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

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organophosphates induce bronchoobstruction in guinea pigs, and salbu-
tamol only transiently reverses this effect, suggesting that it triggers
additional obstructive mechanisms. To further explore this phenom-
enum, in vivo (barometric pleasymography) and in vitro (organ baths,
including ACh and substance P concentration measurement by HPLC
and immunoassay, respectively; intracellular Ca²⁺ measurement in
single myocytes) experiments were performed. In vivo exper-
iments, 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 airflow obstruction rose again as to reach the same intensity
as before salbutamol. Aminophylline caused a decreased decrement
(71%) of the parathion-induced bronchoobstruction. In in vitro stud-
ies, paraoxon produced a sustained contraction of tracheal rings,
which was fully blocked by atropine but not by TTX, õ-conotoxin
(CTX), or epithelium removal. During the paraoxon-induced contrac-
tion, salbutamol caused a temporary relaxation of ~50%, followed by
a partial recontraction. This paradoxical recontraction was avoided by
the M2- or neurokinin-1 (NK1)-receptor antagonists (methoctramine
or AF-DX 116, and L-732138, respectively), accompanied by a
long-lasting relaxation. Forskolin caused full relaxation of the para-
oxon response. Substance P and, to a lesser extent, ACh released from
single myocytes were explored.

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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 after a pentobarbital overdose, and four rings (submitted to different experimental conditions) were obtained from the middle of the trachea. The parathion dose was selected based on previous (35) and new observations that the latter with methoctramine (0.31 μM) or AF-DX 116 (0.3 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 parathion. Likewise, a possible participation of substance P on the effect of salbutamol was evaluated by using L-732138 (10 nM), a neurokinin-1 (NK₁) receptor antagonist (28), added to the organ baths 15 min before parathion. We corroborated that these concentrations of L-732138 do not modify the contractile response to parathion. With the aim to assess if the salbutamol effect was the same during a paraoxon-induced contraction as during a typical cholinergic contractile response, additional tissues were precontracted with 0.32 μM carbachol instead of paraoxon. This difference in the effect of paraoxon is 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.

To evaluate the role of airway epithelium in the parathion-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 paroxon 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) for 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 phosphoramide 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 Gelman Sciences, Ann Arbor, MI) before its storage at −70°C.

AChE activity measurement. The AChE activity was determined in guinea pig 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 of phosphate buffer per 100 mg of tissue) with an homogenizer (Polytron Kinematica PT3100; Brinkmann, Westbury, NY). The homogenate was centrifuged for 15 min at 3,000 g. The supernatant was filtered (Whatman 1 filter paper), and 300 μl of supernatant was added to a cuvette containing 2.5 ml of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB; 0.32 mM) and 300 μl of phosphate buffer (64 mM). The background absorbance per minute was measured at 405 nm at 25°C with a spectrophotometer (DU-640; Beckman, 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

\[
IU = \frac{A \times 3,200}{1.36 \times 10^5 \times 300 \times Co}
\]

where A is the change in absorbance per minute, 1.36 × 10^4 is the extinction coefficient of DTNB, Co is the amount of tissue in the
Enzymatic activity was stopped by adding Leibovitz's solution, the dispersed by mechanical agitation until detached cells were observed. II (neutral protease) for solution containing 1 mg/ml collagenase type I and 4 mg/ml dispase solution to remove the enzyme excess and then placed in a Krebs incubated for 10 min at 37°C. The tissue was washed with Leibovitz's solution containing 2 mg of cysteine and 0.05 U/ml papain and smooth muscle from guinea pig trachea was placed in 5 ml of Hanks' (composition in mM: NaCl 118, KCl 4.6, CaCl$_2$ 2.0, MgSO$_4$ 1.2, CO$_2$ in oxygen, pH 7.4).

Continuous perfusion at a rate of 2–2.5 ml/min with Krebs solution at a 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). Background fluorescence was automatically substracted and determined by removing the cell from the field before starting the experiments. The fluorescence acquisition was obtained according to the formula of Grynkiewicz et al. (20). The fluorescence ratio at 380-nm light excitation in Ca$^{2+}$-saturated cells ($\beta$) was 4.9.

Single myocytes were first stimulated with 10 $\mu$M carbachol, perfused with Krebs solution for 15 min, and then challenged with 10 $\mu$M paraaxon for 4 min, adding 10 $\mu$M carbachol in the last 2 min.

**Drugs.** Parathion (O,O-diethyl O-4-nitrophenyl phosphorothioate) and paraaxon (O,O-dietil O-4-nitrophenyl phosphorothioate) (Riedel-de Haën, Seelze, Germany.) were dissolved in propylene glycol and ethanol (10:1.5 vol/vol). Physostigmine hemisulphate, ACh chloride, carbamylcholine chloride (carbachol), DL-propanol hydrochloride, tetrodotoxin, o-CTX, choline chloride, salbutamol, and forskolin were all purchased from Sigma (St. Louis, MO). Methoctramine tetrahydrochloride was purchased from ICN Pharmaceuticals (Costa Mesa, CA). AF-DX 116 was purchased from Tocris Bioscience (Bristol, United Kingdom). L-732138 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). Forskolin and AF-DX 116 were dissolved in dimethyl sulphoxide and L-732138 in ethanol while the other drugs were dissolved in 0.9% NaCl. Parenteral salbutamol sulphate (Ventolin) was purchased from Glaxo-Wellcome 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 figures as means ± SE, 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) 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 ip) caused a 31% decrease ($P < 0.05$) of the ongoing parathion-induced bronchoconstriction 5 min after its intraperitoneal administration, compared with the averaged Penh values in the previous 10-min period (Fig. 3). After this transient relaxation, Penh was raised again as to reach the same values as before salbutamol administration. A higher salbutamol dose (30 $\mu$g/kg ip) also produced this paradoxical reaction, 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 paraaxon 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 $\mu$M) either paraaxon or physostigmine produced 99.9 ± 0.1% inhibition of AChE activity ($n = 4$ for each group).

Addition of paraaxon 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 $\omega$-CTX or TTX (Fig. 4B), but was fully prevented and reverted...
by atropine (Fig. 4, C 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, 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 pretreatment (188.0 ± 41.7% of KCl contraction).

Salbutamol (100 nM) administered during the paraoxon-induced contraction produced a temporary relaxation of ∼50% (Figs. 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 precontracted 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, favoring 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 shown). Additionally, we found that tissue incubation with the NK1 receptor antagonist L-732138 allowed salbutamol to fully relax the tracheal rings and almost abolished the paradoxical recontraction (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).

ACh 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, range 32.0–79.4), compared with control tissues without salbutamol (median <3.9 pg/ml, range <3.9–9.4; \( P < 0.0001 \); Fig. 10).

**Single cell studies.** Baseline concentration of \([\text{Ca}^{2+}]_i\) in airway myocytes was 49 ± 7 nM, \( n = 4 \). Carbachol addition

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**Fig. 4.** Representative recordings of the effect of paraoxon in in vitro experiments. Paoxon 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 modify the contraction induced by paraoxon but potentiated the relaxation induced by 0.1 \( \mu \)M salbutamol (Sal) (E) and removed the functional antagonism between carbachol (CCh) (0.32 \( \mu \)M) and salbutamol (0.032 \( \mu \)M) (F). Pox, paraoxon (10 \( \mu \)M); Atr, atropine (1 \( \mu \)M); KCl, potassium chloride (60 mM); Met, methoctramine (0.31 \( \mu \)M); TTX, tetrodotoxin (10 \( \mu \)M).

**Fig. 5.** Effect of M2 receptor antagonism on the salbutamol response during the paraoxon-induced contraction in tracheal rings. Paraoxon (\( \bullet \), 10 \( \mu \)M, \( n = 11 \)) induced a contractile response, which was partially reversed by salbutamol (\( \bullet \), 0.1 \( \mu \)M, \( n = 11 \)) 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 favoring a relaxing response of 90% (\( \bullet \), 0.31 \( \mu \)M, \( n = 8 \)). Propranolol (\( \star \), 1 \( \mu \)M, \( n = 6 \)) completely abolished the salbutamol relaxant effect. Symbols represent the means ± SE. \(* P < 0.05\) and \(** P < 0.01\) compared with the paraoxon group and \(\dagger P < 0.01\) compared with the paraoxon-salbutamol group (one-way ANOVA with Bonferroni multiple comparisons test).

**Fig. 6.** Effect of salbutamol and forskolin on the paraoxon-induced contraction in tracheal rings. Paraoxon (\( \bullet \), 10 \( \mu \)M, \( n = 11 \)) induced a contractile response, which was partially reversed by salbutamol (\( \bullet \), 0.1 \( \mu \)M, \( n = 11 \)) with a tendency to contract again from 10 min onward. Forskolin (\( \star \), 1 \( \mu \)M, \( n = 7 \)) completely abolished the paraoxon-induced contraction. The sustained contraction induced by carbachol (\( \bullet \), 0.32 \( \mu \)M, \( n = 8 \)) was moderately reversed by salbutamol following a similar pattern as was observed in the paraoxon group (\( \bullet \), 0.1 \( \mu \)M, \( n = 11 \)). Symbols represent the means ± SE. \(* P < 0.01\) compared with their respective control group; \(\dagger P < 0.01\) compared with the paraoxon-salbutamol group (one-way ANOVA with Bonferroni multiple comparisons test).
caused a transient Ca\(^{2+}\) peak followed by a plateau (Fig. 11). Paraoxon did not modify the [Ca\(^{2+}\)] or 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 \(\omega\)-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 nonneuronal 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 ip) 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-adrenoceptor agonists are capable of stimulating 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 recontraction 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 tachykinin-containing nerve ends (C fibers) is located within and below the airway epithelium, including the trachea (38, 42). Thus one possible explanation of salbutamol-induced tachykinin release is that salbutamol directly stimulates C fibers. However, to our knowledge, the presence of presynaptic 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-day treatment with β2-adrenoceptor agonist (RS- and S-albuterol) induced bronchial hyperresponsiveness (BHR) in sensitized and nonsensitized 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 nonorganophosphate compound), or carbachol, suggesting that increased concentrations of ACh in the micro-environment surrounding the smooth muscle is necessary for the paradoxical effect of salbutamol to take place. Cholinergic contraction of smooth muscle involves activation of both M1 and M2 receptors. The latter is known to be coupled to Gt protein and hence to produce inhibition of adenylate cyclase (33). Thus mechanisms induced by M2 receptor activation are particularly opposed to those produced during β2-adrenoceptor 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 ~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 transplanted with muscarinic M1 receptors. They concluded that these organophosphates act as agonists on M1 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, Fryer et al. (18) and Lein and Fryer (26) recently postulated that organophosphate insecticides induce BHR through a decrease in the neuronal M2 muscarinic receptor function. This presynaptic receptor limits the release of ACh from pulmonary vagal nerves (19) and thus its inhibition by organophosphates would favor 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 nonasthmatic 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 organophosphates 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 wheezing. 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 may even worsen the bronchial obstruction. This last speculation warrants further investigation. Additionally, clinical studies in asthma have found that chronic treatment with β2-adrenoceptor agonists worsens asthma control (34) and induces BHR to a number of bronchoconstrictor stimuli, including methacholine, histamine, exercise, and allergen (10, 23, 34, 40). Therefore, one possible explanation of this
adverse effect of salbutamol could be related to its effect on tachykinin release.

According to our results, we concluded that: 1) organophosphates caused smooth muscle contraction by accumulation of ACh released through a TTX- and α-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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