Short communication

Detection of explosives by olfactory sensory neurons

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\textbf{A B S T R A C T}

The response of olfactory sensory neurons to TNT and RDX as well as to some volatile organic compounds present in the vapors of antipersonnel landmines has been studied both in the pig and in the rat. GC/MS analyses of different plastic components of six different kinds of landmines were performed in order to identify the components of the "perfume" of mines. Studies on rat olfactory mucosa were carried out with electro-olfactogram and calcium imaging techniques, while changes in the cyclic adenosine monophosphate (cAMP) levels following exposure to odorants and explosives were used as a criterion to evaluate the interaction of TNT and RDX with olfactory receptors in a preparation of isolated pig olfactory cilia. These studies indicate that chemical compounds associated with explosives and explosive devices can activate mammalian olfactory receptors.

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1. Introduction

The detection of explosive materials is a highly significant task, which could help in reducing the continued fatalities from landmines among civilians as well as tracking and locating explosives. But since the explosives are chemicals having extremely low vapor pressures, their detection is a very complicated task.

The most efficient method of detecting explosives in current use is sniffer dogs [1]. Rodents are also considered a useful animal model for the detection of buried landmines, as in recent years African giant pouched rats (Crycetomys gambianus) have been trained for use as biodetector of antipersonnel landmines.

The vertebrate nose can detect and discriminate between innumerable low-molecular-weight organic compounds that possess an enormous diversity of chemical structures and properties. Aliphatic and aromatic compounds displaying different functional groups, including aldehydes, esters, ketones, ethers, alcohols, alkenes, carboxylic acids, amines, imines, thiols, halides, nitriles and sulphides can be detected by the olfactory sensory neurons (OSNs) located in the nasal mucosa.

The OSNs are bipolar neurons extending their axons into the olfactory bulb and dendrites toward the nasal lumen; the dendrite tips carry several cilia, which are the sites of odour recognition. Olfactory cilia can be detached and isolated from the olfactory epithelium providing an in vitro model system useful for biochemical studies of olfactory perception. Each OSN expresses one out of a repertoire of some 1000 olfactory receptors (ORs), constituting the largest family of G-protein-coupled receptors (GPCRs) [2]. ORs are located at the membrane of the olfactory cilia and give rise to electrical signals in the olfactory axons through a transduction mechanism, involving the activation of an adenylcyclase and cyclic nucleotide-gated ionic channels (see review [3]).

The functional expression of ORs in yeasts represents one of the most useful biotechnological tools to examine the molecular mechanism of odorant interaction with receptors [4]. Recently an attempt to identify the OR(s) able to bind explosives has been reported in the literature: a library of chimeric rat olfactory receptor proteins was screened in the heterologous yeast expression system to find one that could specifically detect explosive molecules [5].

Alternatively, animal OSN(s), expressing OR(s) specifically activated by explosives, can be identified by calcium imaging analyses of a population of OSNs dissociated from the olfactory mucosa.

Up to now the interaction of explosives and other volatile compounds released by antipersonnel landmines with the olfactory mucosa and with isolated OSNs has not yet been investigated in vitro.

The organic compounds given off from the landmine plastic envelopes and components consist mainly of residues of polymerization solvents, plasticizers and antioxidants.
In the first phase of the present study, we used headspace GC/MS to analyse the organic chemicals given off by the plastic and rubber envelopes of six different kinds of landmines. In the second phase, different experimental models were used to study the interaction of these detected compounds as well as explosives with animal olfactory mucosa and OSNs.

Results show that ORs can be activated by explosive compounds and/or by volatiles released by the landmine casings.

2. Experimental

2.1. Materials

Six different antipersonnel landmines were considered in the present study: TS 50, Aups, MK 2, Valmar, Var 40, Maus 1. Depending on the kind of landmine, one or more of the following plastic and rubber components (22 samples) were analysed: pressure plate, cap, inferior and superior body, o-ring, safety cap. The Military Plant (Baiano di Spoleto, Perugia, Italy) kindly supplied the plastic components of antipersonnel landmines.

Pig nasal turbinates were used as starting material to isolate olfactory cilia. The use of an animal of big size was particularly convenient to increase the yield of membrane preparation. The nasal turbinates were kindly provided immediately after sacrifice by the slaughterhouse of Ruvo di Puglia (Bari, Italy).

As it was not possible (1) to use pig turbinates in EOG measurements for technical reasons and (2) to obtain a good preparation of isolated OSNs from pig, some experiments were performed on rats. The olfactory epithelia, used for the EOG and calcium imaging analyses, were isolated from the 6–8 week-old Sprague–Dawley male and female rats.

Odorants and explosives were usually dissolved in dimethyl sulfoxide (DMSO) giving stock solutions of 20–500 mM; different volumes were added to the appropriate buffer to obtain the final odour concentrations.

The odorants were purchased from Sigma–Aldrich, except the Henkel 100 mixture from Henkel. TNT and RDX came from Supelco. The Cyclic AMP \[^{3}H\] assay system was purchased from Amersham.

2.2. GC/MS analysis

The finely ground plastic components of landmines were analysed by headspace GC/MS analysis. About 1 g of each plastic sample was finely ground in pieces of about 20–50 mg, sealed in a 20 ml headspace vial and heated for 1 h at 40 °C, 60 °C and 80 °C at 10 psi. The analyses were made at such high temperatures in order to facilitate the sample desorption and obtain large enough amounts in the headspace to be detected by the instrument. Then, about 1 ml of the gas phase of the sample was analysed by MS. The GC/MS spectra were obtained with an HP 5890 gas chromatograph and an HP 5988 mass spectrometer. An SE30 capillary column (30 m, 0.32 mm i.d., 0.25 µm film thickness) was used with the following temperature program: 40 °C for 3 min, linear to 240 °C at 20 °C/min, isothermal at 240 °C for 4 min to assure elution of all sample components. Helium (1 ml/min) was used as carrier gas. The MS was operated in electron impact (EI) mode (70 eV). The unknown compounds released by the plastics were identified with the help of an MS library search system.

2.3. Isolation of olfactory cilia

Cilia from pig olfactory epithelia were detached and isolated as described by [6,7]. All operations were carried out at 0–4 °C. Pig olfactory epithelia were dissected and washed in Ringer solution (120 mM NaCl, 5 mM KCl, 1.6 mM K2HPO4, 1.2 mM MgSO4, 25 mM NaHCO3, 7.5 mM glucose, pH 7.4). The olfactory cilia were detached by a calcium shock procedure raising the calcium concentration in the Ringer solution up to 10 mM. After gently shaking (20 min at 4 °C), the deciliated epithelia were removed by centrifugation. The supernatant was collected and the pellet incubated again with the solution containing 10 mM calcium ion for 20 min. After removing the deciliated epithelia by centrifugation, the supernatants containing the detached cilia were combined. Cilia were collected by centrifuging at 27 000 × g for 15 min, and resuspended in a solution containing 3 mM MgCl2, 2 mM EDTA, 10 mM Tris/HCl, 1 mM PMSF buffered at pH 7.4. Samples of concentrated cilia were suspended in a small volume and saved at −80 °C.

2.4. Measurement of changes in cyclic adenosine monophosphate (cAMP) concentration

The procedures described in [8,9] have been modified. All assays were carried out at 37 °C. Briefly 70 µL of cilia suspension (50–100 µg protein/ml) were mixed with 360 µL of stimulating buffer containing 200 mM NaCl, 10 mM EGTA, 50 mM Mops, 2.5 mM MgCl2, 1 mM DTT, 0.05% sodium cholate, 1 mM ATP, 20 µM GTP, 1 mM IBMX buffered at pH 7.4, with or without odorants. Odorants and explosives were usually prepared as 0.1 M stock solutions in ethanol or DMSO. Appropriate volumes were added to the stimulating buffer to obtain the final concentration of 200 µM. Controls were performed using the same solvent concentration and at this concentration neither DMSO nor ethanol affect the reaction. The incubation of cilia with the stimulating buffer was stopped by adding 350 µL of cold 10% perchloric acid. Then samples were centrifuged at 2500 × g for 5 min at 4 °C; 400 µL of the supernatant were added to 100 µL of 10 mM EDTA pH 7.0. The samples were then neutralized by adding 500 µL of a mixture containing 1,1,2-trichlorotrifluoroethane and tri-n-octylamine, mixed and then three phases were obtained by centrifugation: the upper phase contained the water soluble components and was used to estimate the amount of cAMP produced during the incubation of cilia with substrates. The cAMP was determined by radioimmunoassay using an Amersham kit. Protein concentration of cilia was determined using the method of Bradford [10].

2.5. Electro-olfactogram (EOG) recordings

After sacrifice, the rat head was cut open sagitally and the septum was removed to expose the medial surface of the olfactory turbinates. The right half of the head was mounted in a wax dish filled with rat Ringer (see down). The medial surface of turbinates was face up and exposed to the air. A continuous stream of humidified clean air was gently blown on the turbinates through tubing to prevent tissue from drying.

The EOG recording electrode was an Ag/AgCl wire in a capillary glass pipette filled with rat Ringer solution containing 0.6% agarose. The electrode resistance was between 0.5 and 1 MΩ. The recording pipette was placed on the surface of the olfactory epithelium and connected to a differential amplifier (DP-31 Warner Instruments). The EOG potential was acquired using an IT16 interface and acquisition routine written with Igor Pro software (WaveMetrics). All experiments were carried out at room temperature (22–25 °C). Odorant and explosives solutions were prepared as 0.5 M stocks in DMSO and were diluted with water to the final concentration 1 mM; the DMSO concentration in solution was 0.2% (v/v). Responses to DMSO alone were the same as to clean air (<0.5 mV).

Three milliliters of the odorant solution were placed in a 10 ml glass test tube and capped with a silicon stopper. The concentration of volatile odorant was allowed to equilibrate for more than 1 h. Two needles provided the input and output ports for the odorant-containing vapor above solution. For stimulation, a 100- ms pulse of
the odorant vapor at 9 psi was injected into the continuous stream of humidified air.

2.6. Calcium imaging recordings

Single olfactory neurons were isolated from rat olfactory epithelia as follows. Briefly, epithelia were dissected out in oxygenated divalent cation-free rat Ringer’s (145 mM NaCl, 5.6 mM KCl, 10 mM HEPES, 10 mM glucose, 4 mM EGTA, pH 7.4). The tissue was then incubated at 37°C for 45 min in 5 ml of divalent cation-free Ringer containing 5 mg/ml bovine serum albumin (Sigma), 0.5 mg/ml collagenase, 22 U/ml dispase (Gibco BRL) and 50 µg/ml DNase II (Sigma). The tissue was then transferred to a normal rat Ringer’s (138 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1.5 mM MgCl₂, 10 mM HEPES, 10 mM d-glucose, pH 7.4), and the cells were dissociated by tapping the tube containing the tissue. Cells (400 µL) were plated onto Concanaavalin A 10 mg/ml-coated glass cover slips placed in 35 mm Petri dishes and allowed to settle. After 20 min, 2 ml of culture medium was added to each dish, and the dishes were placed at 37°C in a CO₂ incubator for at least 1 h. The culture medium consisted of DMEM/F12 (Gibco BRL) supplemented with 10% FBS, 100 µM ascorbic acid, 1x insulin-transferrin-selenium-X (Gibco BRL), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco BRL). Ca²⁺ recordings were performed as described elsewhere [11]. Briefly, cells were loaded with 5 µM Fura-2/AM plus pluronic acid F127 (80 µg/ml; Molecular probes) in rat Ringer’s at room temperature in the dark for 45 min. Imaging was performed on an inverted fluorescence microscope (IMT-2, Olympus) equipped with a SIT camera (C2400-08, Hamamatsu Photonics) connected to a frame grabber in a Macintosh computer. Recordings were made at 380 nm excitation and 510 nm emission. Images were taken every 4 s, and three frames were averaged. The recording chamber was continuously perfused with oxygenated Ringer by means of peristaltic pump. Odours were applied for 8 s in enough volume to completely replace the solution in the chamber (200 µL). Odorant and explosive solutions were prepared in rat Ringer’s by dilutions from 22 mM stock solutions made in DMSO and applied through syringes connected to the perfusion system with a manifold. The odorant concentrations usually were 30 and 300 µM and stimuli were applied with intervals of at least 1 min. Data are shown as the fractional change in fluorescent light intensity: ΔF/F₀ or (F–F₀)/F₀, where F is the fluorescent light intensity at each point, and F₀ is the value of emitted fluorescent light before the stimulus application (baseline).

3. Results

3.1. GC/MS analyses of organic compounds given off by the plastic components of landmines

The chemical detection of explosive vapours in the immediate vicinity of buried landmines has been examined in a number of literature studies. For example, the level of explosives in the chemical signatures of some types of Yugoslavian landmines buried under the ground was investigated in details [12]. In addition, a recent mathematical model has been used to describe the distribution and diffusion of explosives over buried landmines in different environmental conditions [13].

Here we identified some volatile organic compounds released from the different plastic and/or rubber components of landmine cases. The following types of Italian antipersonnel landmines were considered: (a) Technovar TS 50, (b) Technovar VAR 40, (c) Valmara, (d) Maus 1, (e) MK 2, (f) Aups. All these landmines contain RDX or TNT or a mixture of both explosives. Different plastic components of the different landmines were analysed by headspace GC/MS analysis. In order to evaluate the analyte concentration in the headspace, we estimated the ratio between analytical peak (I) and 40Ar air peak (Iair); by considering that Argon represents 1% of the atmospheric air, the ratio I/Iair equal to 1 corresponds to about 10 µg (for 1 ml injected gas sample). From these assumptions, we estimated that each detected compound was in the range from 0.05 to 20 µg. Table 1 reports the most abundant compounds detected by GC/MS analyses of the various plastic components. Fig. 1 shows the chemical structures of the main organic molecules given off by the different kinds of landmines. Data in Table 1 represent the first attempt to reveal volatiles different from explosives in the signature of plastic cased antipersonnel landmines.

3.2. Activation of the second messenger cAMP pathways in isolated pig olfactory cilia by TNT and RDX

Olfactory cilia were isolated from pig olfactory mucosa and the quality of ciliary preparations used in the present study was assayed by determining both forskolin-stimulated and odorant-stimulated adenylyl cyclase activity (Fig. 2). The enzyme adenylyl cyclase converts the abundant intracellular molecule adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP), the second messenger of the signal transduction cascade.

Citralva, a nitrile substituted terpenoid having a fruity odour quality which was found to be one of the most potent stimulants of the odor-sensitive adenylyl cyclase in frog olfactory ciliary membranes, and eugenol, an aromatic compound having both alcoholic and ether residues on the benzene ring, were selected to assay the odorant-stimulated adenylyl cyclase activity in our cilia preparations.

As can be seen in Fig. 2, the application of 200 µM citralva stimulates the formation of cAMP in isolated pig olfactory cilia, while the same concentration of eugenol resulted in a lower (about 20%) stimulation of adenylyl cyclase.

Interestingly, both the explosives RDX and TNT at the same concentration of 200 µM were able to activate the adenylyl cyclase enzyme in isolated pig olfactory cilia. However, under our experimental conditions, RDX was a much more powerful stimulator than TNT.
Table 1

Volatile organic compounds released from the different components of some landmine cases.

<table>
<thead>
<tr>
<th>Mines</th>
<th>Explosives</th>
<th>N</th>
<th>Sample</th>
<th>Material</th>
<th>Volatile components</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS</td>
<td>RDX</td>
<td>1</td>
<td>Inferior body</td>
<td>Plastic</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>Superior body</td>
<td>Plastic</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>Cap</td>
<td>Rubber</td>
<td>Hexachloroethane</td>
</tr>
<tr>
<td>AUPS</td>
<td>RDX, TNT</td>
<td>4</td>
<td>Detonator button</td>
<td>Plastic</td>
<td>Naphthalene, phenol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>Inferior body</td>
<td>Plastic</td>
<td>Naphthalene, phenol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>Superior body</td>
<td>Plastic</td>
<td>Naphthalene, phenol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>Percussion cap</td>
<td>Rubber</td>
<td>–</td>
</tr>
<tr>
<td>MK</td>
<td>RDX</td>
<td>8</td>
<td>Superior ring</td>
<td>Plastic</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9</td>
<td>Inferior body</td>
<td>Plastic</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>Cap</td>
<td>Rubber</td>
<td>Hexachloroethane, benzothiazole</td>
</tr>
<tr>
<td>VALMARA</td>
<td>RDX, TNT</td>
<td>11</td>
<td>Ignition body</td>
<td>Plastic</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>Outer holder</td>
<td>Plastic</td>
<td>Chlorobenzene, styrene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13</td>
<td>Cap</td>
<td>Plastic</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>Cap o-ring</td>
<td>Rubber</td>
<td>Hexachloroethane</td>
</tr>
<tr>
<td>VAR</td>
<td>RDX</td>
<td>16</td>
<td>Safety cap</td>
<td>Plastic</td>
<td>Diphenylamine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17</td>
<td>Inferior body</td>
<td>Plastic</td>
<td>Diphenylamine, benzene, toluene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18</td>
<td>Superior body</td>
<td>Plastic</td>
<td>–</td>
</tr>
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<td></td>
<td></td>
<td>19</td>
<td>Button cover</td>
<td>Plastic</td>
<td>–</td>
</tr>
<tr>
<td>MAUS</td>
<td>RDX</td>
<td>20</td>
<td>Cap</td>
<td>Rubber</td>
<td>Hexachloroethane</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21</td>
<td>Outer ring</td>
<td>Plastic</td>
<td>–</td>
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<td></td>
<td>22</td>
<td>Inferior body</td>
<td>Plastic</td>
<td>Styrene</td>
</tr>
</tbody>
</table>

All the 22 samples were finely ground and analysed at 40, 60 and 80 °C by headspace GC/MS, as described in Section 2. Most of volatile components could be detected by heating at 80 °C, while naphthalene (in samples 4, 5, 6) was also detected at the lower temperatures 40 °C and 60 °C as well as hexachloroethane (in sample 15) at 60 °C.

3.3. EOG recordings in rat olfactory neurons

The electrophysiological response of olfactory neurons to volatile compounds can be measured by the electro-olfactogram (EOG) technique, which represents a reliable indicator of the stimulus-induced activity of olfactory sensory neurons. EOG is a slow potential, recorded extracellularly from a population of olfactory sensory neurons.

Fig. 2 illustrates the EOG recordings of rat olfactory mucosa exposed to organic compounds previously detected by GC/MS analysis as well as to the explosive compounds, TNT and RDX. While benzene, naphthalene, hexachloroethane, styrene, toluene and chorobenzene elicited a measurable response in the EOG recordings, neither TNT nor RDX were able to stimulate the in toto electrophysiological response of rat olfactory mucosa. It should be noted, however, that this lack of activity could be due to the negligible solubility of both these compounds in the solvents used in our EOG recordings.

To further investigate on the response of rat olfactory epithelium to explosives, we tested the response of single OSNs by the calcium imaging technique.

3.4. Calcium imaging in single olfactory neurons of rat olfactory mucosa

It is known that stimulation by odorants leads to an increase in intracellular calcium in OSNs [3]. The intracellular Ca^{2+} variations

Fig. 3. Representative EOG recordings from rat olfactory epithelium. EOG responses were normalized to the reference odorant, amyl acetate. The experiments were carried out at room temperature (22–25 °C) and all compounds tested at 1 mM.
can be easily monitored, by loading OSNs with compounds whose fluorescence is modulated by Ca^{2+} ion concentrations.

Isolated rat OSNs were stimulated with a panel of explosives and chemical compounds given off by the previously considered mines while responses were monitored with Ca^{2+} imaging. The representative response profiles of three different individual OSNs are shown in Fig. 4: it can be seen that the cell shown in panel a responded only to TNT, that in panel b to RDX and that in panel c to toluene, one of the components of Table 1. In general we found that OSNs were specific to either TNT or RDX. In fact, for example, only TNT but not RDX, toluene, or an odour mixture was found that OSNs were specific to either TNT or RDX. In fact, for example, only TNT but not RDX, toluene, or an odour mixture was able to elicit a calcium intracellular increase in the OSN shown in Fig. 4a. Furthermore, the response of the OSNs to IBMX, an inhibitor of phosphodiesterase, indicated that the observed fluorescence changes were specifically linked to changes in cAMP level. In our calcium imaging study we also found few OSNs responding to styrene and some others to phenol (not shown). The other volatile compounds identified by GC/MS analysis (Table 1) were not tested.

4. Conclusions

Olfactory receptors are considered excellent candidates for the fabrication of sensitive biosensors for explosives and other chemicals of interest.

Here we report that two common explosives, RDX and TNT, increase the intracellular second messengers, cAMP and calcium, in animal olfactory sensory neurons: in particular we show that (1) RDX is an excellent activator of the olfactory transduction mechanism in olfactory sensitive neurons of both pig and rat, (2) TNT is a good activator of the transduction mechanism in isolated rat olfactory sensory neurons, while it is less effective in the preparation of pig isolated cilia, (3) some chemical compounds, commonly found in landmines, produce electrical responses in the isolated olfactory epithelium. All together these results suggest that explosive compounds as well as other chemicals present in landmines interact with and activate olfactory receptors.

In future investigations, on the basis of the present study, RT-PCR of a single olfactory sensory neuron might reveal the olfactory receptors(s) able to specifically interact with explosive molecules, opening the way to the reconstitution of olfactory receptors in artificial membranes as the sensitive element of a bioelectronic nose.

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