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Influence of pinacolyl dimethylphosphinate on soman storage in rats

C. J. VAN DONGEN*, AND J. DE LANGE

Medical Biological Laboratory, P.O. Box 45, 2280 AA Rijswijk, The Netherlands

Atropinized rats pretreated with pinacolyl dimethylphosphinate (PDP) or saline were intoxicated with 6 × LD50 soman and, after 30 and 60 min, the absolute amounts of soman in diaphragms and lungs were measured gas chromatographically. The absolute amounts of free soman in both types of tissues were similar and were decreased after 60 min intoxication compared with the 30 min intoxication period. Pretreatment of rats with PDP reduced the amount of soman in the tissues by 63–78%. Incubation of isolated diaphragms and lungs with sodium fluoride (2·5 mM) increased the amount of free soman in these tissues by $2{\cdot}6 \times 10^3$ and 16×10^3%, respectively. An assay to study the binding of tritiated PDP was developed. Although this assay was suitable to study the binding of tritiated quinuclidinyl benzilate to rat brain, no binding between tritiated PDP and diaphragm or brain tissue could be detected. It was concluded that PDP may counteract soman storage by another mechanism than occupation of binding sites.

One of the problems in the therapy of intoxication with the organophosphorus compound soman, is its persistence in the body. Rats intoxicated with a high dose of soman while initially reacting well to therapy with atropine and the oxime HI-6 (1-(4-aminocarbonylpyridino)methoxymethyl-2-hydroxyiminomethylpyridinium dichloride), died several hours later (Wolthuis & Kepner 1978). These results suggest that at least part of the soman persists in the body, probably reversibly bound to sites other than those for cholinesterase, and which could be called 'a depot' (Van Helden et al 1984). Upon release from these sites, soman could re-inhibit the acetylcholinesterase reactivated by the oxime HI-6.

It has been demonstrated, that the non-toxic compound pinacolyl dimethylphosphinate (PDP) can influence the accumulation of soman in rats (Van Helden et al 1984). Pretreatment with PDP before soman, followed by a therapy with an oxime and atropine, prevented death.

The storage of soman in a depot and the effects of PDP on this storage are not fully understood. Clement (1982) has suggested that plasma aliesterase may be the specific binding site for the soman depot, while other investigators have suggested that specific binding sites for the soman depot may exist in lung tissue or in skin (Kadar et al 1985). In our laboratory evidence was found that at least part of the depot of soman is localized in muscle tissue (Van Helden & Wolthuis 1983). It was demonstrated that pretreatment with PDP reduced the amount of intact soman released by diaphragms isolated from rats intoxicated with soman (Van Dongen et al 1986), which resulted in the hypothesis that the soman-simulator PDP may counteract soman storage in diaphragm tissue probably by occupying binding sites for soman.

In the present study the total amount of soman in diaphragms of rats intoxicated with soman was quantified by gas chromatographic analysis and the effect of PDP on this amount was studied. The amount of soman found in diaphragm tissue was compared with the amount found in lung tissue. In addition, we attempted to demonstrate the existence of specific binding sites for PDP in diaphragm tissue by means of a binding assay with tritium-labelled PDP ([^{3}H]PDP).

MATERIALS AND METHODS

Materials

Soman (pinacolyl methylphosphonofluoridate), [U-^{2}H]pinacolyl methylphosphonofluoridate, and PDP (pinacolyl dimethylphosphinate) were synthesized at the Prins Maurits Laboratory TNO, Rijswijk, The Netherlands. [^{3}H]Quinuclidinyl benzilate ([^{3}H]QNB) was obtained from New England Nuclear Corp., Boston, Mass. The specific activity was 35·2 Ci mmol^{-1}. Atropine sulphate and sodium hexobarbitone were purchased from Brocades Stheeman, Haarlem, The Netherlands. All other chemicals were commercial products of analytical grade.

* Correspondence.

Synthesis of [methyl-^{3}H]pinacolyl dimethylphosphinate ([^{3}H]PDP)

[methyl-^{3}H]Pinacolyl dimethylphosphinate was prepared by reaction of the potassium salt of pinacolyl methylhydrogenphosphite, obtained from the phosphite and potassium hydride in toluene, with ^{3}H-labelled iodomethane (4·2 mg; 0·03 mmol; 925 MBq; Amersham). The yield of [^{3}H]PDP was 4 mg (chemical yield 75%; radiochemical yield 64%) with a total activity of 592 MBq and a specific activity of 26·3 TBq mol^{-1} (712 Ci mol^{-1}).

Absolute amounts of soman in diaphragms and lungs

Male Wistar (WAG/Rij) rats, 180–200 g, bred under SPF conditions in our laboratory, were used. They were anaesthetized with sodium hexobarbitone (175 mg kg^{-1} i.p.) and received atropine sulphate (72 μmol kg^{-1} i.p.) 5 min before the injection of 6 × LD50 soman (2·7 μmol kg^{-1} i.v.). Rats received PDP (36 μmol kg^{-1} i.v.) or saline 10 min before intoxication. The animals were kept alive by artificial ventilation.

Thirty or 60 min after intoxication the rats were killed and diaphragm and lung tissue isolated. The homogenization and extraction procedure was performed according to the method of Benschop et al (1985) used for the analysis of soman in blood, with some minor modifications. Diaphragm and lung tissue were homogenized with a glass–glass Potter Elvehjem homogenizer in 0·2 M acetate buffer, pH 3·85 (1 : 4 w/v) containing 1·25 mM aluminium sulphate and [U-^{2}H]pinacolyl methylphosphonofluoridate as internal standard. In some experiments 2·5 mM sodium fluoride was present instead of aluminium sulphate. The homogenate was sonicated three times during 5 s. After 30 min incubation at 0–4 °C the homogenates were pressed through a Seppak-C18 cartridge. To prevent clogging by the samples, a piece of cotton wool was placed at the top of the cartridge. The analytes were eluted with ethyl acetate (6 mL), after the cartridge had been washed with 0·8 M aqueous sodium hydrogen carbonate (6 mL) to neutralize acetic acid from the buffer. The eluate was cooled to −20 °C until the water was frozen. The organic layer was decanted and concentrated to approximately 200 μL.

The analysis of soman in the concentrate was performed by using a Carlo Erba gas chromatograph equipped with a capillary column coated with CP Sil 8 CB.

Binding assay

The binding of [^{3}H]QNB and [^{3}H]PDP was studied in brain and diaphragm. Rats were decapitated and the tissues were rapidly removed. Tissues from three rats were pooled. The preparation of tissues was performed at 0–4 °C.

Diaphragms were homogenized with a glass–glass Potter-Elvehjem homogenizer in Krebs-Ringer buffer (1 : 10 w/v; NaCl 118, KCl 4·7, KH_2PO_4 1·19, $MgSO_4$ 1·19, $NaHCO_3$ 24, $CaCl_2$ 3 and glucose 10 mM) containing 20 mM HEPES pH 7·4 (Krebs-Ringer-HEPES buffer, KR-HB). The homogenate was passed through polyester monofilament mesh (Monodur, 100 μm).

Brain tissue was homogenized in 0·32 M sucrose (1 : 10 w/v) and centrifuged for 10 min at 1000*g*. The supernatant and the diaphragm homogenate were centrifuged for 30 min at 48 000*g*. The pellets were resuspended in 50 mM sodium phosphate buffer, pH 7·4 (approximately 6 mL, brain tissue) or in KR-HB (approximately 4 mL, diaphragm tissue). The suspensions were stored at −20 °C.

Aliquots of the suspensions 0–2 mg protein as determined by the method of Lowry et al (1951), were incubated with 0·6 and 6 nM of [^{3}H]QNB or [^{3}H]PDP in 50 mM sodium phosphate buffer, pH 7·4 (brain tissue) or in KR-HB (diaphragm tissue). The final volume was 1 mL. For determination of the maximal binding capacity (B_{max}), the dissociation constant (K_d) and the specific binding of [^{3}H]QNB to the muscarinic receptor of the brain, 0·15–6 nM [^{3}H]QNB was incubated with 0·2 mg of the suspension prepared from brain in the presence and absence of 10 μM atropine (Bartholomew et al 1985). After an incubation period of 60 min with gentle shaking at room temperature, the reaction mixture was filtered under reduced pressure through Whatman GF/C glass fibre filters. The filters were washed three times with 3 mL aliquots of cold buffer, placed in vials containing Picofluor as scintillation fluid and were counted for radioactivity after 12 h.

In some experiments, after the incubation period the binding of [^{3}H]PDP to proteins was determined after ultrafiltration. The ultrafiltration procedure was performed using the MPS1 micropartition system (Amicon, USA) with YMT membranes (Zhirkov & Piotrovskii 1984).

RESULTS

In Table 1 the results are shown of the concentrations of soman in diaphragms and lungs isolated from rats 30 and 60 min after injection of 6 × LD50 soman. Thirty min after intoxication about five times more free soman could be detected in diaphragm than

Table 1. The effects of pretreatment with PDP (36 μmol kg^{-1} i.v.) on the absolute amounts of free soman in diaphragm and lung tissues isolated from rats intoxicated with soman (2·7 μmol kg^{-1} i.v.).*

Tissue	Time of intoxication (min)	PDP	Soman (pg mg^{-1} tissues)
Diaphragm	30	−	4·7 ± 0·5 (8)
	30	+	1·7 ± 0·2 (4)**
	60	−	0·91 ± 0·11 (7)
	60	+	0·21 ± 0·08 (11)**
Lungs	30	−	6·1 ± 0·9 (7)
	30	+	1·7 ± 0·3 (4)**
	60	−	0·99 ± 0·12 (6)
	60	+	0·21 ± 0·04 (7)**

* All values represent the mean ± s.e.m.; number of experiments is given in parentheses.
** Significantly different from control values from animals not treated with PDP; Student's *t*-test, $P < 0·05$.

Table 2. The effect of treatment with 2·5 mM sodium fluoride for 30 min at pH 3·85 and 0–4 °C on the soman concentration in diaphragm and lung tissues isolated from rats 60 min after intoxication with soman (2·7 μmol kg^{-1} i.v.).*

Tissue	NaF	Soman (pg mg^{-1} tissue)
Diaphragm	−	0·91 ± 0·11 (7)
	+	24 ± 4 (7)
Lungs	−	0·99 ± 0·12 (6)
	+	161 ± 12 (7)

* All values represent the mean ± s.e.m.; number of experiments is given in parentheses.

60 min after intoxication. The amounts of free soman in diaphragm and lung tissue were about the same. Pretreatment with PDP reduced the amount of soman in diaphragm and lungs by 63–78%, and this effect was already significant after 30 min.

After incubation of the isolated tissues with 2·5 mM sodium fluoride large amounts of soman were found as shown in Table 2. In lung tissue about six times more soman could be detected than in diaphragm tissue. To study the existence of specific binding sites for PDP in rat tissue a binding assay was developed and tested with [^{3}H]QNB and rat brain. The percentage of bound [^{3}H]QNB depended on the amount of tissue used (Fig. 1). After incubation of different amounts of [^{3}H]QNB with 0·2 mg brain suspension plus and minus atropine (10 μM), B_{max} and K_d were determined. Their values were 0·84 pmol [^{3}H]QNB mg^{-1} protein and 0·14 nM, respectively. Using this assay the percentages of [^{3}H]PDP bound to various amounts of diaphragm and brain tissue were measured. The amounts of [^{3}H]PDP measured on the filters were very low (0·5–2%) and were not

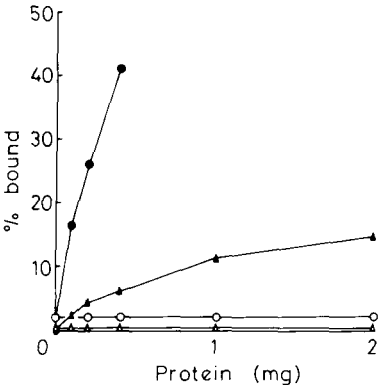

FIG. 1. Binding of 0·6 (●) and 6 (▲) nM [^{3}H]QNB and 0·6 (○) and 6 (△) nM [^{3}H]PDP to tissue. [^{3}H]QNB was incubated with various amounts of brain tissue, while [^{3}H]PDP was incubated with various amounts of either brain or diaphragm tissue. Incubation with both tissues gave similar results. The results are expressed as mean of two independent experiments. Variation between the results of similarly treated preparations was <2%.

affected by the presence of protein (Fig. 1). In addition, the binding of [^{3}H]PDP to whole diaphragm homogenate was studied by incubating homogenate (12·5 mg protein) or buffer with [^{3}H]PDP (5·6, 56, 560 and 5600 nM; final volume 500 μL) and separating bound and free [^{3}H]PDP by ultrafiltration. No difference was found in label in the ultrafiltrates obtained from the incubation mixtures with and without protein.

After in-vivo treatment of rats with [^{3}H]PDP (36 μmol kg^{-1} i.v.) for 30 min and determination of the binding of [^{3}H]PDP to proteins of blood and of diaphragm and lung homogenates using the ultrafiltration method, no binding of [^{3}H]PDP could be detected.

DISCUSSION

In this study we investigated the effect of pretreatment with PDP on the absolute amounts of soman in diaphragms and lung tissues from rats intoxicated with soman. In addition, the existence of specific binding sites for PDP in diaphragm and brain tissue was examined.

The absolute amounts of soman were determined gas chromatographically. The acidification of the homogenization buffer and the addition of aluminium ions to this buffer were necessary to stabilize the soman present in the biological samples. Extraction of internal standard in the presence and absence of tissue homogenate gave similar results. After homogenization and extraction of tissue with addition of a known amount of soman, measurements

have shown that within experimental error no soman was lost. These results proved that the precautions used for the determination of soman in the biological samples were sufficient.

The amounts of free soman in diaphragm and lung tissue were about the same. The amounts of soman found in diaphragm tissue 60 min after intoxication were in agreement with the amounts of soman which were calculated to be present in the diaphragm from the release experiments (Van Dongen et al 1986). Incubation of the tissue homogenates with sodium fluoride leads to an increase in the concentration of soman. This soman may be regenerated from aliesterase as was shown for blood (De Jong & Van Dijk 1984). It has been shown, that lung tissue is enriched in aliesterase activity (Sterri et al 1985). Reynolds et al (1985) have found a higher concentration of pinacolyl methylphosphonyl moieties bound to proteins in lung tissue than in diaphragm tissue after injection of radioactive soman in mice. Kadar et al (1985) who also found a high accumulation of radioactivity in lungs after injecting mice with radioactive soman, suggested that lung tissue may be a site for soman storage. However, this accumulation may be due to the attachment of soman to proteins, from which it cannot be regenerated under physiological conditions as was shown for blood aliesterase (De Jong & Van Dijk 1984).

After pretreatment of rats with PDP before soman intoxication the amount of free soman is reduced in diaphragms and lungs. The effect of PDP can possibly be explained by the assumption that PDP can counteract soman storage by occupying binding sites. However, such sites could not be detected by specific binding of [^{3}H]PDP to diaphragm, brain, blood or lungs. The in-vitro binding assay was checked with the binding of [^{3}H]QNB to the muscarinic receptor of rat brain. The suspension of the pellet obtained after centrifuging tissue homogenate for 30 min at 48 000g was used because after filtration of this fraction all the proteins remained on the filter, while after filtration of whole homogenate only 50% of the proteins remained on the filter. After ultrafiltration of diaphragm homogenate, no proteins could be detected in the ultrafiltrate.

The results obtained in the assays with [^{3}H]QNB and brain tissue (Fig. 1, B_{max}, K_d) were in agreement with the results found by others (Yamamura & Snyder 1974; Schiller 1979; Bartholomew et al 1985). This indicated, that the assay was suitable for studying binding. The difference in amounts of soman found in diaphragms from rats pretreated and non-pretreated with PDP and intoxicated with soman for 60 min (i.e. 0·70 pg soman mg^{-1} tissue = 40 fmol soman mg^{-1} protein), may be equal to the amount of specific bindings sites. If this hypothesis is correct, at least 15% of [^{3}H]PDP would have been bound to the proteins in the binding assay using 0·6 nmol PDP and 2 mg protein (the final volume was 1 mL; Fig. 1). However, since both the binding experiments using the resuspended pellet or the whole homogenate and the experiments performed after in-vivo treatment with [^{3}H]PDP did not show binding, there are probably no binding sites for [^{3}H]PDP.

The results indicate that there are no binding sites for PDP which in turn also suggests that there are no specific binding sites for soman. Interestingly, Benschop has found similar concentrations of soman in blood from rats, with and without pretreatment with PDP, 30 and 60 min after intoxication with 6 × LD50 soman (personal communication). It may be that soman is homogeneously distributed through the whole body of the animal and that there are no specific bindings sites at all. Pharmacokinetic experiments could establish this.

In conclusion, pretreatment with PDP reduced concentrations of free soman in diaphragms and lungs from rats intoxicated with 6 × LD50 soman. Since no specific binding of tritiated PDP could be detected in diaphragm and other tissues, PDP influences soman storage by a mechanism other than occupation of binding sites.

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