injected with MRZ 2593 alone 16, 30 and 60 mg kg^{-1} s.c., 60 min before death also had nociception and transit of the test meal along the small intestine within the drug-free control range. MRZ 2593 however at 30 and 60 mg kg^{-1} reduced gastrointestinal transit when given 20 min before death (Bianchi et al 1982), whereas naloxone up to 1 mg kg^{-1} had no significant effect on either nociception or gastrointestinal transit (Ferretti et al 1981; Bianchi et al 1982).

DISCUSSION

We have tested the assumed low ability of the quaternary narcotic antagonist *N*-allyl levallorphan-bromide (Bianchetti et al 1982) to cross the blood-brain barrier and its selectivity in preventing the peripherally-elicited antipropulsive action of morphine (Manara et al 1980; Bianchi et al 1982) while preserving analgesia.

To ascertain the penetration of narcotic antagonists into the cns, as in a previous study (Tavani et al 1979), we relied on assays of tritiated buprenorphine in cerebral and other tissues of rats given an intravenous tracer dose of this opiate. Lower than control in-vivo binding of buprenorphine indicated that the narcotic antagonist had reached the assayed tissue in 'active' concentrations. The advantage of this approach over conventional direct drug measurements has already been pointed out (Tavani et al 1979).

In the present study we confirmed that the reference drug naloxone is readily available to the cns as shown by consistent reduction of buprenorphine binding therein (Manara et al 1978; Tavani et al 1979). Conversely, we failed to obtain unequivocal evidence for reduced binding of buprenorphine in cerebrum and spinal cord of rats pretreated with several doses of CM 32191 20, 60 or 120 min before the labelled drug. Thus CM 32191 under the test conditions does not seem to enter the cns to any substantial extent.

Although morphine, besides its analgesic action, can centrally inhibit gut motility by acting exclusively at opiate receptors in brain structures (Manara et al 1980), our buprenorphine assays in the spinal cord are of further interest in view of the direct, specific spinal action of narcotics producing an elevation of the pain threshold (Yaksh & Rudy 1976) but possibly constipation too (Porreca et al 1983). The buprenor-

Table 3. Effect of pretreatment with CM 32191 at different intervals on morphine analgesia and gastrointestinal transit inhibition in rats. Morphine 5 mg kg^{-1} was administered i.v. 10, 50 or 110 min after saline or CM 32191 as indicated. Rats were tested with the hot plate, fed the test meal and killed respectively 4, 5 and 10 min after morphine. Hot plate score is given as analgesic index ± s.e.m. (A) and number of rats jumping/number of rats tested (B). Scores of drug-free controls at 10, 50 and 110 min were respectively: A, 0·23 ± 0·10; 0·29 ± 0·8, 0·23 ± 0·07; B, 5/5, 14/14, 12/12. G.I.T. = gastrointestinal transit ± s.e.m. as % of transit in drug-free controls, which at 10, 50 and 110 min was respectively: 46·5 ± 1·7, 45·5 ± 0·3, 39·7 ± 1·8 (% small intestine traversed ± s.e.m. in 5 min).

	Pretreatment (min)								
	10			50			110		
CM 32191	Hot plate score			Hot plate score			Hot plate score		
mg kg^{-1} s.c.	A	B	G.I.T.	A	B	G.I.T.	A	B	G.I.T.
—	1 ± 0	0/6	21 ± 8[2]	1 ± 0	0/14	22 ± 5[2]	0·92 ± 0·08	1/11	12 ± 2[2]
4	1 ± 0	0/3	7 ± 1[2]	1 ± 0	0/11	21 ± 3[2]	1 ± 0	0/6	27 ± 7[2]
8	1 ± 0	0/4	33 ± 16[2]	0·97 ± 0·04	1/12	25 ± 4[2]	1 ± 0	0/11	26 ± 7[2]
16	1 ± 0	0/4	31 ± 10[2]	1 ± 0	0/12	53 ± 7[2,4]	1 ± 0	0/8	55 ± 13[2,4]
30	1 ± 0	0/4	62 ± 13[1,3]	1 ± 0	0/10	54 ± 5[2,4]	0·90 ± 0·07	2/14	75 ± 8[2,4]
60	1 ± 0	0/4	40 ± 12[2]	1 ± 0	0/14	70 ± 6[2,4]	0·95 ± 0·05	1/14	49 ± 10[2,4]

[1] $P < 0·05$. [2] $P < 0·01$ vs drug-free controls. [3] $P < 0·05$. [4] $P < 0·01$ vs saline.

phine assays included measurements in the small intestine longitudinal muscle with attached myenteric plexus, which constantly showed significantly lower than control labelled-drug-binding with naloxone or CM 32191. These findings, like our previous in-vitro binding studies (Monferini et al 1981a, b), support the presence of functionally relevant opiate receptors in the rat small intestine and are consistent with the results of the tests reported here in which, under comparable conditions, naloxone and CM 32191 prevented the intestinal action of morphine.

The selectivity of CM 32191 and of reference narcotic antagonists in preventing the peripherally-elicited antipropulsive action of morphine was evaluated as in our previously established protocol (Bianchi et al 1982), consisting in dual investigation of delay in hot-plate reaction and intestinal transit in individual animals. In agreement with earlier studies from our laboratory (Ferretti et al 1981; Bianchi et al 1982), the antagonism of morphine's intestinal action by naloxone was not selective, since relief of charcoal transit inhibition constantly involved complete loss of analgesia. The *N*-methyl quaternary analogue of naloxone only selectively prevented the slowing of intestinal transit produced by morphine in the lower dose range, without significantly impairing its analgesic action, which was almost suppressed by higher doses, exceeding 16 mg kg^{-1}. Lower doses of *N*-methyl naloxone may significantly impair the analgesic action of morphine when given at shorter intervals after the antagonist than that (i.e. 50 min) selected for the present study (Bianchi et al 1982).

CM 32191 up to the highest dose tested (60 mg kg^{-1} s.c., corresponding to the maximal well tolerated dose), given at three different intervals from morphine ranging from 10 to 110 min, never significantly shortened the opiate-induced delay in the hot-plate reaction. On the other hand from 16 mg kg^{-1} it was definitely effective in preventing the intestinal action of morphine.

These results with CM 32191 support its peripheral selectivity, as expected from the in-vivo binding experiments with buprenorphine, which attested to the poor passage of CM 32191 through the blood-brain barrier. Our extensive tests with CM 32191 over a range of doses and pretreatment intervals were dictated by the notion that these are critical factors for assessing peripheral selectivity of narcotic antagonists (Bianchi et al 1982). The information provided by these tests should prove valuable to those wishing to use CM 32191 as a pharmacological tool for distinguishing the peripheral and central effects of endogenous or exogenous opioids.

Basically, our results support the conclusion that CM 32191 has better peripheral selectivity (Bianchetti et al 1982) and therefore seems preferable to previously available quaternary narcotic antagonists (Bianchi et al 1982) in spite of its relatively low antagonist potency. The findings further support the view that constipation induced in rats and mice by systemically administered morphine is locally-effected rather than centrally elicited (Manara et al 1980; Tavani et al 1980; Ferretti et al 1981; Bianchetti et al 1982).

Acknowledgements

Partly supported by Italian National Research Council (CNR), Contract No. 8000601.04 and 81.01787.04.

REFERENCES

Bianchetti, A., Giudice, A., Picerno, N., Carminati, P. (1982) Life Sci. 31: 2261–2264

Bianchi, G., Fiocchi, R., Tavani, A., Manara, L. (1982) Ibid. 30: 1875–1883

Brown, D. R., Robertson, M. J., Goldberg, L. I. (1983) Neuropharmacology 22: 317–321

Carr, K. D., Simon, E. J. (1983) Ibid. 22: 127–130

Duncan, D. B. (1955) Biometrics 11: 1–42

Ferretti, P., Bianchi, G., Tavani, A., Manara, L. (1981) Res. Commun. Substance Abuse 2: 1–12

Fiocchi, R., Bianchi, G., Petrillo, P., Tavani, A., Manara, L. (1982) Life Sci. 31: 2221–2223

Manara, L., Bianchi, G., Ferretti, P., Monferini, E., Strada, D., Tavani, A. (1980) in: Way, E. L. (ed.) Endogenous and Exogenous Opiate Agonists and Antagonists. Pergamon Press, New York, pp 143–146

Manara, L., Cerletti, C., Luini, A., Tavani, A. (1978) in: Van Ree, J. M., Terenius, L. (eds) Characteristics and Function of Opioids. Elsevier/North Holland, Amsterdam, pp 225–226

Monferini, E., Strada, D., Manara, L. (1981a) Life Sci. 29: 595–602

Monferini, E., Strada, D., Manara, L. (1981b) Ibid. 29: 603–612

Porreca, F., Filla, A., Burks, T. F. (1983) Eur. J. Pharmacol. 86: 135–136

Ramabadran, K., Suaudeau, C., Jacob, J. J. C. (1982) Can. J. Phyiol. Pharmacol. 60: 715–719

Rang, H. P. (1964) Br. J. Pharmacol. 22: 356–365

Rocchetti, M., Recchia, M. (1982) Comput. Progr. Biomed. 14: 7–20

Russel, J., Bass, P., Goldberg, L. I., Schuster, C. R., Merz, H. (1982) Eur. J. Pharmacol. 78: 255–261

Tavani, A., Bianchi, G., Ferretti, P., Manara, L. (1980) Life Sci. 27: 2211–2217

Tavani, A., Bianchi, G., Manara, L. (1979) Eur. J. Pharmacol. 59: 151–154

Yaksh, T. L., Rudy, T. A. (1976) Science 192: 1357–1358