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ANTAGONISM OF MORPHINE-INDUCED RESPIRATORY DEPRESSION BY NOVEL ANTICHOLINESTERASE AGENTS

ESTHER ELMALEM,[1] M. CHOREV[2] and MARTA WEINSTOCK[1]*

[1]Departments of Pharmacology and [2]Medicinal Chemistry, School of Pharmacy, Hebrew University, Ein Kerem, Jerusalem, Israel

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Summary—This study compared the effects of 3 novel antiAChE agents (derivatives of dimethylaminoethyl-phenyl carbamate) with that of physostigmine on the respiratory depression induced by morphine in rabbits. Each drug, RA_6, (1 mg i.v., 2 mg s.c.) RA_7 (1 or 2 mg i.v.); RA_{15} (0.25 or 0.5 mg i.v.), physostigmine (0.05 or 0.1 mg i.v.) or saline (1 ml), was injected simultaneously with morphine (8 mg i.v.) to groups of 6–10 rabbits. Respiration rate, blood gases and pH were monitored for 3 hr. Plasma ChE was measured before and at 15 min intervals after injection. The 4 antiAChE's were given to 40 other rabbits, which were sacrificed at the time of maximal antagonism of the respiratory depressant effect of morphine, in order to measure the activity of AChE in the medulla, cortex and hippocampus.

Physostigmine (0.1 mg) only antagonized the increase in $paCO_2$ induced by morphine at 15 and 30 min. The drugs RA_{15} (0.5 mg), RA_6 (2.5 mg) and RA_7 (2 mg) almost completely prevented the respiratory depression, without obvious signs of peripheral cholinergic hyperactivity, for at least 3 hr. There was no relationship between the degree of antagonism of the effects of morphine with any drug and that of inhibition of ChE in plasma. In contrast, a highly significant correlation ($P < 0.01$) was found between the former and the amount of inhibition of AChE in the medulla.

It is suggested that the novel carbamates may have potential therapeutic application in reducing the respiratory depression of opiates, without impairing analgesia.

Key words—respiratory depression, cholinesterase inhibition in medulla, carbamates, rabbit.

In previous studies in human subjects and experimental animals it was shown that physostigmine could reduce the respiratory depressant effect of morphine, without interfering with the analgesic effect (Snir-Mor, Weinstock, Bahar and Davidson, 1983; Weinstock, Erez and Roll, 1981a; Weinstock, Davidson, Rosin and Schnieden, 1982). However, as potential therapy for concomitant use in patients receiving opiates, physostigmine has a number of serious disadvantages. The most important of these is its relatively high toxicity, which results in the appearance of distressing side effects at therapeutic doses (Christie, Shering, Ferguson and Glenn, 1981). Its low chemical stability and short duration of action also necessitate frequent administration. In an attempt to overcome these drawbacks, a number of novel anticholinesterase agents were synthesized in this laboratory. These agents readily penetrate the central nervous system, have a greater chemical stability and longer duration of action than that of physostigmine and several of them also have significantly higher therapeutic ratios (Weinstock, Razin, Chorev and Tashma, 1986).

*Address correspondence to Professor Marta Weinstock.

The purpose of this study was twofold; to compare the abilities of three of these novel anticholinesterase agents with that of physostigmine to antagonize the respiratory depressant effect of morphine and to determine whether there is a correlation between the degree of such antagonism and the amount of inhibition of acetyl-cholinesterase (AChE) in the medulla oblongata.

METHODS

Antagonism of the cardiovascular and respiratory depressant effects of morphine by the anticholinesterase compounds

Male and female rabbits, weighing 2.5–3 kg, were prepared with catheters in the central ear artery and marginal ear vein, as previously described (Weinstock *et al.*, 1981a). Rectal temperature was monitored on a telethermometer with the aid of a thermistor probe inserted into the rectum. Respiration rate was counted visually for periods of 30 sec. Blood gases and pH were measured on a blood gas analyzer (Instrumentation Laboratories) after correction for the appropriate body temperature from samples of blood taken from the ear artery. Blood pressure and

Each of the following drugs, physostigmine, (0.05 and 0.1 mg/kg); RA_6 (0.5 and 1 mg/kg); RA_7 (1 and 2 mg/kg) and RA_{15} (0.25 and 0.5 mg/kg), was injected intravenously (i.v.) with morphine (8 mg/kg) to groups of 6–10 rabbits per drug. Nine other rabbits were given morphine alone with 0.1 ml/kg saline. An additional group of 6 rabbits received morphine (8 mg/kg) plus RA_6, (2.5 mg/kg) subcutaneously (s.c.). Blood samples were taken for blood gas analysis, at least twice before administration of drug, 5, 15 and 30 min after injection and thereafter at 30 min intervals, for 3 hr.

Measurement of anticholinesterase activity in different areas of the brain of rabbits

Rabbits were injected intravenously with either physostigmine or each of the above drugs, in the doses designated, or with RA_6, (s.c.). A minimum of 4 animals was used for each treatment group. At stated times after the injection, the animals were sacrificed by air embolism. Eight additional rabbits were injected with saline or morphine (8 mg/kg) and sacrificed at the same times as the rabbits treated with the anticholinesterases, i.e. 4 after 60 min and two each after 15 and 30 min. The brain was removed and the frontal cortex, hippocampus and medulla were rapidly dissected out on ice, weighed individually and homogenized in phosphate buffer (0.1 M) pH 8, containing 1% Triton. The mixture was centrifuged at 1000 *g* and the supernatant, which contained most of the solubilized enzyme, was used for the determination of the activity of AChE by the method of Ellman, Courtney, Andres and Featherstone (1961). The percentage inhibition of AChE by the drugs was computed by comparison with the pooled mean value for each of the appropriate saline-treated controls.

Estimation of plasma cholinesterase

Blood (0.5 ml) was withdrawn into a heparinized syringe, during the control period and at 5, 15, 30, 60, 90, 120, 150 and 180 min after injection of the AChE inhibitors. The blood was centrifuged at 4°C for 5 min at 1000 *g* and the activity of AChE of the plasma was measured by the method of Ellman *et al.* (1961).

Drugs

The agents tested were RA_6 (*N*-ethyl-3[1-(dimethylamino)ethyl]phenyl carbamate) HCl. RA_7 (*N*-ethyl, *N*-methyl-3[1-(dimethylamino)ethyl] phenyl carbamate HCl. RA_{15} (*N*-propyl-3(1-dimethylamino)-ethyl]-phenyl carbamate HCl. Physostigmine salicylate (Sigma Ltd); Morphine HCl (Teva Pharmaceuticals, Israel). All drugs were made up freshly in sterile saline, which included an equal weight of sodium metabisulphite, to prevent oxidation. All doses are

RESULTS

Antagonism of the respiratory depressant effect of morphine by antiAChE

Intravenous injection of morphine (8 mg) caused a significant fall in respiration rate of about 50% and a rise in $paCO_2$ of 54% within 15 min, which lasted for 2–3 hr. The paO_2 was significantly reduced from 114 ± 6 to 85 ± 5 at 15 and 30 min, while the pH fell from 7.45 ± 0.007 to 7.27 ± 0.01 at 15–60 min. Morphine also reduced the heart rate by 70–120 beats per min during the first hour and caused a small (5.2 ± 1.8 mmHg) but significant decrease in blood pressure during this period. Although control $paCO_2$ values were very similar in all the treatment groups, the respiration rates varied from 67 ± 4 to 86 ± 6. These differences were normalized by representing the values after administration of drug as a percentage change from the resting rate for each animal.

Physostigmine (0.05) had no significant influence on the respiratory depression induced by morphine at any time after injection, but caused mild salivation in all the rabbits. At a dose of 0.1 mg, physostigmine reduced the elevation in $paCO_2$ only at 15 and 30 min after injection and the fall in respiration rate at 15 min (Fig. 1). Physostigmine potentiated the bradycardia induced by morphine at 15 and 30 min. These effects were accompanied by signs of peripheral cholinergic hyperactivity, including salivation, defaecation and slight muscular twitches.

The drug RA_{15} (0.25 mg) significantly reduced the elevation in $paCO_2$ and the fall in respiratory rate after morphine, only at 15 min after injection (Fig. 2). At a dose of 0.5 mg, both the change in $paCO_2$ and in respiration rate, induced by morphine, were significantly antagonised for 3 hr (Fig. 2) but the brady-

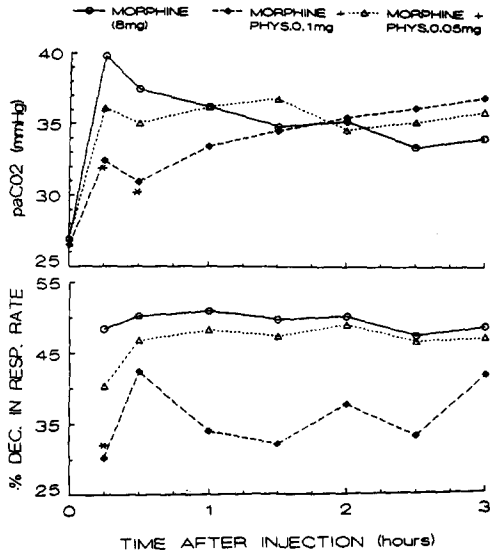

Fig. 1. The influence of physostigmine on the respiratory depressant effect of morphine. Physostigmine was injected

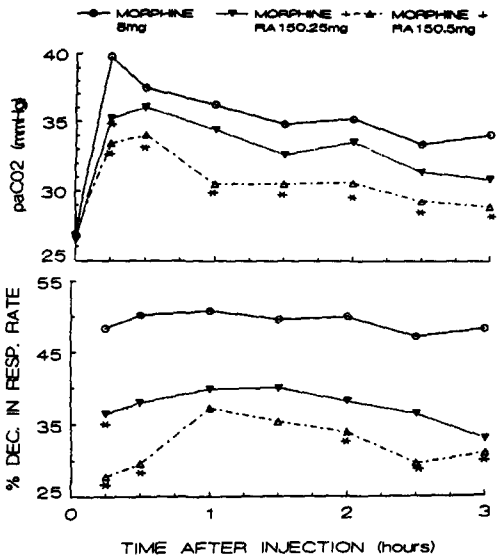

Fig. 2. The influence of RA_{15} on the respiratory depressant effect of morphine. The RA_{15} was injected at the same time as morphine. *Significantly different from morphine alone, $P < 0.05$.

cardia was only increased at 15 min. Peripheral cholinergic activity was present to a lesser degree than with physostigmine. Only a very small antagonistic effect was shown by RA_6 (1 mg) on the respiratory depressant effect of morphine. At a dose of 2 mg (i.v.), it caused marked cholinergic hyperactivity and therefore was not given together with morphine. However, when given subcutaneously, RA_6 (2.5 mg) abolished the effect of morphine on respiration from 1 to 3 hr after injection (Fig. 3). Side effects were only present in a mild degree.

The drug RA_7 had no effect against morphine, at a dose of 1 mg but completely prevented the respiratory depression from 30 min to more than 3 hr when 2 mg were given (Fig. 4). There were no signs of peripheral cholinergic activity at this dose, neither was the bradycardia potentiated. Moreover, the hypotensive effect of morphine was abolished and the reduction in pH markedly attenuated.

Inhibition of plasma cholinesterase by the novel carbamates and physostigmine

The control value for plasma cholinesterase in these rabbits was $24 \pm 2\,\mu M$ acetylcholine (ACh) hydrolysed/ml/hr. Physostigmine caused a maximum inhibition of 40% of this enzyme, 5 min after injection of a dose of 0.1 mg and this declined to 11% by 2 hr (Table 1). In contrast, RA_{15} (0.5 mg) caused more than 70% inhibition of cholinesterase in plasma from 5–90 min and more than 50% after 2 hr. Both RA_6 and RA_7 also caused a maximum inhibition of more than 70% at 15 min, which declined slowly, like that of RA_{15}, over the next 3 hr (Table 1).

The inhibition of AChE by the carbamates in the medulla of rabbits

The antiAChE agents caused between 37 and 63% inhibition in cholinesterase, in the medulla oblongata, depending on the drug, the dose and the time of administration. In order to determine whether there was any correlation between the degree of inhibition of cholinesterase in the brainstem and the extent of

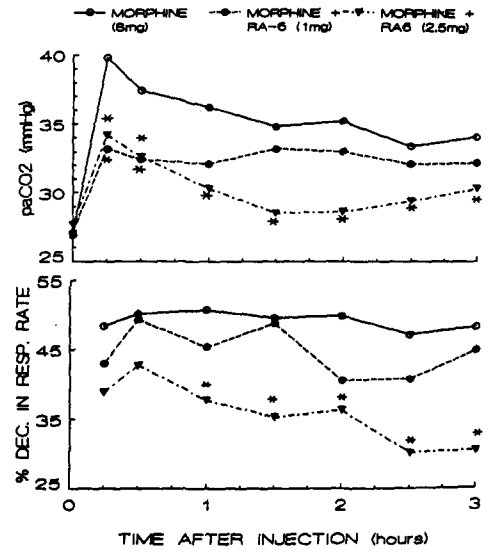

Fig. 3. The influence of RA_6 on the respiratory depressant effect of morphine. The RA_6 (1 mg) was injected intravenously and (2.5 mg) subcutaneously, at the same time as morphine. *Significantly different from morphine alone $P < 0.05$. The values of $paCO_2$ for morphine + RA

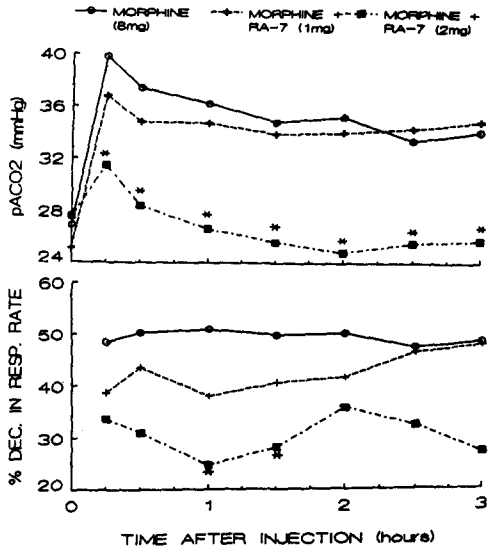

Fig. 4. The influence of RA_7 on the respiratory depressant effect of morphine. The RA_7 was injected intravenously at the same time as morphine. *Significantly different from morphine alone, $P < 0.05$. The values of the $paCO_2$ for morphine + RA_7 (2 mg) was not significantly different from

Table 1. Inhibition of plasma cholinesterase by physostigmine (Physo) and the novel carbamates

Drug	Dose (mg/kg)	Time (min)	Percentage inhibition (±SE)
Physo	0.05	5	32.5 ± 1.6
	0.05	120	7.6 ± 1.8
	0.1	5	43.2 ± 3.6
	0.1	120	11.2 ± 3.5
RA_{15}	0.25	5	56.2 ± 1.2
	0.25	120	31.4 ± 1.1
	0.5	5	78.2 ± 0.9
	0.5	120	51.1 ± 2.2
RA_7	1.0	15	68.0 ± 0.8
	1.0	120	41.7 ± 1.2
	2.0	15	78.1 ± 0.6
	2.0	120	57.5 ± 0.8
RA_6	2.0	180	42.3 ± 1.1
	1.0	15	77.4 ± 0.6
	1.0	120	43.2 ± 1.2
	2.5(s.c.)	30	79.2 ± 1.0
	2.5	120	65.4 ± 1.1
	2.5	180	50.5 ± 0.9

the antagonism of the respiratory depressant effect of morphine, the increase in $paCO_2$ from a mean control value of 26.7–40.1 mmHg after morphine, i.e. 13.4 mmHg, was designated as =100%. Then the effect of each of the antiAChE agents at different times after injection was expressed in relation to the effect of morphine alone; e.g. the combination of morphine and RA_7 (2 mg i.v.) at 30 min resulted in a $paCO_2$ of 28.4 mmHg, i.e. a 16% increase above the control value of 26.7 mmHg.

Since the measurement of enzyme inhibition necessitated the sacrifice of each animal this was only measured at the time of peak antagonism of the effect of morphine and a whole time course was not performed for each drug. The time after administration of drug that the medulla was removed and the activity of AChE was measured is shown in Figure 5. This shows a highly significant correlation ($r = -0.948$; $P < 0.001$) between the percentage reduction in the maximum increase in $paCO_2$ with the antiAChe agents and morphine, and the percentage inhibition of AChE in the medulla of the brainstem. It also shows that this enzyme must be inhibited by at least

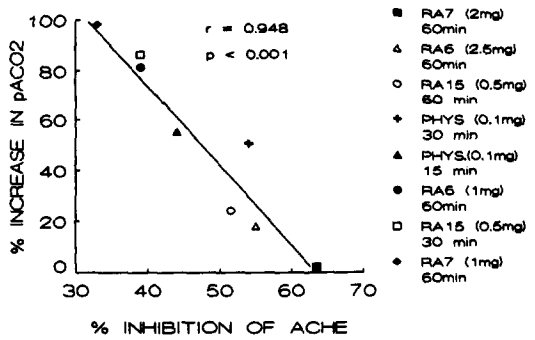

Fig. 5. Relationship between the percentage increase in $paCO_2$, induced by a combination of morphine and antiChEs, to the percentage inhibition of AChE in the medulla,

40% before any significant reduction in the respiratory depressant effect of morphine can be observed and that complete antagonism of the latter was achieved when the enzyme was inhibited by 60%.

The inhibition of AChE by the carbamates in other areas of the brain

The amount of AChE activity in three areas of brain, 30 min after an injection of saline or morphine (8 mg) is shown in Table 2. Morphine did not appear to cause any significant inhibition. The degree of enzyme inhibition by the carbamates in the frontal cortex, hippocampus and medulla of rabbits, sacrificed at the time of peak antagonism of morphine, is shown in Table 3. While no difference was seen in the 3 areas of brain with physostigmine, 15 min after injection, the inhibition became significantly greater in the hippocampus and medulla at 30 min and had almost completely worn off by 90 min. On the other hand, RA_{15} showed a greater effect in the latter areas of the brain than in the cortex at 15 min, which became similar in them all at 30 min.

At 60 min, RA_6 (1 and 2.5 mg) caused similar degrees of inhibition in the cortex and hippocampus but the smaller dose had a much smaller effect in the medulla. The inhibition of enzyme, induced by RA_7 at 60 min, was dose-related and similar in all three regions.

DISCUSSION

Hypercapnia stimulates respiration by increasing the release of ACh from neurones in the medulla (Dev and Loeschcke, 1979; Metz, 1966). Acetylcholine, applied directly to this area (Miller, 1949) and centrally acting cholinomimetics or anticholinesterases, administered parenterally, also stimulate respiration (Weinstock, 1981; Weinstock, Roll and Zilberman, 1981b). Conversely, narcotic analgesics are thought to depress respiration by inhibiting the release of ACh (Domino and Wilson, 1973), thereby reducing the sensitivity of the respiratory centre to CO_2 (Florez, McCarthy and Borison, 1968). Since cholinomimetics and physostigmine have analgesic activity, when given alone to experimental animals and man (Pleuvry and Tobias, 1971; Sitaram, Buchsbaum and Gillin, 1977) and, unlike naloxone, do not impair that of morphine, physostigmine was tested for its ability to reverse narcotic-induced respiratory depression in post-operative patients (Weinstock *et al.*, 1982; Bourke, Rosenberg and Allen, 1984). A significant but short-lived antagonism was observed in some but not in all subjects. Failure to obtain a consistent effect of adequate duration may have been

Table 2. Activity of AChE in areas of the brain in rabbits injected with saline or morphine (μM ACh hydrolysed/g/hr)

Area	Saline (8)	Morphine (2)
Cortex	45 ± 2	40 ± 4

Table 3. Inhibition of AChE by physostigmine and novel carbamates in different areas of the brain

Drug dose (mg/kg)	Time (min)	Percentage inhibition (±SE) Cortex	Hippocampus	Medulla
Physo (0.1)	15	37.0 ± 3.4	44.1 ± 2.1	44.0 ± 1.5
	30	35.3 ± 2.9	55.0 ± 1.9*	54.1 ± 4.5*
	90	17.0 ± 1.8	10.0 ± 5.3	16.5 ± 5.1
RA_{15} (0.5)	15	40.2 ± 0.9	59.9 ± 1.3*	53.2 ± 1.9*
	30	40.0 ± 3.3	38.2 ± 3.8	39.2 ± 3.7
RA_6 (1.0)	60	62.5 ± 0.9	56.8 ± 3.4	38.8 ± 2.4*
(2.5) s.c.	60	56.3 ± 2.6	61.1 ± 3.4	55.4 ± 1.0
RA_7 (1.0)	60	37.9 ± 6.8	48.5 ± 2.6	33.2 ± 4.0
(2.0)	60	66.1 ± 3.9	71.9 ± 4.6	63.5 ± 3.1

due to the short half-life of the drug (Giacobini, Somani, McIlhany, Downen and Hallak, 1987) and its narrow therapeutic window (Christie *et al.*, 1981).

In the present study, physostigmine (0.1 mg/kg), administered together with morphine to rabbits, significantly decreased the rise in $paCO_2$ for 30 min but intensified the reduction in blood pH and bradycardia. The acidosis may have been due to production of lactic acid in muscle in response to stimulation of nicotinic receptors by the increased levels of ACh (Weinstock *et al.*, 1981b). Muscle fasciculations, of a small intensity, were seen at this dose in some of the rabbits and these became pronounced at 0.2 mg/kg. Physostigmine inhibited cholinesterase in plasma by a maximum of 40%, 5 min after injection, which rapidly declined to about 10% within 2 hr. Peak inhibition of AChE (54%) did not occur in the medulla until 30 min.

All three novel carbamates produced a long-lasting antagonism of the respiratory depressant effect of morphine, with only very mild signs of peripheral cholinergic hyperactivity. This was minimized with RA_6, through ensuring a slower absorption, by administering the drug subcutaneously. In contrast to physostigmine, RA_7 completely prevented the respiratory depression from 30 to 180 min and also reduced the acidosis, bradycardia and hypotensive effect of morphine. This suggests that the degree of inhibition of AChE in the heart and skeletal muscle was relatively less with this drug than with physostigmine and that the increase in central cholinergic activity and resultant sympathetic stimulation, overcame the cardiovascular depressant effects of morphine (Weinstock, Zavadil, Chieuh and Kopin, 1979). The data agree with previous findings with RA_7 in the rat, in which it was demonstrated that AChE was inhibited, to a significantly greater extent in the brain, than

Unlike reports with physostigmine in other species (Giacobini *et al.*, 1987; Hallak and Giacobini, 1986), there was no direct relationship between the degree of inhibition of cholinesterase in plasma in the rabbit and the amount of antagonism of the respiratory depression or of the severity of peripheral cholinergic side effects. Both RA_6 and RA_7 (1 mg) showed only minimal antagonism of morphine and no cholinergic symptoms, in spite of more than 60% inhibition of cholinesterase in plasma, which lasted for at least 1 hr. In contrast, physostigmine only reduced the activity of cholinesterase by a maximum of 40%, for a few minutes but produced peripheral cholinergic symptoms and antagonism of morphine.

A comparison of the relative degrees of inhibition by the four drugs in these areas of brain also revealed significant differences between them. While physostigmine and RA_{15} were more active in the hippocampus and medulla, RA_6 (1 mg) caused a greater inhibition in the cortex and hippocampus. This suggested that the drugs may either be distributed to these areas at different rates, or that the enzymes within these regions may show unequal sensitivity to the inhibitors. There is some evidence in favour of the former suggestion from the studies of Hallak and Giacobini (1986) and Giacobini *et al.* (1987), who showed that the concentration of physostigmine in the brain of the rat varied considerably in different areas and this produced differences in the degrees of inhibition of AChE and in the resultant levels of ACh. It was also found that RA_7 showed a greater inhibition *in vitro*, in solubilized preparations of AChE, prepared from the cortex and hippocampus, than from the striatum (Weinstock, Kay, Razin and Enz, 1987). This indicates that there may also be differences in the sensitivity of the enzymes to the antiChE agents in various regions of the brain.

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