

Identification and characterization of the bombykal receptor in the hawkmoth

Manduca sexta

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Summary statement

The receptor complex MsexOr1 and MsexOrco responds to the sex pheromone bombykal when expressed in heterologous systems (*Xenopus* oocytes, HEK293 cells, CHO cells).

Abstract

Manduca sexta females attract their mates with the release of a species-specific sex-pheromone blend with bombykal (*E,Z*)-10,12-hexadecadienal and (*E,E,Z*)-10,12,14-hexadecatrienal being the two major components. Here, we searched for the hawkmoth bombykal receptor in heterologous expression systems. The putative pheromone receptor MsexOr1 co-expressed with MsexOrco in *Xenopus* oocytes elicited dose-dependent inward currents upon bombykal application (10 - 300 μ M), and coexpressed in HEK293 and CHO cells caused bombykal-dependent rises in the intracellular free Ca²⁺ concentration. Also the bombykal receptor of *Bombyx mori* BmOr3 coexpressed with MsexOrco responded to bombykal (30 - 100 μ M) with inward currents. In contrast, MsexOr4 coexpressed with MsexOrco neither responded to bombykal (30 - 100 μ M) nor to the (*E,E,Z*)-10,12,14-hexadecatrienal mimic. Thus, MsexOr1, but probably not

MsexOr4, and also not MsexOrco, is the bombykal-binding pheromone receptor in the hawkmoth. Finally, we obtained evidence that phospholipase C- and protein kinase C- activity is involved in the hawkmoth's bombykal-receptor-mediated Ca^{2+} signals in HEK293 and CHO cells.

Introduction

Crepuscular and nocturnal moths developed exquisitely sensitive olfaction to locate their mates (review: (Kaissling, 2014)). Females of the nocturnal hawkmoth *Manduca sexta* release pulses of a species-specific sex-pheromone blend of eight aliphatic aldehydes, while calling for their mates. Bombykal (*E,Z*)-10,12-hexadecadienal (BAL) and (*E,E,Z*)-10,12,14-hexadecatrienal are the two major blend components necessary to evoke male reproductive behavior (Starrat et al., 1979); (Tumlinson et al., 1989); (Tumlinson et al., 1994). Conspecific males detect their species-specific sex-pheromone blend with olfactory receptor neurons (ORNs) on their antennae (review: (Homborg et al., 1989); (Kaissling et al., 1989)). The ORNs express odorant receptors (Ors) in their dendritic cilia that innervate hair-like, long trichoid sensilla (Altner and Prillinger, 1980); (Keil and Steinbrecht, 1984). Odorant-binding Ors (OrX) in insects belong to a new family of 7TM proteins with inverted membrane topology, with intracellular N-terminus. They heteromerize with a larger, ubiquitous, inverted 7TM protein termed olfactory receptor coreceptor (Orco) that locates and maintains Ors in the ciliary membranes of ORNs (Clyne et al., 1999); (Vosshall et al., 1999); (Larsson et al., 2004); (Benton et al., 2006); (Vosshall and Hansson, 2011). In addition to this "chaperon-like function" Orco forms a non-specific, spontaneously opening Ca^{2+} -permeable cation channel in heterologous expression systems (Sato et al., 2008); (Wicher et al., 2008); (Jones et al., 2011); (Sargsyan et al., 2011); (Nolte et al., 2013). Since ORNs in Orco mutant flies showed strongly diminished spontaneous action potential activity, Orco controls the membrane potential and, thus, spontaneous activity of ORNs as prominent leak current (Larsson et al., 2004); (Benton et al., 2007); (Deng et al., 2011). Orco's property as leaky ion channel controlling spontaneous membrane potential oscillations was termed previously "pacemaker channel function" to distinguish it from the "chaperon function" of Orco (Stengl, 2010). Furthermore, experiments with heterologously expressed OrX/Orco

complexes suggested that heteromers of OrX and Orco are able to form ligand-gated ion channels (German et al., 2013); (Nakagawa et al., 2012); (Sato et al., 2008); (Wicher et al., 2008) that promote ionotropic primary transduction (Sato *et al.* 2008; Wicher *et al.* 2008), while there is slower metabotropic transduction at a later stage of the fruitfly's odor response (Wicher *et al.* 2008). Whether and how Orco contributes to odor transduction *in vivo* in different insect species is still under debate (Nakagawa and Vosshall, 2009); (Nolte et al., 2013); (Nolte et al., 2016).

Sensitization of odor responses in intact fruitflies relied on proper Orco function which was impaired with insufficient Orco phosphorylation by protein kinase C (PKC) (Sargsyan et al., 2011). Thus, for sensitization to occur, first, Orco needed to be phosphorylated via PKC, before it could be opened directly via cAMP-binding. Furthermore, *D. melanogaster* Orco can already be gated via phospholipase C (PLC) activation, even in absence of cAMP or cGMP (Sargsyan et al., 2011). It remained unknown whether odor stimulation first activates PLC for proper Orco phosphorylation or whether background activity of PLC keeps Orco phosphorylated. Patch clamp studies on primary cell cultures of *M. sexta* ORNs as well as tip recordings of pheromone-sensitive sensilla in intact hawkmoths indicated that different PLC and PKC dependent ion channels in ORNs play important roles in pheromone transduction, but it remained un-discerned whether any of these many ion channels is Orco (Stengl, 1993); (Stengl, 1994); (Stengl, 2010); (Stengl et al., 1992). In addition, it was not rigorously examined yet, whether also in heterologous expression OrX/Orco-dependent odor responses were mediated via Ca²⁺-dependent enzymes such as PLC and PKC.

As a prerequisite to answer open questions concerning hawkmoth pheromone transduction, first, it needs to be determined whether any of the two putative hawkmoth pheromone receptor proteins, MsexOr1 and MsexOr4 responds to the main sex pheromone component BAL (Grosse-Wilde et al., 2011). It was noticed before by different research groups that it was very difficult to get functional membrane insertion of MsexOrco and MsexOrX proteins, even more difficult than in other insect species. Therefore, the present study was dedicated to identify the BAL receptor in *M. sexta* while employing different expression systems. Furthermore, since MsexOrco is Ca²⁺-permeable and increases intracellular Ca²⁺ levels in the cells where it opens, and since

MsexOrX and MsexOrco proteins possess putative PKC phosphorylation sites, we asked whether manipulation of the Ca²⁺-dependent enzymes PLC and PKC may modify BAL response in different heterologous expression systems.

Materials and Methods

Odor stimulation

For the de-orphanization experiments (*E,Z*)-10,12-hexadecadienal (BAL) was employed at concentrations of 100 nM to 300 μM. To test the specificity of the hawkmoth pheromone receptor the female silkworm sex pheromone bombykol (10*E*,12*Z*)-hexadeca-10,12-dien-1-ol (BOL) was used at a concentration of 100 μM for comparison. Since the trienal (*E,E,Z*)-10,12,14-hexadecatrienal is a relatively unstable substance a more stable chemical mimic, (*E,Z*)-11,13-pentadecadienal (*E*11,*Z*13-15:AL) (*C*15) was employed (Christensen and Hildebrand, 1997).

Cell culture and calcium imaging

Human embryonic kidney (HEK293) cells or Chinese hamster ovary (CHO) cells (Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were grown in a 1:1 mixture of Dulbecco's Modified Eagle Medium and Ham's F-12 medium with L-glutamine (PAA, Cölbe, Germany) containing 10 % FBS (PAA) at 37°C and 5 % CO₂ content. HEK293 cells (Fig. 2, 3) or CHO cells (Fig. 3) were plated on poly-L-lysine (0.01 %, Sigma-Aldrich, Steinheim, Germany) coated coverslips in 24 well plates and cultured at a density of ~10⁵ per well. The *M. sexta* OR constructs MsexOrco and MsexOr1 (Grosse-Wilde et al., 2010) were transfected at a 0.5 - 0.7 μg/well concentration using Transficient (MBL International Corporation, Woburn, USA).

For calcium imaging, cells were incubated in bath solution containing 5 μM fura-2 acetomethylester (Molecular Probes, Invitrogen, Eugene, USA) for 30 min. Excitation of fura-2 at 340 and 380 nm was performed with a monochromator (Polychrome V, T.I.L.L. Photonics, Gräfelfing, Germany) coupled via an epifluorescence condenser into an Axioskop FS microscope (Carl Zeiss, Jena, Germany) with a water immersion objective (LUMPFL 40xW/IR/0.8; Olympus, Hamburg, Germany). Emitted light was separated by

a 400 nm dichroic mirror and filtered with a 420 nm long-pass filter. Free intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was calculated according to the equation

$$[\text{Ca}^{2+}]_i = K_{eff} \frac{R - R_{min}}{R_{max} - R} \cdot K_{eff}, R_{min}, \text{ and } R_{max} \text{ were determined as described in Mukunda et al. (2014).}$$

Fluorescence images were acquired using a cooled CCD camera controlled by TILLVision 4.0 software (T.I.L.L. Photonics). The resolution was 640x480 pixels in a frame of 175x130 μm (40x/IR/0.8 objective). Image pairs were obtained by excitation for 150 ms at 340 nm and 380 nm; background fluorescence was subtracted. Cells were continuously perfused with bath solution in the recording/perfusion chamber (RC-27, Warner Instruments Inc., and Hamden, CT, USA). The bath solution contained (in mM): 135 NaCl, 5 KCl, 1 CaCl_2 , 1 MgCl_2 , 10 HEPES, 10 glucose; pH = 7.4; osmolarity = 295 mOsmol/l. BAL and the modulators were applied using either a bath perfusion system (BPS4 from ALA, New York, USA) or a rapid solution changer (RSC160 from Biologic, Claix, France). BOL was obtained from Pheropbank (Wijk bij Duurstede, Netherlands); U73122, U73343 and Gö6976 were obtained from Calbiochem (Darmstadt, Germany). BAL was synthesized by the working group “Mass Spectrometry/Proteomics” of the Max-Planck Institute for Chemical Ecology (Jena, Germany). BAL, BOL and C15 were dissolved and applied in 0.01 % dimethylsulfoxide (DMSO). For the analysis only responding cells were used. Responses are characterized by a clear BAL-stimulation related increase in $[\text{Ca}^{2+}]_i$.

Functional analysis of ORs in *Xenopus laevis* oocytes

cRNA synthesis, oocyte microinjection, and two- electrode voltage-clamp recording were performed as previously described (Hopf et al., 2015). Briefly, oocytes were microinjected with 6.25 ng cRNA encoding a ligand tuning OR (MsexOr1, MsexOr4 or BmOr3) and 6.25 ng cRNA encoding Orco cRNA (MsexOrco). Currents were recorded and amplified using an OC-725C amplifier (Warner Instruments, Hamden, CT, USA). Before electrophysiological recording, BAL was prepared in dimethylsulphoxide as 10 mM to 300 mM stock solutions and C15 as 1 mM to 10 mM stock solutions, which were then diluted into the bath solution at 0.1% to give the final desired concentration.

Results and Discussion

To identify *M. sexta*'s BAL receptor, the pheromone receptor candidates MsexOr1 and MsexOr4 (Grosse-Wilde et al., 2010) were coexpressed with MsexOrco first in *Xenopus* oocytes and then in HEK293 and CHO cells. Responses to different pheromone components were examined with two electrode voltage clamp recordings or with Ca²⁺ imaging.

Only MsexOr1 and BmOr3 but not MsexOr4 respond to BAL

In *Xenopus* oocytes expressing MsexOrco and MsexOr1 BAL stimulation activated inward currents concentration-dependently with a threshold concentration of 30 µM (**Fig. 1A**). In addition, co-expression of MsexOrco with the BAL receptor BmOr3 from the silkworm *Bombyx mori* also allowed for BAL-induced inward currents (**Fig. 1B**). The maxima of BmOr3-mediated BAL-responses were higher than the MsexOr1-dependent responses, hinting for better membrane insertion of the silkworm BAL receptor. In contrast to MsexOr1, co-expression of the second pheromone receptor candidate MsexOr4 with MsexOrco did not elicit BAL-induced inward currents (**Fig. 1C**). Since only the highest BAL concentration tested elicited very small responses it is likely that MsexOr4 is indeed expressed in the plasma membrane, but that it is not specific for BAL. To examine whether MsexOR4 expressed in oocytes responds to minor pheromone components such as (*E,E,Z*)-10,12,14-hexadecatrienal it was stimulated with the more stable mimic (*E,Z*)-11,13-pentadecadienal at concentrations of 1 µM and 10 µM. However, no specific responses were obtained at all concentrations tested (**Fig. 1D**).

Thus, further investigations focused on MsexOr1 expression. To further examine the sensitivity of the hawkmoth BAL-receptor, MsexOrco and MsexOr1 were expressed in HEK 293 cells and Ca²⁺ imaging was employed, to monitor receptor activation. While for femto- to nanomolar BAL concentrations the percentage of cells showing a response to stimulation did not significantly differ from the response to solvent control (0.1 % DMSO, median: 0.0 %), for 1 µM BAL there was a significantly higher percentage of responding

cells (median: 6.2 %)(**Fig 2A,B**). To examine the selectivity of MsexOrco and MsexOr1 to the hawkmoth pheromone, the receptor complex was stimulated with bombykol (BOL), the sex pheromone of female *Bombyx mori*. Application of 100 μ M BOL produced only weak responses in comparison to 100 μ M BAL (**Fig. 2C**).

Since only coexpression of MsexOrco with MsexOr1 but not with MsexOr4 conferred BAL sensitivity, MsexOr1 is the BAL-ligand binding subunit. Since it was very difficult to obtain any plasma membrane expression of all subunits tested it was not examined whether MsexOr1 expression alone is sufficient to elicit BAL responses, as was found for OrX proteins from other species (Wetzel et al., 2001); (Sakurai et al., 2004); (Nakagawa et al., 2005); (Neuhaus et al., 2005); (Grosse-Wilde et al., 2006); (Smart et al., 2008); (Deng et al., 2011). It still remains elusive whether MsexOr4 would detect minor components of the *M. sexta* pheromone blend. Since application of (*E,Z*)-11,13-pentadecadienal (up to 10 μ M), which mimics (*E,E,Z*)-10,12,14-hexadecatrienal, did not activate a current in oocytes expressing MsexOr4 + MsexOrco constructs, the respective pheromone receptor of the second behaviorally relevant main pheromone compound still needs to be identified in the hawkmoth (Tumlinson et al., 1989); (Tumlinson et al., 1994); (Kaissling et al., 1989). Alternatively, the lack of odor responsiveness could also indicate that MsexOr4 was not inserted into the plasma membrane. However, the weak response to high BAL concentration (Fig. 1C) indicates at least a minimum of membrane expression of MsexOr4.

MsexOrco and MsexOrX proteins need additional molecular compounds for stable plasma membrane insertion

As compared to heterologous expression of Orco and OrX proteins from other species it was considerably more difficult to express *M. sexta* receptors in the plasma membrane of the different heterologous expression systems employed (**Fig. 1A-B**). Apparently, more molecules next to the chaperon Orco are necessary to insert MsexOrX proteins in the plasma membrane. In *D. melanogaster*, pheromone receptors are composed of the Orco/OrX construct and a sensory neuron membrane protein (SNMP) (Benton et al., 2007; Jin et al., 2008; Li et al., 2014). Furthermore, interactions between SNMP-1 and

ligand binding Ors, but not with Orco were found in FRET- experiments (German et al., 2013). The antennal transcriptome of *M. sexta* contains the message of SNMP-1 (Grosse-Wilde et al., 2011) which is also known to be expressed in pheromone receptor expressing sensory neurons in *A. polyphemus* and *H. virescens* (Forstner et al., 2008). This could indicate that SNMP-1 might be part of the *M. sexta* BAL receptor complex, necessary for plasma membrane insertion. Coexpression of MsexOr1 + MsexOrco with SNMP-1 in HEK293 cells allowed for stronger Ca^{2+} responses upon stimulation with the synthetic Orco agonist VUAA1 (Jones et al., 2011) as compared to cells expressing only MsexOrco (Nolte et al., 2013). However, still in the presence of SNMP together with MsexOrco and MsexOr1 only few HEK293 cells appeared to express these molecules in the plasma membrane, allowing for Orco activation via its agonist VUAA1. Therefore, there are still more, unknown molecular components necessary for stable plasma membrane insertion and maintenance.

PLC and PKC dependent regulation of BAL responses in heterologous expression systems

Next, a possible dependence of the BAL response on PLC or PKC activity was examined, as reported previously for hawkmoth pheromone transduction (Stengl, 1993); (Stengl, 1994); (Stengl, 2010); (Dolzer et al., 2008) or for fruitfly Orco (Sargsyan et al., 2011); (Getahun et al., 2013). Thus, Ca^{2+} imaging experiments were performed in HEK293 cells expressing MsexOrco and MsexOr1. Application of 100 μM BAL induced an immediate, transient rise of the free intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) from 100 to 500 nM (**Fig. 3A**). It should be noted that the response to BAL in HEK293 is much slower than that in oocytes.

The rise in $[\text{Ca}^{2+}]_i$ in response to BAL application was strongly reduced and delayed by the PLC inhibitor U73122 (**Fig. 3A, B**). Within 250 s after BAL stimulation, there was a slow rise in $[\text{Ca}^{2+}]_i$ without reaching a clear peak in the presence of U73122. By contrast, the inactive analog U73343 had only minor effects (**Fig. 3A, B**). Also, PKC inhibition with Gö6976 significantly reduced and delayed BAL-induced $[\text{Ca}^{2+}]_i$ responses (**Fig. 3A, B**). Thus, BAL responses depended on PLC, as well as on PKC activity.

To exclude the possibility that the effect of PLC/PKC inhibition on BAL-induced Ca^{2+} responses was a specific property of the expression system, comparative experiments were performed in CHO cells. As shown for HEK293 cells BAL application elicited a robust Ca^{2+} response in CHO cells expressing MsexOrco and MsexOr1. Preincubation of cells with U73122 or Gö6976 nearly abolished the BAL-dependent rise in $[\text{Ca}^{2+}]_i$ (**Fig. 3C, D**).

Thus, PLC/PKC activity is a prerequisite to BAL receptor mediated Ca^{2+} signals in HEK293 and CHO cells. This finding could be explained by different hypotheses. It could be hypothesized that MsexOrs and MsexOrco need to be phosphorylated, before they can be gated by BAL as an ionotropic odor receptor-ion channel complex, as suggested previously in *D. melanogaster* (Sargsyan et al., 2011); (Getahun et al., 2013). Alternatively, it could be hypothesized that MsexOrs couple to $G_{\alpha q}$ -proteins requiring activation of PLC for pheromone transduction, as suggested by previous findings in moths and cockroaches (Breer et al., 1990); (Boekhoff et al., 1993);(Boekhoff et al., 1994); review: (Stengl, 2010). More experiments in different insect species are necessary to resolve the still unsolved puzzle of insect odor/pheromone transduction.

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Competing interests

No competing interests declared.

Author contributions

S.M., NF and LH performed the experiments, DW, MS and KT analyzed the data, DW and MS wrote the manuscript. All authors contributed to the design of the study and to the final version of the manuscript.

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Figures

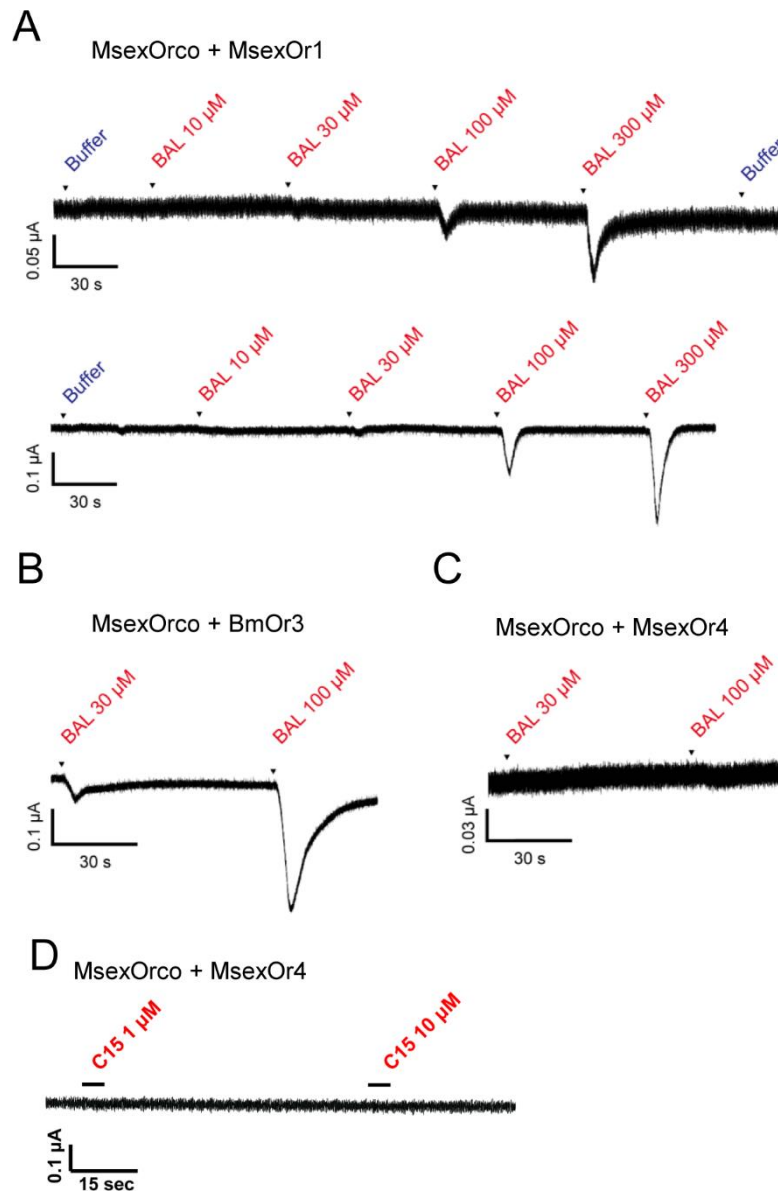


Fig. 1. Bombykal (BAL) stimulation activates a dose-dependent inward current in *Xenopus* oocytes expressing MsexOrco + MsexOr1. Representative current traces of oocytes injected with the indicated cRNAs at a holding potential of -80 mV. The arrowheads mark the application of BAL (10 μ M, 30 μ M, 100 μ M, 300 μ M) or control solution (buffer). **(A)** Oocytes coexpressing MsexOrco and MsexOr1 showed a dose-

dependent inward current in response to BAL stimulation with a threshold concentration of about 30 μM . **(B)** Even stronger responses to BAL stimulation were recorded from oocytes coexpressing MsexOrco and BmOr3, the BAL receptor of the silkworm *B. mori*. **(C)** In contrast, coexpression of MsexOrco and the putative pheromone receptor MsexOr4 did not allow for BAL responses. **(D)** Oocytes coexpressing MsexOrco and MsexOr4 showed no response to C15 (1 and 10 μM).

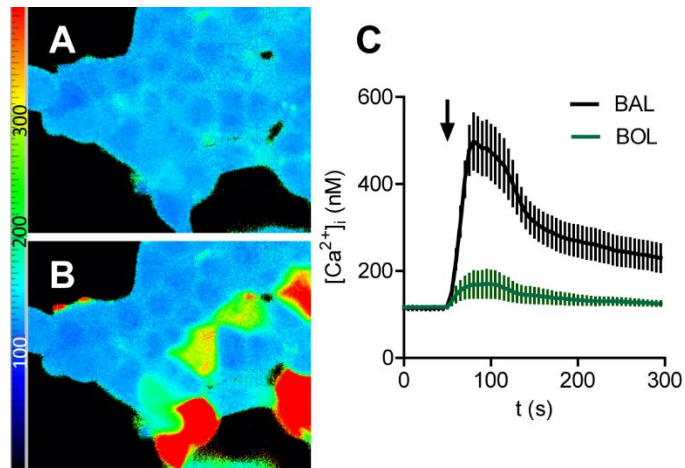


Fig. 2. Test for functional expression of MsexOrco and MsexOr1 in HEK293 cells by means of Ca^{2+} imaging. **(A, B)** Images of $[\text{Ca}^{2+}]_i$ in HEK293 cells expressing the pheromone receptor before (A) and after (B) stimulation with $100 \mu\text{M}$ BAL. Color code gives nM $[\text{Ca}^{2+}]$. **(C)** Time course of the free intracellular Ca^{2+} concentration in response to $100 \mu\text{M}$ BAL ($n = 24$) or $100 \mu\text{M}$ BOL ($n = 50$) stimulation at 50 s. Mann Whitney test, *** $p < 0.001$.

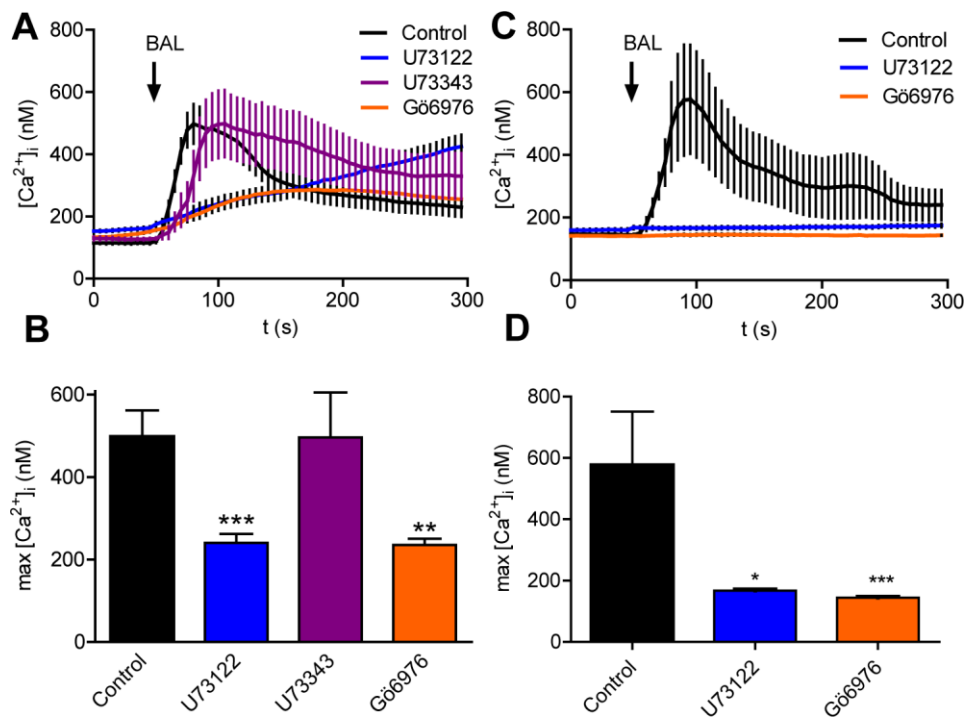


Fig. 3. Inhibition of PLC and PKC affects the BAL response in HEK293 (**A, B**) or CHO (**C, D**) cells coexpressing MsexOrco + MsexOr1. (**A**) Ca^{2+} responses in HEK 293 cells obtained by stimulation of MsexOr1 + MsexOrco with 100 μ M BAL (arrow) under control conditions ($n = 24$) and in the presence of the PLC inhibitor U73122 (10 μ M, $n = 44$), its inactive analog U73343 (10 μ M, $n = 14$) or of the PKC inhibitor Gö6976 (1 μ M, $n = 34$). $[Ca^{2+}]_i$ was determined with Ca^{2+} imaging using the dye fura2. (**B**) The maximum Ca^{2+} rise within 50 s after stimulation with 100 μ M BAL under control conditions and in the presence of PLC/PKC inhibitors. PLC inhibition and PKC inhibition reduces the fast BAL-induced rise in $[Ca^{2+}]_i$. Kruskal-Wallis test with Dunn's Multiple Comparison Test against Control; *** $p < 0.001$; ** $p < 0.01$; ns $p > 0.05$, not significant. (**C**) Ca^{2+} responses in CHO cells obtained by stimulation of MsexOr1 + MsexOrco with 100 μ M BAL (arrow) under control conditions ($n = 14$) and in the presence of the PLC inhibitor U73122 (10 μ M, $n = 24$) or of the PKC inhibitor Gö6976 (1 μ M, $n = 36$). (**D**) The maximum of the Ca^{2+} rise upon stimulation with 100 μ M BAL under control conditions and in presence of PLC/PKC inhibitors. PLC inhibition and PKC inhibition reduces the

fast BAL-induced rise in $[Ca^{2+}]_i$. Kruskal-Wallis test with Dunn's Multiple Comparison Test against Control; *** $p < 0.001$; * $p < 0.05$.