Pharmacology Biochemistry & Behavior, Vol 24, pp 733–736, 1986 © Ankho International Inc Printed in the U S A 0091-3057/86 $3 00 + 00

Cholinergic and Opiate Involvement in the Antinociceptive Effect of Diisopropylfluorophosphate

LUCIO G. COSTA AND SHELDON D MURPHY

Department of Environmental Health, SC-34, University of Washington, Seattle, WA 98195

Received 2 April 1985

COSTA, L G AND S D MURPHY *Cholinergic and opiate involvement in the antinociceptive effect of diisopropylfluorophosphate* PHARMACOL BIOCHEM BEHAV **24**(3) 733–736, 1986 —The organophosphate diisopropylfluorophosphate (DFP, 3 or 6 mg/kg, IP) caused a dose-related antinociception in mice which was antagonized by the muscarinic antagonist scopolamine The opiate antagonist naloxone antagonized the antinociceptive effect of the highest dose of DFP, but did not affect the antinociception caused by 3 mg/kg DFP Twenty-four hours after the administration of DFP, reaction time in animals which received a 3 mg/kg dose did not differ from control However, reaction time was still significantly higher than control in mice administered 6 mg/kg DFP twenty-four hours earlier This residual antinociception was antagonized by naloxone but not by scopolamine, suggesting that it was opioid in nature These results suggest that antinociception induced by a low dose of DFP is primarily due to a cholinergic mechanism, while higher doses appear to affect also the opiate system Since we have previously shown that DFP (6 mg/kg) increases met-enkephalin levels in brain, it is possible that high doses of DFP might interfere with enkephalin metabolizing enzymes This conclusion cannot be extended to the organophosphate disulfoton, whose antinociception, even at high doses, appears to involve only an interaction with the cholinergic system

Diisopropylfluorophosphate Disulfoton Organophosphate Antinociception Cholinergic-opiate interactions Mechanisms of analgesia

THE irreversible acetylcholinesterase inhibitor diisopropylfluorophosphate (DFP) has been shown to exert a dose-dependent antinociceptive and hypothermic effect in mice and rats [9, 10, 11, 14] While hypothermia was antagonized only by centrally active muscarinic antagonists, such as atropine and scopolamine, antinociception was also antagonized by the opiate antagonist naloxone [9, 10, 14] DFP was also shown to increase met-enkephalin levels in brain [15] These results suggested that the opiate system, in addition to the cholinergic system, is involved in the antinociceptive effect of DFP In the course of experiments aimed at further characterizing DFP-antinociception, we found that the involvement of the opiate system was dependent upon the dose of DFP administered The result described in the present study suggests that DFP-induced antinociception is due to a cholinergic mechanism at low doses, and to a cholinergic-opiate mechanism when higher doses are administered We also report that another cholinesterase inhibitor, the organophosphate disulfoton, causes antinociception which does not appear to involve any interaction with the opiate system

METHOD

Male Swiss-Webster mice (25–35 g, Tyler Laboratories Inc , Bellevue, WA) were used throughout this study Animals were housed five per cage under standard laboratory conditions and had food and water available ad lib Diisopropylfluorophosphate (Sigma Chemical Co , St Louis, MO) was dissolved in corn oil and injected IP at doses of 3 0 or 6 0 mg/kg, corresponding to 1/3 and 2/3 of its LD_{50} value, respectively, as previously determined (Costa, unpublished result) Disulfoton (0,0-diethyl S-[2-(ethylthio)-ethyl] phosphorodithioate, technical grade, 97%, Mobay Chemical Corp , Kansas City, MO) was injected in corn oil at the dose of 10 mg/kg, corresponding to 2/3 of its IP LD_{50} [5] Control mice were injected with corn oil only (5 ml/kg) Scopolamine hydrochloride (Sigma Chemical Co) and naloxone hydrochloride (Research Biochemicals Inc , Wayland, MA) were dissolved in distilled water and administered by IP injection at doses of 1 0 and 2 0 mg/kg, respectively, 20 min before the antinociception test This short interval was chosen since the effect of naloxone was shown to wear off between 1 and 2 hr following administration [10]

Antinociception was assessed by the tail immersion test, as previously described [6] Briefly, mice were placed in a plastic restraint cage and their tails immersed, to a constant depth, in a water bath maintained constant at 50±0 25°C by a thermostatically controlled heating block The time (in seconds) between immersion and the animal withdrawing its tail was recorded The nociceptive end-point was a violent jerk of the tail An arbitrary 30 sec "cut-off" time was imposed as a maximal antinociceptive response The control response time ranged from 4 to 6 sec

In the hypothermia experiments [6], colonic temperature

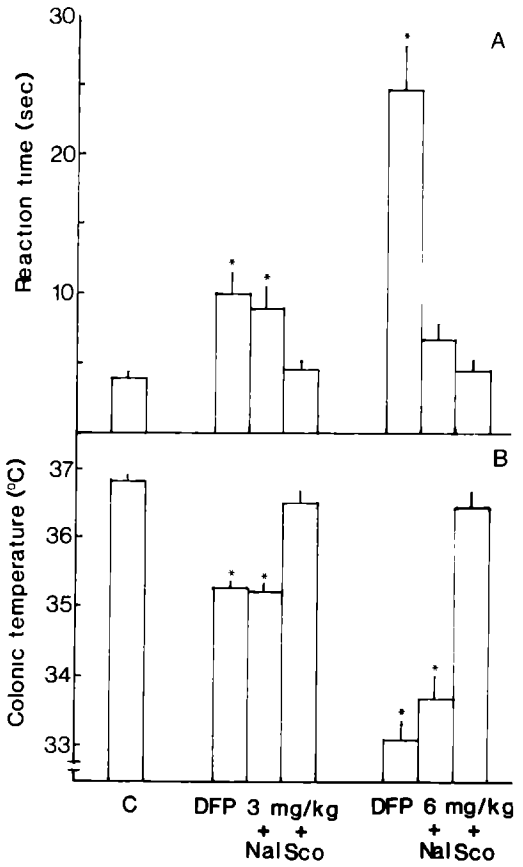

FIG 1 Effect of scopolamine (Sco, 1 0 mg/kg) and naloxone (Nal, 2 0 mg/kg) on the antinociceptive (A) and hypothermic (B) effects of DFP DFP was dissolved in corn oil and administered IP at the doses of 3 0 or 6 0 mg/kg Reaction time and colonic temperature were measured 1 hr after DFP Scopolamine and naloxone were administered 40 min after DFP Each bar represents the mean (±SEM) of six mice *Significantly different from control (C), p<0 01

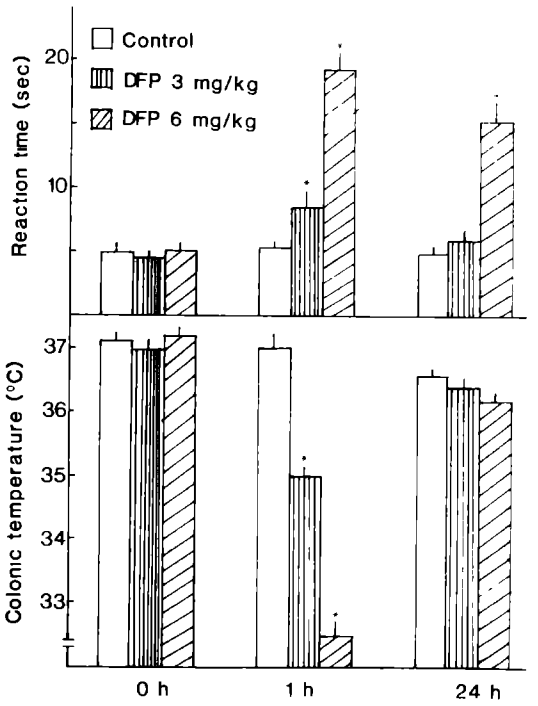

FIG 2 Colonic temperature and reaction time in mice 1 hr or 24 hr after administration of DFP (3 0 or 6 0 mg/kg) Control animals were injected with corn oil Each bar represents the mean (±SEM) of six mice *Significantly different from control, $p<0$ 01

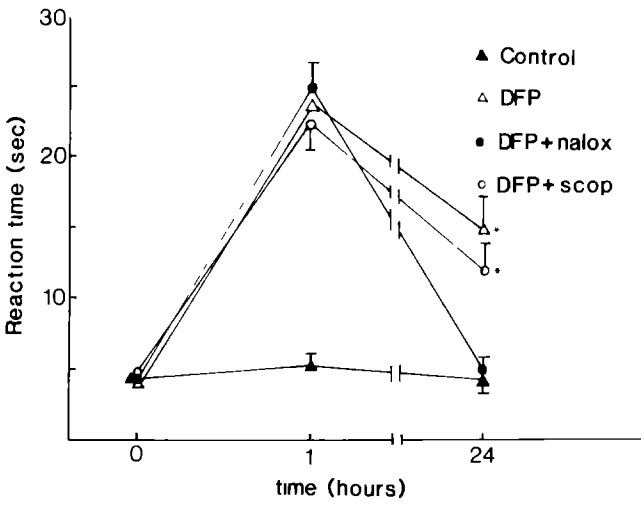

FIG 3 Effect of scopolamine (scop, 1 0 mg/kg) and naloxone (nalox, 2 0 mg/kg) on the antinociceptive effect of DFP (6 0 mg/kg), 24 hr after DFP administration Reaction time was measured 1 hr or 24 hr after DFP Scopolamine or naloxone were injected 20 min before the measurement at 24 hr Each bar represents the mean (±SEM) of six mice *Significantly different from control, $p<0$ 01

was taken as an index of body temperature and was measured by a thermistor mounted in a rectal probe connected to a Telethermometer (Yellow Springs Instrument Company, Yellow Springs, OH) The flexible thermistor probe was inserted 25 mm deep into the rectum The average of two measurements taken during an interval of 30 min before treatment was considered as the initial temperature at 0 time During temperature measurements, mice were kept in a plastic restrainer and the probe was retained in the rectum until a constant temperature reading was obtained

Brain cholinesterase (ChE) activity was measured by the method of Ellman [8], as modified by Benke *et al* [1] Mice were killed by cervical dislocation and the brain rapidly removed on ice, and homogenized in sodium phosphate buffer (0 1 M, pH 8 at 25°C) An aliquot of tissue homogenate (equivalent to approximately 0 8 mg tissue), 50 μl of 0 1 M 5,5′-dithiobis (2-nitrobenzoic acid) and 5 μl of 1 0 M acetylthiocholine (both from Sigma Chemical Co), were added to an appropriate volume of phosphate buffer to make a final volume of 5 ml The absorbance (at 412 nm) was read immediately after the addition of the substrate acetylthiocholine and after 30 min incubation at 27°C The initial absorbance, as well as a reagent blank absorbance was subtracted from the final reading

Results were analyzed for statistical significance by analysis of variance followed by Newman-Keuls test [13]

RESULTS

Administration of DFP (3 0 or 6 0 mg/kg) caused an

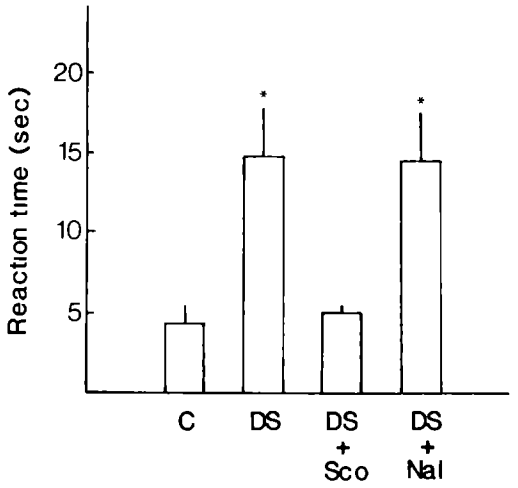

FIG 4 Effect of scopolamine (sco, 1 0 mg/kg) and naloxone (nal, 2 0 mg/kg) on the antinociceptive effect of disulfoton (DS, 10 mg/kg IP, corn oil) Reaction time was measured 4 hr after disulfoton Scopolamine and naloxone were injected 20 min before the 4 hr measurement, i e , 220 min after disulfoton Each bar represents the mean (±SEM) of five mice *Significantly different from control, $p<0$ 01

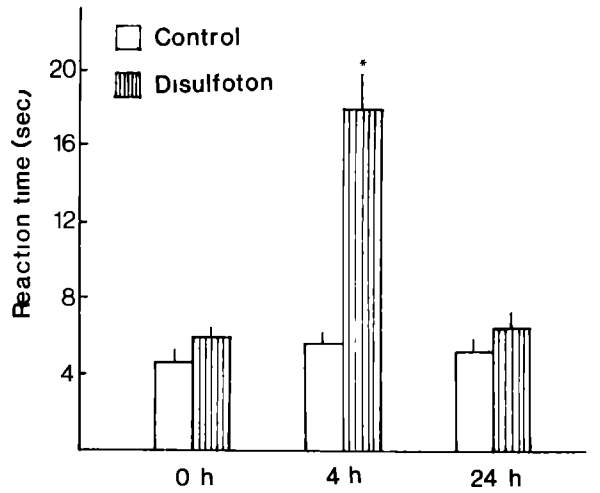

FIG 5 Antinociceptive effect of disulfoton (10 mg/kg IP) 4 hr and 24 hr after administration Control mice were administered corn oil only Each bar represents the mean (±SEM) of six mice *Significantly different from control, $p<0$ 01

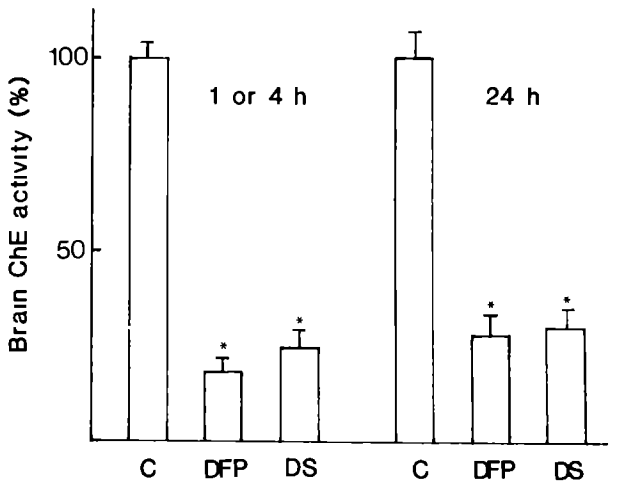

FIG 6 Effect of DFP (6 0 mg/kg) and disulfoton (DS, 10 mg/kg) on brain cholinesterase activity Cholinesterase activity was measured 1 hr and 24 hr after DFP and 4 hr and 24 hr after disulfoton Each bar represents the mean (±SEM) of five–six mice Control cholinesterase activity ranged from 10 9 to 11 5 μmol acetylthiocholine hydrolized per min per g wet tissue *Significantly different from control, $p<0$ 01

hypothermic and antinociceptive effect in mice (Fig 1) Hypothermia was antagonized by scopolamine but not by naloxone Scopolamine also antagonized antinociception induced by both doses of DFP, whereas naloxone was effective only towards the highest dose of the organophosphate (6 0 mg/kg)

Twenty-four hours after the administration of DFP, colonic temperature had returned to control values (Fig 2) Reaction time in mice injected with 3 0 mg/kg DFP was also not different from control On the other hand, reaction time was still significantly higher than controls in mice which were injected with 6 0 mg/kg DFP 24 hr earlier (Fig 2) In order to determine the cholinergic and/or opiate nature of this residual antinociception, groups of mice injected with 6 0 mg/kg DFP, were administered scopolamine or naloxone 20 min before the 24 hr time-point Figure 3 shows that scopolamine caused only a slight reduction in reaction time, while naloxone completely abolished this residual antinociception

In order to determine whether this apparent involvement of the opiate system in antinoception was common to other organophosphates, a series of experiments was performed with disulfoton This compound had been previously shown to cause antinociception in mice [7] Antinociception induced by disulfoton (10 mg/kg) was antagonized by scopolamine but not by naloxone (Fig 4) Furthermore, reaction time had returned to control values 24 hr after disulfoton administration (Fig 5)

Although the dose of disulfoton is comparable to the highest dose of DFP, in that they are both 2/3 of their LD_{50} value ([5], Costa, unpublished results), these two organophosphates might have a different access to the central nervous system To assess this possibility, we measured ChE activity in brain from mice treated with DFP and disulfoton, at the times of maximal antinociception and 24 hr after treatment Results of these experiments, summarized in Fig 6, indicated that the extent of brain ChE inhibition was similar with both organophosphates at all time-points

DISCUSSION

Data obtained in the present study suggest that the antinociceptive effect of DFP is due to a cholinergic-opiate mechanism or to solely a cholinergic mechanism, depending on the dose of DFP On the contrary, the hypothermic effect of DFP appears to be due only to activation of cholinergic muscarinic receptors, independent of the dose Antinociception induced by 3 0 mg/kg DFP was not antagonized by naloxone, suggesting a lack of opiate receptor involvement On the other hand, a higher dose of DFP (6 0

mg/kg) induced a naloxone-sensitive antinociception, as previously reported [14] Twenty-four hours following administration of either dose of DFP, body temperature had returned to control values Similarly, reaction time did not differ from control 24 hr following administration of 3 0 mg/kg DFP This recovery had been previously observed [11,12] and might be due to a short term desensitization of muscarinic receptors [7], since ChE activity remains significantly inhibited (this study, [11,12])

Reaction time was still significantly higher than control 24 hr after administration of 6 0 mg/kg DFP, although it had lowered from that observed at 1 hr (Fig 3) This residual antinociception was antagonized by administering naloxone, but was not affected by scopolamine, suggesting that it was primarily due to an opiate mechanism

Similarly, Clement and Copeman [3] recently observed that high doses of the organophosphates soman or sarin produced a long lasting analgesia in mice, as measured by the hot-plate test Naloxone (10 mg/kg) but not atropine (2 2 mg/kg) reversed this analgesia, 24 hr after administration of the organophosphates [3] We had previously shown that DFP (6 0 mg/kg) increase the content of met-enkephalin in brain 1 hr following administration [15] Although the mechanism of this effect has not been investigated, it might involve inhibition of enkephalin metabolism by DFP For example, acetylcholinesterase has been shown to act as a peptidase and to hydrolize enkephalins *in vitro*, and this peptidase activity is inhibited by high concentrations of DFP [2] In addition, DFP has been shown to inhibit, *in vitro*, the hydrolysis of various neuropeptides involved in antinociception (reviewed by Costa, 1985) Although met-enkephalin levels were not measured in this study, it is possible that these or other opioid peptides, like endorphins [3], are still elevated 24 hr after administration of DFP (6 0 mg/kg) This would explain the specific sensitivity to naloxone of this residual antinociception On the other hand, the lowest dose of DFP (3 mg/kg), which causes a naloxone-insensitive antinociception, would be expected not to alter met-enkephalin levels Further biochemical studies are needed in order to confirm these hypotheses

We had previously shown that a carbamate cholinesterase inhibitor, physostigmine, causes antinociception which is not antagonized by naloxone [14] Furthermore, physostigmine did not alter brain met-enkephalin levels following *in vivo* administration [15], and did not inhibit the peptidase activity of acetylcholinesterase *in vitro* [2] Thus, physostigmine-induced antinociception appears to be due only to cholinergic stimulation To assess whether other organophosphates act similarly to DFP, we investigated the antinociceptive effect of disulfoton The antinociceptive profile of this compound was similar to that of physostigmine and the lower dose of DFP, suggesting that its antinociceptive effect is solely due to a cholinergic mechanism

Since DFP interacts with the opiate system only at high doses, it is also possible that the dose of disulfoton was not sufficiently high for such interaction to occur This possibility, however, seems unlikely The doses of DFP and disulfoton used (6 0 and 10 mg/kg) correspond to 2/3 of their LD_{50} ([5], Costa, unpublished) Furthermore, these two organophosphates caused a similar inhibition of brain ChE at the time of maximal antinociception (1 or 4 hr after administration of DFP and disulfoton, respectively) and at 24 hr (Fig 6)

Thus, DFP antinociception differs from that induced by the organophosphate disulfoton or the carbamate physostigmine but is similar to that induced by Soman and Sarin [3] It is possible that only such potent and irreversible cholinesterase inhibitors are able to phosphorylate the peptidasic site of acetylcholinesterase and/or enzymes, thus altering the metabolism of neuropeptides

ACKNOWLEDGEMENTS

This study was supported in part by research grant ES-03424 from the National Institute of Environmental Health Sciences We thank Mrs Ruth Larsen and Ms Azure M Skye for secretarial assistance

REFERENCES

1 Benke, G M , K L Cheever, R E Mirer and S D Murphy Comparative toxicity, anticholinesterase action and metabolism of methylparathion and parathion in sunfish and mice *Toxicol Appl Pharmacol* **28**. 97–109, 1974

2 Chubb, I W , E Raimieri, G H White and A J Hodgson The enkephalins are amongst the peptides hydrolized by purified acetylcholinesterase *Neuroscience* **4**. 1369–1377, 1982

3 Clement, J G and H T Copeman Soman and Sarin induce a long-lasting naloxone reversible analgesia in mice *Life Sci* **34** 1415–1422, 1984

4 Costa, L G Organophosphorus compounds In *Toxicology of the Nervous System*, edited by C L Galli, L Manzo and P S Spencer New York Plenum Press, 1985, in press

5 Costa, L G , B W Schwab, H Hand and S D Murphy Reduced [^{3}H]-quinuclidinyl benzilate binding to muscarinic receptors in disulfoton-tolerant mice *Toxicol Appl Pharmacol* **60** 441–450, 1981

6 Costa, L G , S V Doctor and S E Murphy Antinociceptive and hypothermic effect of trimethyltin *Life Sci* **31**. 1093–1102 1982

7 Costa, L G , B W Schwab and S D Murphy Differential alterations of cholinergic muscarinic receptors during chronic and acute tolerance to organophosphorus insecticides *Biochem Pharmacol* **31** 3407–3413, 1982

8 Ellman, G R , K D Courtney, V Andres and R M Featherstone A new and rapid colorimetric determination of acetylcholinesterase activity *Biochem Pharmacol* **7** 88–95, 1961

9 Koehn, G L and A G Karczmar Effect of diisopropylphosphofluoridate analgesia and motor behavior in the rat *Prog Neuropsychopharmacol* **2** 169–177, 1978

10 Koehn, G L , G Henderson and A G Karczmar Diisopropylphosphofluoridate-induced antinociception possible role of endogenous opioids *Eur J Pharmacol* **61** 167–172 1980

11 Overstreet, D H , G D Schiller and T A Day Failure of cycloheximide to alter rate of recovery of temperature following acute DFP treatment *Eur J Pharmacol* **44**. 187–190, 1977

12 Overstreet, D H , S C Helps, A M Prescott and G D Schiller Development and disappearance of subsensitivity to pilocarpine following a single administration of the irreversible anticholinesterase agent, DFP *Psychopharmacology (Berlin)* **52** 263–269, 1977

13 Snedecor, G W and W G Cochran *Statistical Methods* Ames, IA Iowa University Press, 1974

14 Zorn, S H , L G Costa and S D Murphy Diisopropylfluorophosphate- and physostigmine-induced antinociception in mice *Toxicologist* **3**. 14, 1983

15 Zorn, S H , L G Costa and S D Murphy Interaction between diisopropylfluorophosphate and the opiate system in mice *Toxicologist* **4**· 171, 1984