

Apoptosis induced by prolonged exposure to odorants in cultured cells from rat olfactory epithelium

Sebastian Brauchi^{a,b,1}, Christian Cea^{a,1}, Jorge G. Farias^c,
Juan Bacigalupo^{d,e}, Juan G. Reyes^{a,*}

^aInstituto de Quimica, Pontificia Universidad Catolica de Valparaiso, Casilla 4059, Valparaiso, Chile

^bUniversidad Austral de Chile, Valdivia, Chile

^cInstituto de Estudios de la Salud, Universidad Arturo Prat, Iquique, Chile

^dDepartamento de Biologia, Facultad de Ciencias, Universidad de Chile, Santiago, Chile

^eCentro de Investigacion de Dinamica Celular y Biotechnologia, Universidad de Chile, Santiago, Chile

ABSTRACT

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Multicellular organisms undergo programmed cell death (PCD) as a mechanism for tissue remodeling during development and tissue renewal throughout adult life. Overdose of some neuronal receptor agonists like glutamate can trigger a PCD process termed excitotoxicity in neurons of the central nervous system. Calcium has an important role in PCD processes, especially in excitotoxicity. Since the normal turnover of olfactory receptor neurons (ORNs) relies, at least in part, on an apoptotic mechanism and odor transduction in ORNs involves an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), we investigated the possibility that long-term exposures to odorants could trigger an excitotoxic process in olfactory epithelial cells (EC). We used single-cell $[Ca^{2+}]_i$ determinations and fluorescence microscopy techniques to study the effects of sustained odorant exposures in olfactory EC in primary culture. Induction of PCD was evaluated successively by three independent criteria: (1) measurements of DNA fragmentation, (2) translocation of phosphatidylserine to the external leaflet of the plasma membrane, and (3) caspase-3 activation. Our results support the notion of an odorant-induced PCD in olfactory EC. This odorant-induced PCD was prevented by LY83583, an odorant response inhibitor, suggesting that ORNs are the main epithelial cell population undergoing odorant-induced PCD.

1. Introduction

Considerable progress has been made on elucidating the mechanisms of olfactory transduction over the past decade. Thus, several models have been proposed to explain how odors are detected (Dionne and Dubin, 1994; Morales and Bacigalupo, 1996; Madrid et al., 2005; Schild and Restrepo,

1998). In the best-established model, binding of an odorant to its G-protein-coupled odorant receptor triggers a cyclic AMP cascade (Schild and Restrepo, 1998). Cyclic AMP opens a cyclic nucleotide-gated cation channel (CNGC; Nakamura and Gold, 1987). This event increases intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Restrepo et al., 1993; Leinders-Zufall et al., 1998), which in turn gates a calcium-dependent chloride channel

* Corresponding author. Fax: +56 32 273422.

E-mail address: jreyes@ucv.cl (J.G. Reyes).

¹ These authors contributed equally to this work.

(Kleene and Gesteland, 1991; Lowe and Gold, 1993; Kurahashi and Yau, 1993). Both conductances are responsible for a depolarizing (excitatory) receptor potential, leading to an increase in action potential discharge frequency. Calcium has also been shown to mediate odor inhibition by opening Ca^{2+} -dependent K^+ channels, causing a hyperpolarizing receptor potential that leads to a reduction in firing rate (Morales et al., 1994; Sanhueza et al., 2000; Madrid et al., 2005).

As part of its normal turnover, ORN population density is controlled by the balance between neurogenesis and apoptosis. Apoptosis occurs in mature ORNs under normal conditions (see Cowan and Roskams, 2002; Suzuki, 2004 for reviews), but the molecular entities that trigger this normal turnover apoptotic process have not been identified. Sensory deprivation by naris occlusion (Meisami, 1976) causes a reduction in the turn over rate of ORNs, without changing the total number of mature ORNs in the epithelium (Farbman et al., 1988). Thus, the delay observed in normal apoptotic process was attributed to a protective effect from airborne odorants.

Because a rise in $[\text{Ca}^{2+}]_i$ is essential for the odorant response (see Schild and Restrepo, 1998), we reasoned that a prolonged exposure to high levels of odorants may be equivalent to a sustained presence of an excitotoxic agent, which can lead to programmed cell death (PCD) in other cell types through processes that involve an increase in intracellular Ca^{2+} (Said et al., 1996; Mattson, 2000). In this work, we show, for the first time that sustained over-exposition to odorants causes apoptosis in a large fraction of cultured olfactory epithelial cells (EC), including olfactory receptor neurons (ORNs). The CNGC blocker LY83583 prevented the rise in $[\text{Ca}^{2+}]_i$ and the odorant induction of apoptosis, presumably because it impeded an abnormally sustained high intracellular Ca^{2+} level by abolishing the Ca^{2+} influx through its channel, strongly suggesting that ORNs underwent an excitotoxicity-like PCD in culture.

2. Results

We investigated whether prolonged odorant exposition of olfactory neurons in culture induces programmed cell death. Apoptosis was determined by three different approaches, all of which supported the notion that odorants induce PCD during extended exposures.

2.1. Olfactory epithelial cells in culture

In order to estimate the proportion of ORNs in our cultures, we used an anti-OMP antibody which identifies mature ORNs. Immune staining of the dissociated cells with this antibody gave $47 \pm 5\%$ of stained cells ($N = 2$, 4 coverslips, 3 fields per coverslip examined). Immune staining with the anti-OMP antibody of the cultured cells after 48 h culture with change of medium at 24 h, gave an average value of $77 \pm 5\%$ stained cells ($N = 2$, 5 coverslips, 3 fields per coverslip examined; Fig. 1B). Immunoassaying using an antibody against neurofilaments renders a good correlation with OMP distribution (Fig 1A). Thus, our dissociation and culture procedure allowed us to obtain olfactory EC with a large proportion of ORNs. Some of these ORNs retained their morphology in primary cultures (Fig. 1D). The viability of the cultured olfactory EC was approximately 70%.

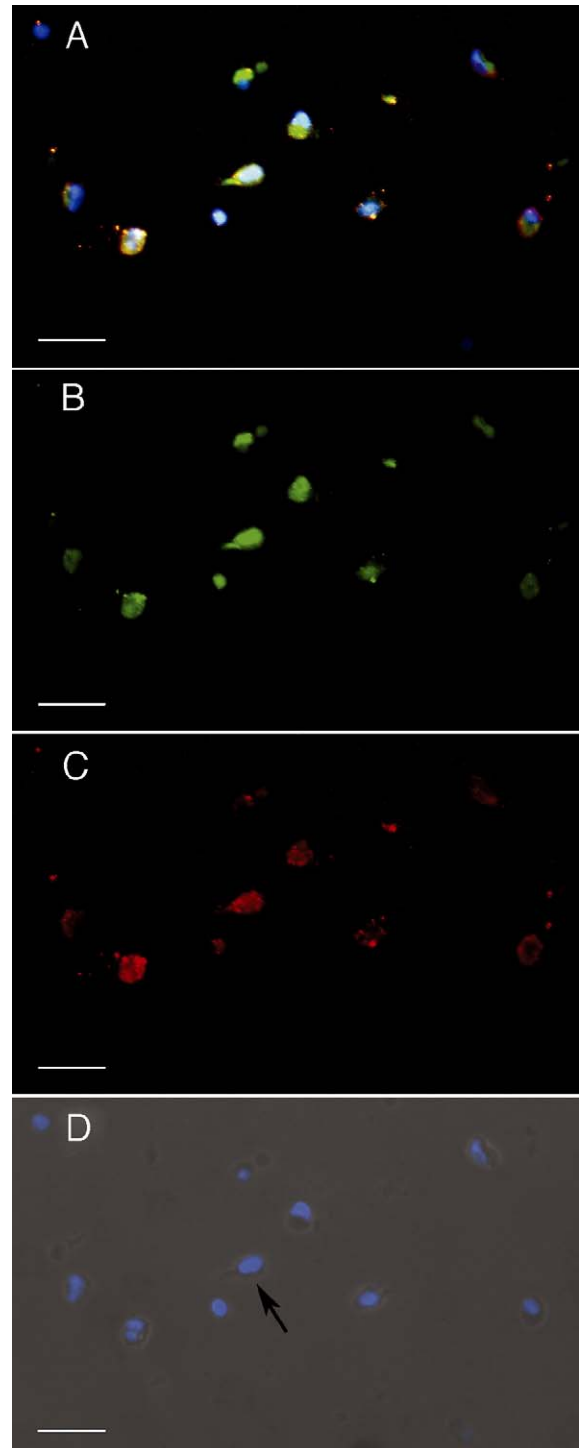


Fig. 1 – Rat olfactory epithelial cells in culture. Olfactory EC were obtained by enzyme digestion of olfactory epithelia followed by mechanical disruption to separate adult ORNs. (A) The image shows a triple labeling of the cultured cells after 48 h of culture (green = OMP, red = neurofilament, blue = nucleus). A large proportion of cells showing both OMP and neurofilament staining could be observed. Individual channels are shown in the panels below, (B) Anti-OMP, (C) anti-neurofilament, (D) merged image of bright field and Hoescht 33342. Notice that some of the positive labeled cells conserved their neuronal morphology (arrow). The scale bar indicates 25 μm .

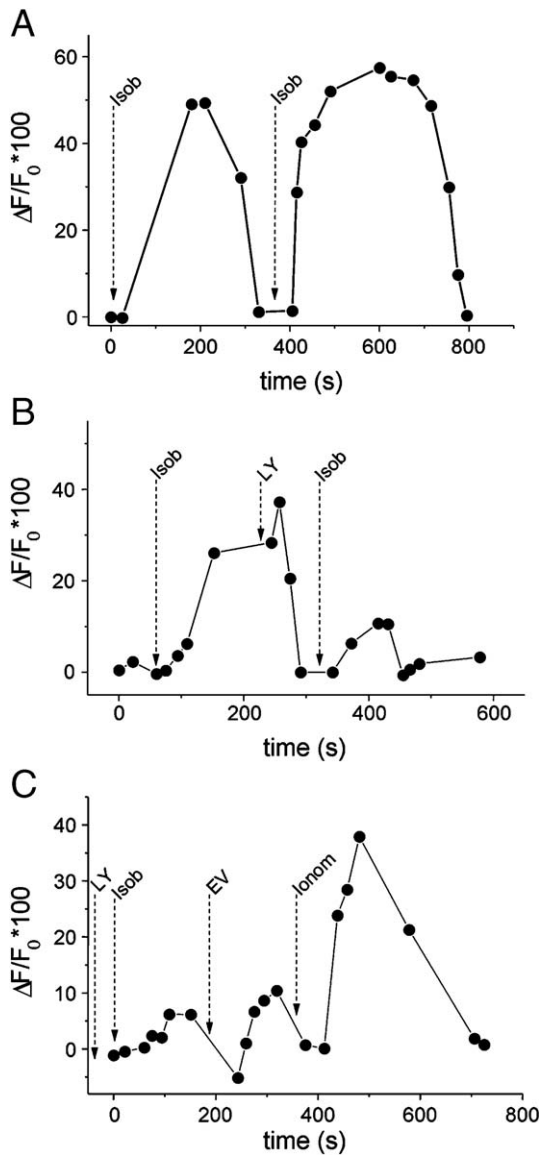


Fig. 2 – Fractional fluorescence change of Fluo-3 AM loaded ORNs in primary culture upon odor stimulation. (A) Responses of an ORN to 200 s stimuli of 150 μM isobutyrate (Isob). (B) At the indicated times, the cell was sequentially exposed to 150 μM isobutyrate (200 s), 150 μM isobutyrate + 10 μM LY83583, and back to isobutyrate. (C) The cells were incubated for 5 min in the presence of 10 μM LY83583, inhibitor of the odorant transduction cascade, and then they were sequentially exposed to isobutyrate and ethyl vanillin (EV, 150 μM each). The ability of Fluo-3 loaded cells to respond with $[\text{Ca}^{2+}]_i$ changes was tested with 5 μM ionomycin.

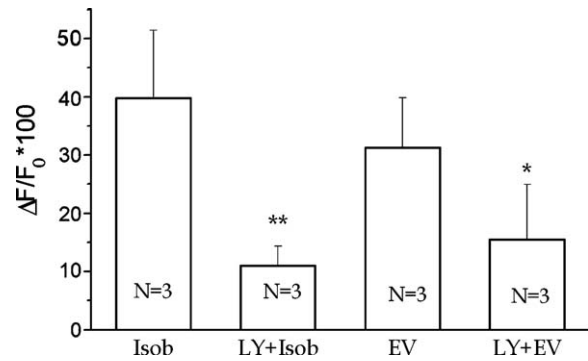


Fig. 3 – Single-cell peak fractional fluorescence change of Fluo-3 AM loaded ORNs in primary culture upon odor stimulation. LY83583 inhibits the $[\text{Ca}^{2+}]_i$ response to isobutyrate (Isob) or EV. Preincubation with 10 μM LY83583 significantly diminished the $[\text{Ca}^{2+}]_i$ increase induced by 150 μM Isob or EV ($P < 0.01$, * $P < 0.05$; ANOVA-Bonferroni test, N is the number of cell preparations tested).**

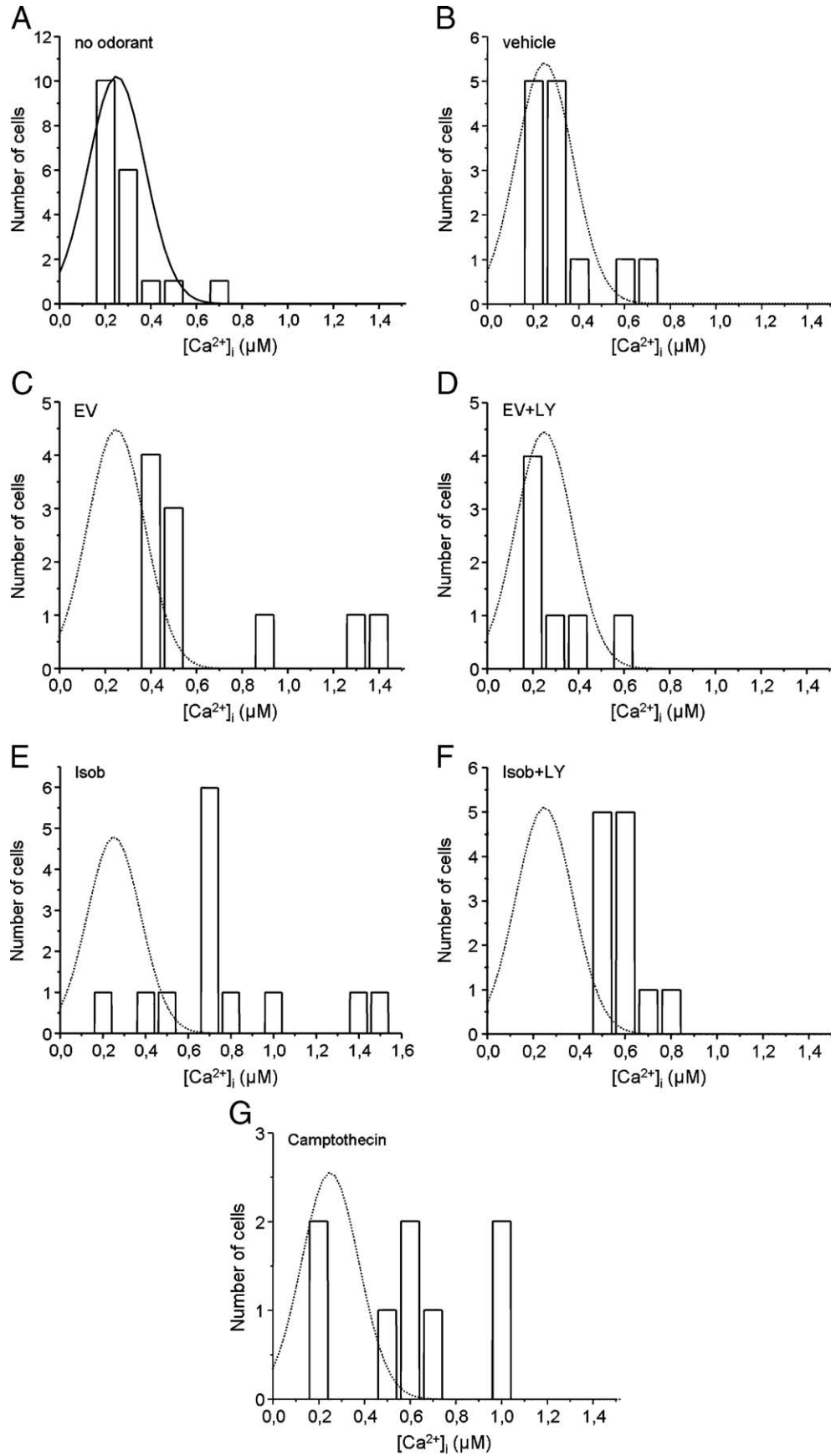
2.2. $[\text{Ca}^{2+}]_i$ responses to odorants in ORNs inhibited by LY83583

The $[\text{Ca}^{2+}]_i$ response of ORNs to odorants was studied in order to determine, first, the functionality of these cells (selected by their morphological characteristics), second, whether the odorant concentration used was adequate to generate a substantial raise in $[\text{Ca}^{2+}]_i$, and third, whether LY83583, a blocker of the transduction channel CNGC, effectively inhibited the odorant-induced Ca^{2+} response. A specific ORN was able to respond repeatedly and reproducibly to an odorant (Fig. 2A). Some cells responded to different odorants (Fig. 2C). We were unable to determine the early time course of the $[\text{Ca}^{2+}]_i$ response due to limitations in the time resolution of our image acquisition system. We observed that the total time period that $[\text{Ca}^{2+}]_i$ stayed high varied from 100 to 300 s in the different cells tested. The size of the response (peak value, $\Delta F / F_0 * 100$) varied from 18 to 49% in different cells. Incubation with LY83583 changed the response amplitude significantly (Figs. 2B and C and 3). Fig. 2C also shows that ionomycin was able to induce a prominent $[\text{Ca}^{2+}]_i$ raise even in the presence of LY83583, indicating that the fluorescence probe was clearly sensitive to changes in $[\text{Ca}^{2+}]_i$ and that the magnitude of the $[\text{Ca}^{2+}]_i$ response was not limited by probe sensing capabilities. Statistical analysis of the magnitudes of $[\text{Ca}^{2+}]_i$ responses for EV and isobutyrate showed a significant difference ($P < 0.01$, ANOVA followed by Bonferroni t test) in the presence and absence of LY83583 (Fig. 3).

2.3. $[\text{Ca}^{2+}]_i$ in ORNs under sustained odorant exposure

An elevation of $[\text{Ca}^{2+}]_i$ appears to be a key factor in neuronal excitotoxic PCD induction (Mattson, 2000). Using fluorescence

Fig. 4 – Single-cell $[\text{Ca}^{2+}]_i$ measurements on Fluo-3 loaded olfactory EC continuously exposed to odorants for 3 h. (A) No odorant addition (control), (B) vehicle, (C) ethyl vanillin (EV), (D) LY83583 + ethyl vanillin, (E) isobutyrate (Isob), (F) LY83583 + Isob, (G) camptothecin (positive control). Continuous line in panel A indicates a Gauss fitting to the data. Dotted lines in all other panels were drawn for comparison purposes, and correspond to a Gaussian curve with variable amplitude (arbitrarily set according to the plotted data), but similar position and width parameters as the control Gaussian curve.



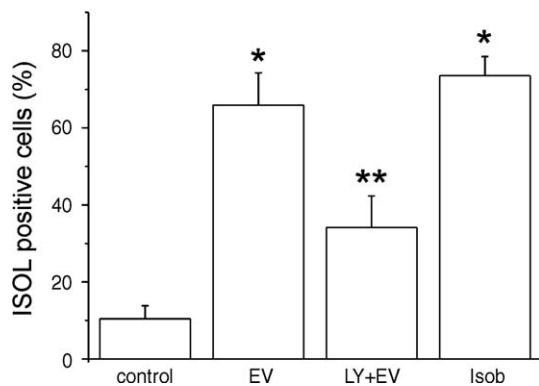


Fig. 5 – DNA fragmentation analysis. The effects of odorants on DNA strand breakage were evaluated using an ISOL assay after 3 h incubation under the experimental conditions shown in the figure (odorant-free, EV, EV + LY38538, and Isob). Results shown in the graphs are presented as the percentage of positive labeled cells in the culture. Kruskal–Wallis–Dunn statistical analyses are shown (* $P < 0.05$ vs. control, ** $P < 0.05$ vs. EV).

microscopy and Fluo-3 AM as a $[Ca^{2+}]_i$ probe, we studied whether a continuous exposure to an odorant was capable of inducing a sustained rise in $[Ca^{2+}]_i$. The values of $[Ca^{2+}]_i$ in cultures subjected to diverse experimental conditions for 3 h are plotted in Fig. 4: (A) no odorant addition, (B) vehicle, (C) ethyl vanillin (EV), (D) LY83583 + ethyl vanillin, (E) isobutyrate (Isob), (F) LY83583 + isobutyrate, (G) camptothecin (positive control). As seen in Fig. 4, 94% of control cells presented $[Ca^{2+}]_i$ values below 400 nM. In the presence of EV some cells presented $[Ca^{2+}]_i$ as high as 1.3 μ M. LY83583 prevented this $[Ca^{2+}]_i$ rise in EV treated cells, showing a frequency profile very similar to the control values. Isobutyrate was also able to induce an increase in $[Ca^{2+}]_i$, with >75% of the cells showing $[Ca^{2+}]_i$ values larger than 400 nM. In contrast to EV, LY83583 partially prevented the rise in $[Ca^{2+}]_i$ in olfactory EC treated with isobutyrate. Cells treated with camptothecin, a topoisomerase inhibitor, showed increased cell death and release of cells from the culture plates. The remaining cells presented relatively high $[Ca^{2+}]_i$ (Fig. 4G).

2.4. Odorant-induced apoptosis in olfactory epithelial cells revealed by DNA fragmentation

Because the different procedures to estimate apoptosis in olfactory EC were not compatible with the simultaneous determination of ORNs identity, we expressed our apoptosis measurements as percentages of total olfactory EC. These olfactory EC consisted of at least 77% ORNs (see above).

Continuous exposure to ethyl vanillin or isobutyrate for 3 h induced apoptotic DNA strand breakages, of 66 ± 8 and $74 \pm 5\%$ in the presence of ethyl vanillin and isobutyrate, respectively. Considering spontaneous apoptosis of 10% of the cells, the odorant-induced apoptosis was approximately 56 and 64% for ethyl vanillin and isobutyrate, respectively. LY83583 reduced the odorant-induced apoptosis to approximately 24% of the cells (Fig. 5).

2.5. Odorant-induced apoptosis revealed by phosphatidylserine presence in the external plasma membrane leaflet

DNA strand breakage, estimated by the ISOL assay, suggested that an odor-induced apoptotic process was taking place in olfactory EC. In order to confirm this observation, we independently used an Annexin-V assay, which detects exposure of PS on the external leaflet of the plasma membrane. High annexin-V reactivity was found in all odorant-exposed cultures (Fig. 6). Because of the variability in background annexin V values (3–30%) between different cell preparations, we have expressed the effects of the odorants as the relative changes with respect to the control in each preparation. When the cells were incubated with the odorants isobutyrate or ethylvanillin in the presence of the olfactory response inhibitor LY83583, the levels of apoptosis were not significantly different ($P > 0.05$; Kruskal–Wallis–Dunn test) from those under control condition (in the absence of odorant; Fig. 6). The effect of LY83583 of abolishing odorant-induced annexin-V exposure to the external leaflet of the plasma membrane strongly suggests that the apoptotic process observed in olfactory EC was triggered by a receptor-mediated odorant-induced mechanism.

2.6. Odorant-induced apoptosis revealed by caspase-3 activation

Activation of a caspase cascade is a clear and definitive indication that a cell has entered a PCD process. Specifically, caspase-3 activation (an executor caspase) can be considered as a no-return step in the apoptotic process. Because caspase activation is a definitive apoptotic index, we performed a more exhaustive study of this parameter, including controls with the different agents with which the cells were challenged. The percentage of cells exhibiting caspase-3 activation was

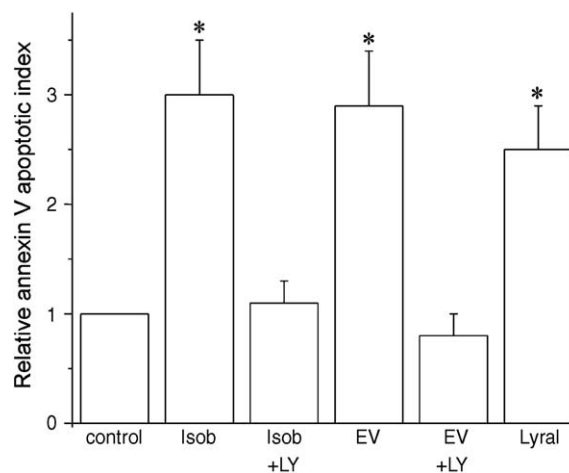


Fig. 6 – Apoptosis revealed by the presence of phosphatidylserine (PS) in the plasma membrane external leaflet. Effects of odorants (150 μ M) and 10 μ M LY83583 on PS translocation to the external leaflet of the plasma membrane was estimated by the Annexin V assay. The data are expressed as the relative apoptotic index of each treatment with respect to the control without odorant. Kruskal–Wallis–Dunn statistical analyses are shown (* $P < 0.05$).

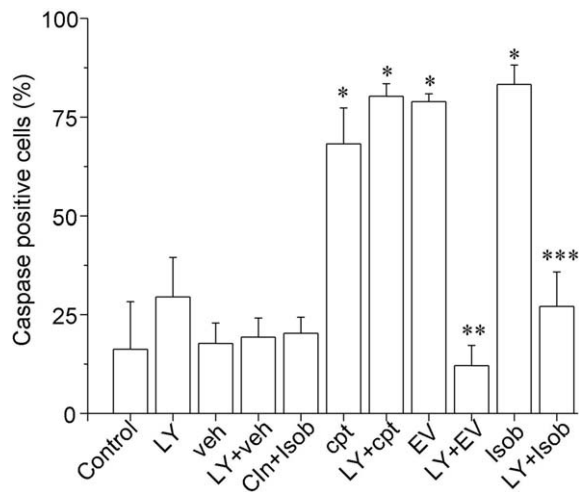


Fig. 7 – Apoptosis revealed by caspase-3 activation. The data were obtained after 3 h of exposure to the different conditions. Odorant, camptothecin, LY83583, and caspase inhibitor concentrations were (in μM) 150, 0.15, 10, and 2, respectively. The bars show the control (C) in the absence of odorant, LY83583, vehicle addition (veh), the reaction in the presence of caspase-3 inhibitor (Cln), LY83583 + veh, camptothecin (cpt), LY 83583 + camptothecin; ethyl vainillin (EV), LY83583 + EV, isobutyrate (Isob), LY83583 + Isob. Kruskal-Wallis-Dunn statistical analyses are shown (* $P < 0.05$ vs. control, ** $P < 0.05$ vs. EV, * $P < 0.05$ vs. Isob).**

significantly larger in odorant-exposed than in the control cultures ($P < 0.05$, Kruskal-Wallis-Dunn test) (Fig. 7). LY83583 prevented odorant-induced apoptosis in all odorant-exposed cell cultures. No significant differences between odorant supplemented with LY83583 treated cultures and control cultures were observed. LY83583 by itself had no effect on caspase-3 activation. Furthermore, LY83583 had no effect on caspase-3 activation induced by camptothecin, a well known inducer of apoptosis. These results confirmed that PCD was induced by odorants in olfactory EC, as suggested by DNA fragmentation and Annexin-V assays.

3. Discussion

The olfactory epithelium is a remarkable tissue, where a continuous neuronal cell renewal takes place throughout the life span of an individual (Mackay-Sim, 2003). The cellular response to odorants and the molecular mechanisms underlying it have been explored in a number of recent studies (e.g., Schild and Restrepo, 1998; Gibson and Garbers, 2000). However, less attention has been given to the association of odorants and cell death and renewal in this neuroepithelium. PCD has been described in the olfactory epithelium, especially as a consequence of the removal of the olfactory bulb (Nakagawa et al., 1996; Michel et al., 1997) or due to normal turn over (Cowan and Roskams, 2002; Suzuki, 2004). The relationship between cellular turnover and environmental odorant exposure has been previously explored (Maruniak et al., 1989; Farbman et al., 1988; Hinds et al., 1984). Although it seems clear that ORNs can die by the action of external noxious

stimuli, the role of extended odorant exposure on inducing PCD in ORNs and its possible relationship with excitotoxicity had not been previously investigated.

We were able to obtain a cultured cell preparation with $77 \pm 5\%$ of mature ORN cells, as judged by their reactivity to the anti-OMP antibody; $\sim 40\%$ of the OMP-positive cells retain their neuronal characteristics. It has been described that 12–33% of ORNs responded to a specific odorant (Sato et al., 1994; Duchamp-Viret et al., 1999; Gomez et al., 2000). However, in our experiments approximately 40% (range 32–45%) of the cells responded to a pure odorant, an observation that was likely due to the relatively high odorant concentration used in our protocols. We found that prolonged exposure to odorants triggered apoptosis in 57–83% of the cultured olfactory EC. Because we used three independent criteria to estimate PCD induction in these cells, we think that our results show unequivocally the existence of an apoptotic mechanism induced by odorants in olfactory EC. The fact that the fraction of apoptotic cells (approximately 80%) was much larger than the fraction of non-ORNs (approximately 25%) in culture strongly suggests that apoptosis was induced in an important fraction of ORNs. In fact, these numbers are in agreement with the idea that ORNs are the main apoptotic target of prolonged odorant exposure. This idea was confirmed both by the ability of LY83583 to significantly diminish the apoptotic action of the odorants, and the fact that apoptosis induced by camptothecin, an inhibitor of mammalian topoisomerase I (Hsiang et al., 1985), was not affected by LY83583. The effects of LY83583 strongly suggest that the odorant-induced PCD we observed is mediated by the transduction mechanism of the sensory neurons. Thus, our *in vitro* data provide good evidence that olfactory EC can be induced to undergo apoptosis by prolonged and continuous odorant exposure. Given the fact that LY83583 can decrease the odorant-, but not the camptothecin-induced apoptosis, our results can only be explained if an important fraction of the apoptotic cells are ORNs.

It has been shown that $[\text{Ca}^{2+}]_i$ higher than 350 nM can trigger a PCD process in neurons (Collins et al., 2001; Ferri and Kroemer, 2001). Hence, the existence of a population of cells that presented an odorant-dependent high $[\text{Ca}^{2+}]_i$ change among the cells exposed to the odorants suggests that an increase in $[\text{Ca}^{2+}]_i$ was involved in the odorant-induced PCD observed in our studies. This odorant-induced $[\text{Ca}^{2+}]_i$ increase was inhibited by LY83583, also hinting that the odorant induced PCD and odorant induced increase in $[\text{Ca}^{2+}]_i$ were linked. We attempted to test the hypothesis that $[\text{Ca}^{2+}]_i$ increases were responsible for the induction of apoptosis by loading the cells with the Ca^{2+} chelator BAPTA (using BAPTA-AM), but failed due to the cytotoxic effects of this compound on olfactory EC.

In vivo, Watt et al. (2004) have shown that ORNs over-expressing an octanal receptor-GFP protein are protected from death by intermittent exposure to octanal. Instead, Carr et al. (2001) have shown that exposure to continuous high odorant concentrations induced death in both ORNs and supporting cells. Our data strongly indicate that ORNs undergo an excitotoxic-like apoptotic death *in vitro*. We think that our results can be taken as a basis to understand the apparently more complex and sometimes contradictory results obtained *in vivo*, that most likely are reflecting differences between intermittent and continuous odorant exposure as well as the complexity of cell-cell interactions in the olfactory epithelium.

Furthermore, it has been observed that following long-term odorant exposures (days), several days are needed for *in vivo* recovery of odor-sensitivity. This observation has been interpreted as odor-induced adaptation, a phenomenon defined as a decrease in odor sensitivity following repetitive or continuous exposure to the same odorant (Dalton, 2000, Yee and Wysocki, 2001). Our finding that ORNs undergo apoptosis during prolonged odor exposures offers an alternative explanation for this phenomenon in terms of death and replacement of functional ORNs, particularly considering that the recovery time of odor sensitivity *in vivo* is similar to the time that germinal epithelial basal cells take to proliferate and differentiate into a new (naive) population of cells after damage (Dalton, 2000; Herzog and Otto, 1999).

4. Experimental procedures

4.1. Cell preparation and culture

Nasal olfactory mucosa was obtained from two or three adult male Wistar rats after cervical dislocation and decapitation, according to the guidelines of the Animal Ethics Committee of the University of Chile, Santiago, Chile. The epithelia were dissected and placed in Krebs–Henseleit buffer (KH; in mM: 144 NaCl, 4.2 KCl, 1.6 MgCl₂, 1.6 KH₂PO₄, 10 HEPES, 300 mosM, pH 7.4) supplemented with 1.8 mg/ml glucose. A cell preparation suitable for giving a high yield of ORNs was developed. In brief, the epithelial tissue was placed in a Hanks balanced solution with dispase II (4.8 U/ml, Sigma-Aldrich, USA). The tissue after the enzyme digestion was mechanically disrupted to separate adult ORNs. Large pieces of tissue were removed with tweezers and the cell suspension was pipetted repeatedly with a fire polished Pasteur pipette. The cell suspension was pelleted at 340 × *g* for 5 min and the resulting pellet was suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine fetal serum, penicillin (100 U/ml) and streptomycin (0.1 mg/ml). The cells were cultured on 12 mm cover slips pre-coated with pegotone (a bioadhesive, Bios Chile, Santiago, Chile), at 2–2.5 million cells per cover, with three cover slips per well for 48 h at 33 °C in 95% air/5%CO₂. This preparation gave a high percentage of OMP positive cells (77%, see Fig. 1 and Results).

4.2. Immunocytochemistry

The proportion of mature ORNs was estimated using a polyclonal anti-olfactory marker protein (OMP) goat-anti-rat antibody (Wako Chemicals USA Inc., Richmond, USA). The OMP is present only in mature ORNs (Farbman and Margolis, 1980). After cell culture, the cells were fixed in 5% paraformaldehyde for 15 min at 4 °C. The fixative was washed out and -on specific sites were blocked with 3% BSA for 3 h. Subsequently, the ORNs were incubated with the primary antibody in the presence of BSA (3%) at 4 °C for 1 h. After washing them with PBS, the cells were incubated with a secondary antibody in the presence of 3% BSA at 4 °C for 30 min. Fluorescence was analyzed using a Zeiss Axiovert 200 M epifluorescence microscope (Oberkochen, Germany) (excitation wavelength 340–380 nm, emission wavelength >410 nm) and a 40× objective lens. Primary antibodies used were polyclonal goat anti-rat OMP (1:10,000) and monoclonal mouse anti-rat neurofilament

200 (1:500; Sigma-Aldrich, USA). Secondary antibodies used were rabbit anti-goat IgG (1:500) and rabbit anti-mouse IgG (1:200) coupled with AlexaFluor-488 and AlexaFluor-594, respectively (Molecular Probes, USA). The nucleus was counterstained using Hoescht 33342 (H₃₃₃₄₂, Molecular Probes, USA). Controls were performed omitting the primary antibody and treating the sample with pre-immune goat serum.

4.3. Apoptosis induction

Cultured cells were exposed to odorants for 3 h. We utilized the odorants isobutyrate (2-methylpropanoic acid and ethylvanillin (3-ethoxy-4-hydroxybenzaldehyde) (Sigma Chemical Co., St Louis, MO, USA), a putrid and floral odorant, respectively, at final concentrations of 150 μM. This value was chosen in order to expose the olfactory EC to a high odorant concentration, but within the concentration range commonly used in physiological studies (e.g., Ma and Shepherd, 2000; Zufall and Leinders-Zufall, 2000). The odorant stock solutions were prepared in ethanol (EtOH) (ethyl vanillin) or KH buffer (isobutyrate) and added to the culture medium at a final EtOH concentration of 0.4%. Several controls were designed for caspase measurements: (1) no addition, (2) vehicle addition (EtOH 0.4%), and (3) positive control (camptothecin, 0.15 μM, Sigma Chemical Co., St Louis, MO, USA) (Hsiang et al., 1985). The odorant response inhibitor LY83583 (10 μM) was used in order to test the specificity of odorant PCD induction. This compound effectively inhibits the cyclic nucleotide-gated channel ($K_d = \sim 1.4 \mu\text{M}$), as well as guanylyl cyclase III (Leinders-Zufall and Zufall, 1995), two essential components of the odorant transduction cascade.

4.4. Determination of cellular DNA fragmentation

In order to detect the type of DNA fragmentation that is specific in apoptosis, we utilized the *In Situ* Oligo Ligation method (ApoTag ISOL, Q-BIOgene, UK). The ISOL method is based upon the specificity of the enzyme T4 DNA ligase (Weiss et al., 1968); in this method, synthetic nucleotides labeled with biotin selectively react with the specific types of genomic DNA ends (blunt and short single base ends) characteristics of apoptotic cells. This reaction is followed by addition of a streptavidin–peroxidase conjugate that binds tightly to the biotin on the oligonucleotide. Lastly, the addition of DAB (diaminobenzidine), a chromogenic peroxidase substrate, causes a brown precipitate that in this work was visualized using bright field microscopy. The ISOL reaction leads to a significantly smaller number of false-positive results in comparison to terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) and *in situ* end labeling (ISEL) (Lesauskaite et al., 2004). DNA fragmentation estimated by TUNEL assay has been utilized previously as an apoptotic criterion in olfactory epithelium (Michel et al., 1997). In these experiments, we utilized two cell preparations, 2 plates (coverslips) per preparation and selected at least 3 fields per plate, counting 50–87 cells/field. This procedure was repeated for every condition tested.

4.5. Annexin-V labeling assay

An early indicator of apoptosis is the translocation of phosphatidylserine (PS), from the internal to the external

leaflet of the plasma membrane. In order to detect phosphatidylserine exposition to the outer leaflet, we used an Annexin-V-FLUOS kit for apoptosis detection (Boehringer-Mannheim, Werk Penzberg, Germany). After carefully washing out the culture medium, the cells were incubated with annexin-V solution and propidium iodide for 15 min at room temperature. After this incubation, the fluorescent cells were analyzed using a NIKON Diaphot microscope with the appropriate set of filters for fluorescein and propidium iodide. This method allowed us to differentiate apoptotic from lysed cells. Apoptotic cells presented green membranes, and lysed cells presented green membranes and red nuclei (Fadok et al., 1992). Non-apoptotic cells did not fluoresce. Because lysed cells could have previously undergone apoptosis, the annexin V assay gives a minimum estimate of apoptosis. In these experiments, we utilized 3 cell preparations, 3 plates (coverslips) per preparation and selected at least 3 fields per plate, counting 28–47 cells/field, a procedure that was repeated for every condition.

4.6. Determination of caspase-3 activation

A protease (caspase) cascade is one of the essential aspects of PCD. Activation of caspase-3 implies an irreversible commitment to cell death in the PCD process (e.g., Adrain and Martin, 2001). We estimated caspase-3 activation using a specific substrate (PhiPhilux™ G₂D₂) from Calbiochem (CA, USA). In this assay, a seven residue long peptide is linked to a fluorescent probe that yields an intense red fluorescence signal when it is cleaved by the enzyme (Komoriya et al., 2000). After 60 min of incubation in culture conditions with the caspase-3 substrate, the cells were carefully washed and their fluorescence was analyzed using a NIKON Diaphot epifluorescence microscope (excitation wavelength 510–550 nm, emission wavelength >570 nm). In some measurements, a caspase-3 inhibitor (Z-DEVD-FMK, Calbiochem, USA) was used to estimate the specificity of the caspase-3 reaction. In these experiments, we utilized 3 cell preparations, 3 plates (coverslips) per preparation and selected at least 3 fields per plate, counting at least 43–310 cells/field. Such a procedure was repeated for every condition.

4.7. Dynamic changes of [Ca²⁺]_i induced by odorants in olfactory EC

In order to correlate the effects of the odorants on apoptosis and the changes in [Ca²⁺]_i, it was important to characterize first if the isolated cells [Ca²⁺]_i responded to the addition of odorants. Fluo-3 AM (Molecular Probes, Eugene, OR) was used to analyze the acute response of [Ca²⁺]_i in ORNs to 150 μM odorant addition, and the effect of LY83583 on this response. Freshly isolated cells were incubated with Fluo-3 AM (5 μM) for 30 min at 33 °C. The probe was washed out from the dish and fluorescence images were acquired with a cooled CCD camera (SpectraSource, Los Angeles, USA) (excitation wavelength 440–480, emission wavelength > 510) in an inverted Olympus IX-70 fluorescence microscope (40× objective lens). For the LY83583 assays, the cells were incubated with 10 μM LY83583 between 30 and 120 s prior to odorant addition. The measurements are presented as the percentage of Fluo-3 fluorescence changes ($\Delta F/F_0$), where ΔF corresponds to the difference between the time-dependent fluorescence after odorant addition and the basal (F_0) average value 1 min before odorant addition. In

single-cell [Ca²⁺]_i measurements, photo bleaching of the fluorescent probe was reduced by using a 25% transmittance neutral density filter and, when present, it was corrected using a first order exponential decay function. The measurements were performed in three different cell preparations and 10–15 cells were tested per condition.

4.8. [Ca²⁺]_i measurements in cells chronically exposed to odorants

In order to analyze the steady state levels of [Ca²⁺]_i in cells exposed to the odorants for a prolonged time, we conducted single-cell [Ca²⁺]_i measurements. After being exposed to the odorants for 3 h, the cells were incubated with 5 μM Fluo-3 AM for 30 min. Following wash-out, calibration of the Fluo-3 signal was performed as described by Kao et al. (1989). In brief, addition of ionomycin (5 μM) was followed by Mn²⁺ (2 mM) and ended with digitonin to permeabilize the cells. These experimental conditions allowed us to estimate F_{\max} , F_{\min} , and $F_{\text{background}}$, as described by Kao et al. (1989). In these experiments, we utilized two cell preparations, 2 coverslips per preparation and selected at least 3 fields per plate, determining [Ca²⁺]_i in 7–19 cells/coverslip. This procedure was repeated for every condition.

4.9. Data acquisition and analysis

Calcium imaging was accomplished using an Olympus IX-70 epifluorescence inverted microscope with a cooled 16 bit CCD camera model MCD-220 (SpectraSource Instruments, CA, USA), attached to the videoport of the microscope. Images were digitized with SpectraSource software and analyzed with Scion Image for Windows (Scion Corporation, USA). Pictures were acquired by an Axiocam photcamera (Zeiss, Oberkochen, Germany), and processed with Adobe Photoshop (Adobe Systems Incorporated, USA). Data analysis was performed with Microcal Origin (Microcal Software Inc., MA, USA). GraphPAD InStat version 1.1 (GraphPAD software, USA) was used for statistical analysis. This analysis included ANOVA and Bonferroni test. Kruskal–Wallis non-parametric test followed by Dunn's multiple comparison test was applied to the data in order to determine differences in apoptosis. Each cell preparation was derived from at least two rats.

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