Paradoxical effect of salbutamol in a model of acute organophosphates intoxication in guinea pigs: role of substance P release

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ABSTRACT

Organophosphates induce bronchoobstruction in guinea pigs, and salbutamol only transiently reverses this effect, suggesting that it triggers additional obstructive mechanisms. To further explore this phenomenon, in vivo (barometric plethysmography) and in vitro (organ baths, including ACh and substance P concentration measurement by HPLC and immunoassay, respectively; intracellular Ca$^{2+}$ measurement in single myocytes) experiments were performed. In in vivo experiments, parathion caused a progressive bronchoobstruction until a plateau was reached. Administration of salbutamol during this plateau decreased bronchoobstruction up to 22% in the first 5 min, but thereafter airway obstruction rose again as to reach the same intensity as before salbutamol. Aminophylline caused a sustained decrement (71%) of the parathion-induced bronchoobstruction. In in vitro studies, paraoxon produced a sustained contraction of tracheal rings, which was fully blocked by atropine but not by TTX, ω-conotoxin (CTX) or epithelium removal. During the paraoxon-induced contraction, salbutamol caused a temporary relaxation of ~50% followed by a partial re-contraction. This paradoxical re-contraction was avoided by the M$_2$- or NK$_1$-receptor antagonists (methoctramine or AF-DX 116, and L-732,138, respectively), accompanied by a long lasting relaxation. Forskolin caused full relaxation of the paraoxon response. Substance P, and at lesser extent ACh, released from tracheal rings during 60-min incubation with paraoxon or physostigmine, respectively, were significantly increased when salbutamol was administered in the second half of this period. In myocytes, paraoxon did not produce any change in the intracellular Ca$^{2+}$ basal levels. Our results suggested that: 1) organophosphates caused smooth muscle contraction by accumulation of ACh released through a TTX- and CTX-resistant
mechanism, 2) during such contraction, salbutamol relaxation is functionally antagonized by the stimulation of $M_2$ receptors, and 3) after this transient salbutamol-induced relaxation, a paradoxical contraction ensues due to the subsequent release of substance P.

**Key words:** salbutamol, albuterol, $\beta_2$-adrenoceptor agonist, organophosphates, parathion, paraoxon, tachykinins, substance P, physostigmine, airway smooth muscle
INTRODUCTION

Parathion is one of the main representatives of organophosphates, a family of compounds synthesized since the 1940s and still widely used all around the world in agriculture and veterinary medicine as insecticides and antihelmintics (12). In order to achieve biological activity, parathion must be biotransformed in the liver and other tissues into paraoxon. The latter metabolite has a very strong inhibitory capacity on acetylcholinesterase (AChE) activity (27, 36), leading to an increase in ACh effects. The use of organophosphates and carbamates are responsible for over 1,000,000 poisoning victims per year worldwide (17). This figure, however, might well underestimate the problem due to the amount of non-reported cases, mainly in developed countries (37).

In a previous study using an in vivo model of organophosphate poisoning we found that guinea pigs with an ongoing parathion-induced bronchoobstruction responded to salbutamol administration with a transient bronchodilation, which was followed by a returning of the bronchoobstruction (35). Those results allowed us to speculate that salbutamol triggered some obstructive mechanism that rapidly counteracted its relaxing action. In this regard, some studies have demonstrated that salbutamol can facilitate neurotransmitters release, such as ACh release from parasympathetic nerves, through pre-junctional β2-adrenoceptor stimulation (11, 44-46). Because such an effect might well explain the airway obstruction induced by salbutamol, in the present work this hypothesis and some other possible mechanisms explaining the paradoxical effect of salbutamol were explored.
MATERIALS AND METHODS

Animals and experimental design

Male Hartley guinea pigs (500-600 g) bred in conventional conditions in our institutional animal facilities (filtered conditioned air, 21 ± 1°C, 50-70% humidity, sterilized bed) and fed with Harlan® pellets and sterilized water were used. The protocol was approved by the Scientific and Bioethics Committees of the Instituto Nacional de Enfermedades Respiratorias. The experiments were conducted in accordance with the published Guiding Principles in the Care and Use of Animals approved by the American Physiological Society.

Most experiments were performed using either barometric plethysmography for freely-moving guinea pigs or the organ bath technique for tracheal tissue. The sequence of drugs administered in these experiments is shown in Table 1. In addition, intracellular Ca²⁺ concentration was measured in enzymatically-dispersed tracheal myocytes.

In vivo experiments: Barometric plethysmography

Guinea pigs were studied in a whole body, barometric plethysmography chamber for freely moving animals (Buxco Electronics Inc., Troy, NY), and responses were recorded by a specialized software (Biosystem XA v1.5.4, Buxco). The underlying principles of this technique have been already described by others (13, 15, 21). The main variable in this method is the “enhanced pause” (Penh), which has been shown to be correlated with total lung resistance in BALB/c mice (2, 21) and with specific airway...
resistance in guinea pigs (4, 9). The software was adjusted to only include breaths with a tidal volume of 1 ml or more, with a minimal inspiratory time of 0.15 s, a maximal inspiratory time of 3 s and a maximal difference between inspiratory and expiratory volumes of 10%. A constant air flow (10 ml·s⁻¹) was continuously supplied to the plethysmographic chamber without affecting the signal.

After each guinea pig was put inside the plethysmographic chamber, recording was initiated 5 min later and a basal measurement of Penh was done. Approximately 20 min later, parathion (10 mg·Kg⁻¹) or vehicle (propylenglycol:ethanol, 10:1.5 v/v) was administered by the intraperitoneal route, and from this point onward respiratory parameters were recorded at minutes 5, 10, and every 10 min thereafter. The parathion dose was selected based on previous (35) and new experiments, in order to obtain the desired effect on airways without significant neurological effects. Because respiratory parameters were calculated by the computer in each breath, adjustments were made to the software in order to average values from all breaths occurring during 15 s, and then to average those values during the last 5 min of each period. In order to evaluate whether a contraction of the airway smooth muscle was a component of the pulmonary toxicity of parathion, the bronchodilator effect of salbutamol (10 µg·Kg⁻¹, i.p.) or aminophylline (40 mg·Kg⁻¹, i.p.) was assessed during the plateau of the parathion-induced airway obstruction, while control animals received i.p. saline solution. These salbutamol and aminophylline doses corresponded to the therapeutic dosage used in humans, and both of them were applied 90 min after parathion administration.

**In vitro experiments: Organ baths**
Guinea pigs were euthanized by a pentobarbital overdose and four rings (submitted to different experimental conditions) were obtained from the middle of the trachea and studied in a 5 ml organ bath system, as previously described (7). Tissues were stimulated three times with KCl (60 mM), and then paraoxon (10 μM) or physostigmine (10 μM) were added to the organ bath. Contractile responses to these drugs were expressed as percentage of the third KCl response. We corroborated that at these concentrations both paraoxon and physostigmine produced comparable contractile responses (91.9 ± 9.4 vs 89.3 ± 3.3 % of KCl contraction, respectively, p=0.83). In some experiments, tissues were preincubated with 10 μM tetrodotoxin (TTX), 0.32 μM ω-conotoxin GVIA (ω-CTX), or 1 μM atropine during 15 min before addition of paraoxon. In a separate set of tissues, during the plateau of the paraoxon- or physostigmine-induced maximum contraction, salbutamol (0.1 μM) or forskolin (1 μM an adenylate cyclase activator) were added with the aim to evaluate their relaxing effect.

In order to explore the role of β2- and M2-receptors in the effect of salbutamol, the former were blocked with propranolol (10 μM), and the latter with methoctramine (0.31 μM) or AF-DX 116 (0.1 μM), added 15 min before salbutamol. We corroborated that these concentrations of propranolol, methoctramine and AF-DX 116 do not modify the plateau of tracheal contraction induced by paraoxon. Likewise, a possible participation of substance P on the effect of salbutamol was evaluated by using L-732,138 (10 nM), an NK1 receptor antagonist (28), added to the organ baths 15 min before paraoxon. We corroborated that these concentration of L-732,138 does not modify the contractile response to paraoxon. With the aim to assess if the salbutamol effect was the same during a paraoxon-induced contraction than during a typical
cholinergic contractile response, additional tissues were precontracted with 0.32 μM carbachol instead of paraoxon. Finally, to demonstrate a possible functional antagonism between M₂- and β₂-receptors (6, 16), methoctramine was also administered after a lower dose of salbutamol (32 nM) in tracheas precontracted with 0.32 μM carbachol.

In order to evaluate the role of airway epithelium in the paraoxon-induced responses, guinea pig tracheal smooth muscle strips (in which epithelial layer and connective tissue surrounding smooth muscle were removed by dissection under stereoscopic microscopy) were used instead of tracheal rings. These smooth muscle strips were submitted to 10 μM paraoxon stimulation, with or without 10 μM TTX preincubation.

In experiments aimed to obtain samples of bath fluid for substance P measurement, tracheal rings were incubated with paraoxon (10 μM) during 1 h, with or without addition of salbutamol (100 nM) in the second half of this period. Afterwards, the bath liquid was recovered and stored in vials at -70°C until its study. These experiments were performed with 3.2 μM phosphoramidon in order to avoid the enzymatic degradation of substance P by neutral endopeptidases. The same procedure was used to obtain samples for ACh measurement, excepting that physostigmine (10 μM) was used instead of paraoxon (because the organophosphate spoils the analytic column for ACh and choline measurement used in the HPLC system), and three concentrations of salbutamol were separately tested (1, 10, or 100 μM). In addition, these last samples were filtered through 0.2 μm nylon membrane Acrodiscs® (Pall Co. Gelman Sciences, Ann Arbor, MI) before its storage at -70°C.
Acetylcholinesterase activity measurement

The AChE activity was determined in rabbit lung lobe and plasma samples using a colorimetric method based on the Ellman reaction (14), and expressed as percentage of inhibition. Briefly, the lung tissue was homogenized in phosphate buffer (1 ml phosphate buffer per 100 mg tissue) with an homogenizer (Brinkmann Polytron Kinematica PT3100, Westbury, NY). The homogenate was centrifuged during 15 min at 3000 g. The supernatant was filtered (22 µm PTFE filter), and 300 µl of supernatant were added to a cuvette containing 2.5 ml of 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB, 0.32 mM) and 300 µl phosphate buffer (64 mM). The background absorbance per minute was measured at 405 nm at 25°C with a spectrophotometer (Beckman DU-640, Fullerton, CA). Afterwards, 100 µl of AChE substrate (42 mM acetylthiocholine) was added to the cuvette, and the change in absorbance per minute was measured. Once the background absorbance was subtracted, the AChE activity was calculated as International Units (IU) by means of the following equation:

\[
\text{IU} = \frac{A \times 3200}{1.36 \times 10^4 \times 300 \times \text{Co}}
\]

where \( A \) is the change in absorbance/min; \( 1.36 \times 10^4 \) is the extinction coefficient of DTNB; \( \text{Co} \) is the amount of tissue in the supernatant (mg tissue/ml buffer); 3200 and 300 are the total volume (µl) of the cuvette and the volume (µl) of the supernatant, respectively.

Regarding plasma samples, after 1:25 dilution in phosphate buffer, 100 ml were added to a cuvette containing 2.5 ml of DTNB and 500 ml phosphate buffer. The
remaining steps were the same as those described for lung homogenates. The formula was changed (100 ml of sample instead of 300 ml), and IU corresponded to moles of substrate hydrolyzed/min/ml of plasma.

**Acetylcholine measurement by HPLC**

ACh and choline concentrations in the physostigmine-containing samples were measured by cation-exchange HPLC-electrochemical detection method as described by Potter et al. (30). In this technique, an analytic column for ACh and choline (MF-6150, Bionalytical Systems, West Lafayette, IN), an immobilized enzyme reactor (IMER; Bionalytical Systems) and an electrochemical detector (Coulochem II, ESA Inc., Chelmsford, MA) were coupled to the HPLC (model 9012, Varian Co., Walnut Creek, CA). Mobil phase (50 mM Tris/NaClO₄ plus 1% ProClin® reagent, pH 8.5) was pumped at a rate of 1 ml·min⁻¹. Standard curves of ACh and choline (1 nM to 100 nM) were used for calibration. The detection limit of our HPLC system was ~0.1 nM in a 15 µl sample. Data were stored in a microcomputer and analyzed using a data acquisition and analysis software (Star Chromatography Workstation v4.01, Varian Co.). ACh production in the samples was expressed as the sum of ACh plus choline detected by the HPLC system and expressed as nmol·L⁻¹.

**Substance P measurement**

Substance P concentration was measured through a competitive enzyme immunoassay kit (Cayman Chem., Ann Arbor, MI). Samples were read at 405 nm using a Multiskan MS photometer (Labsystems Oy, Helsinki, Finland). Substance P concentration was expressed as pg·ml⁻¹ after comparing with a standard curves made
with the same kit. The working range of the substance P assay was from 3.9 to 500 pg·ml⁻¹.

**Ca²⁺ measurement in guinea pig tracheal myocytes**

Airway smooth muscle from guinea pig trachea was placed in 5 ml Hanks solution containing 2 mg cysteine and 0.05 U·ml⁻¹ papain, and incubated for 10 min at 37°C. The tissue was washed with Leibovitz’s solution to remove the enzyme excess, and then placed in a Krebs solution containing 1 mg·ml⁻¹ collagenase type I and 4 mg·ml⁻¹ dispase II (neutral protease) during ~10 min at 37°C. The tissue was gently dispersed by mechanical agitation until detached cells were observed. Enzymatic activity was stopped by adding Leibovitz’s solution, the cells were centrifuged at 600 rpm during 5 min and the supernatant was discarded. This last step was repeated once again. Afterward, cells were loaded with 0.5 µM fura 2/AM in low Ca²⁺ (0.1 mM) at room temperature (22-25°C). After 1 h, cells were allowed to settle down into a heated perfusion chamber with a glass cover in the bottom. This chamber was mounted on an inverted microscope (Diaphot 200, Nikon, Tokyo, Japan) and the cells adhered to the glass were continuously perfused at a rate of 2-2.5 ml·min⁻¹ with Krebs solution (composition in mM: NaCl 118, KCl 4.6, CaCl₂ 2.0, MgSO₄ 1.2, NaHCO₃ 25, KH₂PO₄ 1.2, glucose 11; 37°C, equilibrated with 5% CO₂ in oxygen, pH 7.4).

Cells loaded with fura 2 were exposed to alternating pulses of 340 and 380 nm excitation light, and emission light was collected at 510 nm using a microphotometer (Photon Technology International, Princeton, NJ). Background fluorescence was automatically subtracted and determined by removing the cell from the field before starting the experiments. The fluorescence acquisition rate was 0.5 s. Intracellular Ca²⁺
concentration ([Ca^{2+}]_i) was calculated according to the formula of Grynkiewicz et al. (20). The Kd of fura 2 was assumed to be 386 nM (24). The mean 340/380 fluorescence ratios for R_{max} and R_{min} were obtained by exposing the cells to 10 mM Ca^{2+} in presence of 10 μM ionomycin and in Ca^{2+}-free Krebs with 1.11 mM EGTA, respectively. R_{max} was 8.99 and R_{min} 0.35. The fluorescence ratio at 380 nm light excitation in Ca^{2+}-free Krebs solution and Ca^{2+} saturated cells (β) was 4.9.

Single myocytes were first stimulated with 10 μM carbachol, perfused with Krebs solution for 15 min, and then challenged with 10 μM paraoxon during 4 min, adding 10 μM carbachol in the last two minutes.

**Drugs**

Parathion (O,O-diethyl O-[4-nitrophenyl] phosphorothioate) and paraoxon (O,O-dietil O-[4-nitrophenyl] phosphate) (Riedel-de Haën, Seelze, Germany.) were dissolved in propylene glycol and ethanol (10:1.5 v/v). Physostigmine hemisulphate, acetylcholine chloride, carbachol, carbamylcholine chloride (carbachol), (±)-propranolol hydrochloride, tetrodotoxin, ω-conotoxin, choline chloride, salbutamol, and forskolin were all purchased from Sigma Chem. Co. (St Louis, MO). Methoctramine tetrahydrochloride was purchased from ICN Pharmaceuticals Inc. (Costa Mesa, CA). AF-DX 116 was purchased from Tocris Bioscience (Bristol, UK). L-732,138 was purchased from Biomol Research Laboratories Inc. (Plymouth Meeting, PA). Forskolin and AF-DX 116 were dissolved in dimethyl-sulphoxide and L-732,138 in ethanol while the other drugs were dissolved in 0.9% NaCl. Parenteral salbutamol sulphate (Ventolin®) was purchased
from Glaxo-Wellcome, Mexico, and aminophylline (Aminofilin®) from Laboratorios Pisa SA, Guadalajara, Mexico.

**Statistical analysis**

Most data were evaluated through one-way or repeated measures ANOVA, followed by Bonferroni multiple comparisons test or Dunnett’s test. Substance P detection was analyzed through Mann-Whitney U test. Statistical significance was set at two-tailed \( p<0.05 \), and assessed using GraphPad InStat v3.05. Data are expressed in the text and illustrations as mean ± SEM, excepting substance P concentration, which was expressed as median and range.

**RESULTS**

**In vivo studies**

We corroborated that the parathion dose used in our *in vivo* experiments (10 mg·kg\(^{-1}\)) caused (90 min later) a 97.8 ± 0.8\% and 67.3 ± 4.9\% inhibition of AChE activity in guinea pig lung homogenates and plasma, respectively (n=5 for each group, Fig. 1A).

Administration of parathion to control male guinea pigs caused a progressive increase of the basal Penh values in up to 250\%, from 0.213 ± 0.010 to 0.566 ± 0.080, n=7, and remained unchanged until the end of the recording period (Fig. 2).

Salbutamol (10 \( \mu \)g·kg\(^{-1}\), i.p.) caused a 31\% decrease (p<0.05) of the ongoing parathion-induced bronchoconstriction 5 min after its intraperitoneal administration, as compared with the averaged Penh values in the previous 10 min period (Fig. 3). After
this transient relaxation, Penh raised again as to reach the same values as before salbutamol administration. A higher salbutamol dose (30 μg·kg\(^{-1}\), i.p.) also produced this paradoxical re-contraction, though somewhat delayed in time (data not shown). By contrast, aminophylline produced a progressive decrement (p<0.05 to p<0.001) of the parathion-induced bronchoconstriction that was maximal at 20 min (77% decrement) and remained relatively unchanged thereafter.

**In vitro studies**

In guinea pig lung homogenates we confirmed that paraoxon and physostigmine caused (3 min later) a concentration-dependent inhibition of AChE activity (Fig. 1B). Specifically, at the concentration used in the present study (10 μM) either paraoxon or physostigmine produced 99.9 ± 0.1% inhibition of AChE activity (n=4 for each group).

Addition of paraoxon to organ baths produced a sustained smooth muscle contraction of guinea pig tracheal rings (Fig. 4A). This response was not modified by previous incubation with ω-CTX or TTX (Fig. 4B), but was fully prevented and reverted by atropine (Fig. 4C and D). In some tissues we corroborated that these ω-CTX and TTX concentrations were enough to abolish or significantly diminish, respectively, the contractile response induced by electrical field stimulation (data not shown). Elimination of epithelium and connective tissue from the airway preparation not only did not interfere with the contractile effect of paraoxon, but it caused an increased response to this organophosphate, as compared with whole tracheal rings (190.3 ± 39.3 vs 102.1 ± 11.4 % of KCl contraction, respectively, p<0.02). This last response was not modified by TTX pre-treatment (188.0 ± 41.7 % of KCl contraction).
Salbutamol (100 nM) administered during the paraoxon-induced contraction produced a temporary relaxation of ~50% (Fig. 5 and 6) 10-20 min after its administration. From this point onwards, tissues began to contract again but without reaching the initial maximal paraoxon-induced contraction. A similar pattern was observed when tissues were pre-contracted with carbachol instead of paraoxon (Fig. 6). We corroborated that the relaxation induced by salbutamol was due to β-adrenoceptor stimulation, since preincubation with propranolol completely abolished this response (Fig. 5).

The M₂ antagonist methoctramine did not modify the ongoing paraoxon-induced contraction (Fig. 4E), but fully prevented the paradoxical contraction occurring after the transient salbutamol-induced relaxation, favouring a sustained relaxing response of ~90% (Fig. 5). In this same context, a lower concentration of salbutamol (32 nM) induced a partial relaxation of the carbachol-induced contraction, and methoctramine (administered during the plateau of such relaxing response) was able to completely relax the tissue (Fig. 4F). Likewise, AF-DX 116 did not affect the paraoxon-induced contraction in tracheal rings, but permitted a complete relaxing effect of salbutamol (140.4 ± 27.1% relaxation, not illustrated). Additionally, we found that tissue incubation with the NK₁ receptor antagonist L-732,138 allowed salbutamol to fully relax the tracheal rings, and almost abolished the paradoxical re-contraction (Fig. 7). By contrast, forskolin completely relaxed the paraoxon response from 10 min ahead (Fig. 6). Some of these phenomena were essentially reproduced when physostigmine was used instead of paraoxon (Fig. 8).
Acetylcholine and substance P measurements

As can be seen in Fig. 9, control levels of ACh released from tracheal rings during a 60-min incubation with physostigmine had a tendency to increase when 1, 10, or 100 nM salbutamol was administered in the second half of this period, reaching statistically significant difference at the intermediate concentration. On the other hand, addition of 100 nM salbutamol significantly increased the concentration of substance P recovered in the organ bath fluid (median 34.9 pg·ml⁻¹, range 32.0 to 79.4), as compared with control tissues without salbutamol (median <3.9 pg·ml⁻¹, range <3.9 to 9.4, p<0.0001, Fig. 10).

Single cell studies

Baseline concentration of [Ca²⁺]ᵢ in airway myocytes was 49 ± 7 nM, n=4. Carbachol addition caused a transient Ca²⁺ peak, followed by a plateau (Fig. 11). Paraoxon did not modify the [Ca²⁺]ᵢ nor the response to a superimposed stimulation with carbachol.

DISCUSSION

In the present study we found that under conditions of AChE inhibition salbutamol induced the release of substance P, and to a lesser extent ACh, with a subsequent contraction large enough to overcome its relaxing effect.

Parathion is an organophosphate pesticide with a well known and potent inhibitory effect on AChE activity (8, 35) mediated through its active metabolite, paraoxon. One toxicological consequence of the resulting ACh accumulation is airway obstruction, which in in vivo conditions has been postulated to be mediated by a
combination of airway smooth muscle contraction, augmented mucous secretion and edema (35). In the present work we found that the main physiopathological mechanism involved in the organophosphate-induced airway obstruction was the smooth muscle contraction, since in in vivo conditions aminophylline rapidly reverted much of this obstruction (71%, Fig. 3), while forskolin caused a rapid and complete relaxation of the paraoxon-induced contraction in vitro (Fig. 6). The cholinergic nature of this response was confirmed by the total abolishment of the contraction by atropine in the in vitro preparation. This response, however, was TTX- and ω-CTX-resistant, suggesting that spontaneous ACh release involves mechanisms other than those classically occurring during neuronal depolarization. Such additional mechanisms have been already postulated by others (1). Because there is good evidence that minute amounts of ACh may also be produced by non-neuronal sources (27, 29), an alternative possibility is that ACh was not coming from nerves but from other airway cell types. In separate experiments we ruled out a major role of airway epithelium as additional source of ACh in the organophosphate-induced contraction, but further experiments are needed to discard other sources such as airway smooth muscle (41).

Contrasting with forskolin and aminophylline, salbutamol was unable to induce a sustained relaxation (in vitro) or bronchodilation (in vivo) and, furthermore, its moderate and transient relaxing effect was rapidly overlapped by an additional contraction. At least in the in vivo experiments, the dose used in our study (10 μg·kg⁻¹ i.p.) was close to the intravenous dose recommended for the acute relief of severe asthmatic bronchospasm in children (5). Additionally, in a previous work (35) we found that aerosolized salbutamol at therapeutic doses used in humans (2 mg/ml, 2 min) also
produced the paradoxical effect. Thus, the paradoxical effect of salbutamol in guinea pigs occurred at doses equivalent to those used in humans.

In addition to their main pharmacological effect on airways (relaxation of smooth muscle), several studies have demonstrated that salbutamol and other β2-adrenoceptors agonists are capable to stimulate vagal nerve ends to induce ACh release (11, 44-46). This might explain the increment of ACh concentration observed in the organ bath fluids after salbutamol administration. Nevertheless, this increment was small enough as to raise doubts about its potential role in the paradoxical re-contraction induced by salbutamol.

Contrasting with the ACh results, salbutamol was able to induce a significant increase of substance P concentration. This last tachykinin may explain the secondary contraction observed during the salbutamol response, which is in agreement with the almost full relaxation occurring during the antagonism of NK1 receptors. It is well known that an extensive network of tachykinins-containing nerve ends (C- fibers) is located within and below the airway epithelium, including the trachea (38, 42). Thus, one possibility to explain the salbutamol-induced tachykinins release is that salbutamol directly stimulates C-fibers. However, up to our knowledge the presence of pre-junctional β2-adrenoceptors in C-fibers has not been described so far, and thus this possibility requires further demonstration. The possible role of C-fibers is in agreement with a recent work by Keir et al. (25). They demonstrated that a 10-days treatment with β2-adrenoceptor agonist (RS- and S-albuterol) induced bronchial hyperresponsiveness (BHR) in sensitized and non-sensitized guinea pigs. The development of BHR was prevented by capsaicin treatment, suggesting a role of sensory nerves (C- fibers) in this phenomenon.
In our *in vitro* studies we found that the transitory character of the relaxation induced by salbutamol was similarly observed during the contraction induced either by paraoxon, phystostigmine (a non-organophosphate compound), or carbachol, suggesting that increased concentrations of ACh in the microenvironment surrounding the smooth muscle is necessary for the paradoxical effect of salbutamol to take place. Cholinergic contraction of smooth muscle involves activation of both M₃ and M₂ receptors. The latter is known to be coupled to Gᵢ protein and hence to produce inhibition of adenylate cyclase (33). Thus, mechanisms induced by M₂ receptors activation are particularly opposed to those produced during β₂-adrenoceptors stimulation. This functional antagonism between M₂ and β₂ receptors has been already reported (6, 16) and might be largely involved in our results, inasmuch as the blockade of M₂ receptors by methoctramine or AF-DX 116 enhanced the salbutamol-induced relaxation and abolished the paradoxical effect. We were able to demonstrate the existence of this functional antagonism by using a slightly lower concentration of salbutamol (32 nM). In these experiments salbutamol caused an approximately 50% relaxation of the carbachol-induced contraction, and the subsequent blockade of M₂ receptors by methoctramine produced a rapid and complete relaxation of the tissues (Fig. 4F). In summary, our results strongly suggest that at least two mechanisms are involved in the paradoxical effect of salbutamol during an acute organophosphate intoxication: 1) the functional antagonism between M₂- and β₂-receptors, and 2) the salbutamol-induced release of substance P.

However, additional mechanisms might also be involved. For example, van den Beukel et al. (39) studied the effect of parathion and paraoxon on Chinese hamster ovary cells transfected with muscarinic M₃ receptors. They concluded that these
organophosphates act as agonists on M₃ receptors (which in airway smooth muscle mediate the ACh contractile effect). In this context, we did not find a direct effect of paraoxon in single airway smooth muscle cells. On the other hand, the group of Fryer (18, 26) recently postulated that organophosphate insecticides induce BHR through a decrease in the neuronal M₂ muscarinic receptor function. This pre-junctional receptor limits the release of ACh from pulmonary vagal nerves (19), and thus its inhibition by organophosphates would favour the release of this neurotransmitter if a subsequent neural stimulation takes place. To what extent these mechanisms are participating in the paradoxical effect of salbutamol remains to be elucidated.

Exposure to organophosphate pesticides may worsen symptoms in asthmatic patients or induce respiratory complaints such as wheezing in non-asthmatic subjects (3, 22, 32, 43). In the case that an organophosphate poisoning is recognized, the appropriate treatment with oximes and atropine is likely to be started. Nevertheless, in many cases organophosphate poisoning is low-grade in nature and the exposure history to organophosphate might not always be evident (22, 37). In this last setting, it is expected that a β₂-adrenoceptor agonist such as salbutamol is administered for the treatment of wheezings. In this context, our current results in these in vivo and in vitro models of organophosphate pesticide intoxication allow us to speculate that effectiveness of such treatment might be less-than-expected or even worsen the bronchial obstruction. This last speculation warrants further investigation. Additionally, clinical studies in asthma have found that chronic treatment with β-adrenoceptor agonists worsen asthma control (34) and induces BHR to a number of bronchoconstrictor stimuli, including methacholine, histamine, exercise and allergen
Therefore, one possible explanation to this adverse effect of salbutamol could be related to its effect on tachykinins release.

According to our results, we concluded that: 1) organophosphates caused smooth muscle contraction by accumulation of ACh released through a TTX- and ω-CTX-resistant mechanism, 2) during such contraction, salbutamol relaxation is functionally antagonized by the stimulation of M2 receptors, and 3) after this transient salbutamol-induced relaxation, a paradoxical contraction ensues due to substance P release. Thus, further studies on the potential adverse effect of salbutamol during organophosphate-induced bronchoobstruction in humans are warranted.

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REFERENCES


40. **van Schayck CP, Graafsma SJ, Visch MB, Dompeling EvWC, van Herwaarden CLA.** Increased bronchial responsiveness after inhaling


FIGURE LEGENDS

Figure 1. Inhibition of acetylcholinesterase (AChE) activity by organophosphates and physostigmine in guinea pigs. Panel A shows the AChE activity in lung homogenates and plasma measured 90 min after intraperitoneal administration of parathion (10 mg·kg\(^{-1}\), n=5), and expressed as percentage inhibition of AChE activity in lung homogenates (1.00 ± 0.06 IU) and plasma (3.13 ± 0.25 IU) from control animals, respectively. Panel B illustrates in vitro non-cumulative concentration-response curves to paraoxon and physostigmine on AChE activity in lung homogenates (n=4 experiments) and expressed as percentage inhibition of its respective control basal AChE activity (1.00 ± 0.08 IU). Symbols and data correspond to average ± SEM.

Figure 2. Effect of parathion in in vivo experiments. Parathion (10 mg·kg\(^{-1}\) i.p.) induced a progressive increase of the Penh value in male guinea pigs and remained relatively unchanged until the end of the recording period. Symbols represent the average ± SEM of n=7 animals.

Figure 3. Effect of bronchodilators on the parathion-induced bronchoobstruction in guinea pigs submitted to barometric plethysmography. The arrow indicates the intraperitoneal administration of salbutamol (●, 10 µg·kg\(^{-1}\)), aminophylline (■, 40 mg·kg\(^{-1}\)) or saline solution (○, control group) during the ongoing bronchoobstruction induced by parathion (10 mg·kg\(^{-1}\)). Basal values were obtained by averaging the Penh values from the last 10 min before bronchodilators administration. Symbols represent the average ± SEM of n=7 animals in each group. *P<0.05, and **P<0.01 compared
Paradoxical salbutamol effect in organophosphates intoxication

with their respective basal value (repeated measures ANOVA with Bonferroni multiple comparisons test).

**Figure 4.** Representative recordings of the effect of paraoxon in *in vitro* experiments. Paraoxon produced a sustained smooth muscle contraction of the guinea pig tracheal rings (A). This response was not modified by previous incubation with TTX (B), but was completely blocked (C) and reverted (D) by atropine. Methoctramine did not modified the contraction induced by paraoxon, but potentiated the relaxation induced by 0.1 μM salbutamol (E), and removed the functional antagonism between carbachol (0.32 μM) and salbutamol (0.032 μM) (F). Pox=paraoxon (10 μM), Atr=atropine (1 μM), KCl=potassium chloride (60 mM) and Met=methoctramine (0.31 μM), TTX=tetrodotoxin (10 μM).

**Figure 5.** Effect of M₂ receptors antagonism on the salbutamol response during the paraoxon-induced contraction in tracheal rings. Paraoxon (○, 10 μM, n=11) induced a contractile response which was partially reversed by salbutamol (●, 0.1 μM, n=11), with a tendency to contract again from 10 min onward. The paradoxical contraction occurring after the transient salbutamol-induced relaxation was fully abolished by the M₂ antagonist methoctramine favouring a relaxing response of ~90% (■, 0.31 μM, n=8). Propranolol (▼, 1 μM, n=6) completely abolished the salbutamol relaxant effect. Symbols represent the average ± SEM. *P<0.05, **P<0.01 compared with the paraoxon group and †P<0.01 compared with the paraoxon-salbutamol group (one-way ANOVA with Bonferroni multiple comparisons test).
Figure 6. Effect of salbutamol and forskolin on the paraoxon-induced contraction in tracheal rings. Paraoxon (O, 10 μM, n=11) induced a contractile response which was partially reversed by salbutamol (●, 0.1 μM, n=11), with a tendency to contract again from 10 min onward. Forskolin (▲, 1 μM, n=7) completely abolished the paraoxon-induced contraction. The sustained contraction induced by carbachol (□ 0.32 μM, n=8) was moderately reversed by salbutamol following a similar pattern as was observed in the paraoxon group (■, 0.1 μM, n=11). Symbols represent the average ± SEM. *P<0.01 compared with their respective control group, and †P<0.01 compared with the paraoxon-salbutamol group (one-way ANOVA with Bonferroni multiple comparisons test).

Figure 7. Effect of an NK₁ antagonist on the salbutamol response during the paraoxon-induced contraction in tracheal rings. The paradoxical contraction occurring after the transient salbutamol-induced relaxation (●, n= 10) was fully abolished by the NK₁ receptor antagonist (L-732,138, O, n= 11) favouring a complete relaxing response. Symbols represent the average ± SEM. *P<0.05, **P<0.01 compared with the paraoxon-salbutamol group (non-paired Student’s t test).

Figure 8. Effect of physostigmine on guinea pig tracheal rings. Physostigmine administration (O, 10 μM, n=9) induced a contractile response. Salbutamol addition (●, 0.1 μM, n=9) induced a temporary relaxation, but forskolin (▲, 1 μM, n=5) completely reversed it. Symbols represent the average ± SEM. *P<0.01 compared with
phystostigmine group, and †P<0.01 compared with salbutamol group (one-way ANOVA with Bonferroni multiple comparisons test).

**Figure 9.** Effect of salbutamol on ACh release from tracheal rings incubated with phystostigmine during 60-min. Tissues were incubated during the last 30 min with different concentrations of salbutamol, and compared with control tissues. Bars and vertical lines represent the average ± SEM of n=11 animals. *p<0.05 (repeated measures ANOVA with Dunnett’s test).

**Figure 10.** Effect of salbutamol on substance P release from tracheal rings. Symbols represent the substance P concentration accumulated during 60 min incubation with 10 mM paraoxon without (control group, n=7) or with salbutamol (100 nM, n= 13) during the last 30 min. DL=detection limit. *p<0.0001 (Mann-Whitney U test).

**Figure 11.** Effect of paraoxon on the carbachol induced intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)](i)) changes in single airway smooth muscle cells. Carbachol addition caused a transient Ca\(^{2+}\) peak, followed by a plateau (filled bar, n=4). Paraoxon incubation did not modify the response to a superimposed stimulation with carbachol (hatched bar). W=washing.
**TABLE 1. Experimental design**

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<th><em>Plethysmography experiments</em></th>
<th><em>Organ baths experiments</em></th>
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AF=AF-DX 116, Ami=aminophylline, Atr=atropine, CCh=carbachol, For=forskolin, L-732=L-732,138, Phy=physostigmine, Pox=paraoxon, Pro=propranolol, Pth=parathion, Sal=salbutamol, TTX=tetrodotoxin, ω-CTX=ω-conotoxin GVIA. *=separate experiments without epithelium (n=5 for each group) were also performed.
FIG. 1

A

AChE Inhibition (%)

Lung

Plasma

B

-Log[M]

Paraoxon

Physoptigmine

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FIG. 2
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FIG. 3

Time (min)

Penh (% of increase)
FIG. 9

Control

Salbutamol (nM)

ACh (nmol · L⁻¹)

*
Chávez et al.
FIG. 11

- **Carbachol 10 μM**
- **Paraoxon 10 μM**

[Diagram showing changes in intracellular calcium ([Ca^2+]) levels over time with 100 s time scale]