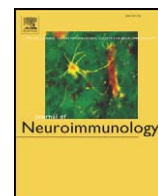




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Immunoglobulin-free light chains mediate antigen-specific responses of murine dorsal root ganglion neurons

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ABSTRACT

Immunoglobulin-free light chains (IgLC) secreted by B lymphocytes, have been shown to mediate hypersensitivity by inducing antigen-specific mast cell activation. Although both mast cells and sensory neurons contribute to the hypersensitivity response, the role of IgLC in relation to sensory neurons is unknown. We therefore aimed to investigate the effects of IgLC on cultures of murine dorsal root ganglion (DRG) neurons. Immunohistochemistry demonstrated that IgLC and IgE could specifically bind to DRG neurons, on which the presence of FcεRI, the specific receptor for IgE, was demonstrated by western blotting. Further, optical recordings with Fluo-4 showed that application of the corresponding antigen to IgLC- or IgE-sensitized DRG neurons induces a sustained increase in intracellular Ca²⁺ in about half of these neurons. These results show that IgLC and IgE can mediate antigen-specific responses in murine neurons. Our findings present a novel way of antigen-specific neuronal activation.

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1. Introduction

Immunoglobulins are synthesized by B lymphocytes and function as potent effector molecules (antibodies) in the humoral immune response. Based on heavy chain classes, immunoglobulins can be divided into five isotypes (IgA, IgD, IgE, IgG and IgM), each of which with different biological activity. Apart from two heavy chains an antibody also possesses two light chains. Antigen specificity based on antigen binding sites in antibodies is determined by the variable domains of both light and heavy chains. Immunoglobulin-free light chains (IgLC) have been shown to exhibit antigen binding by themselves (Painter et al., 1972; Yoo et al., 1967). For instance, IgLC purified from a monoclonal antibody to vasoactive intestinal polypeptide displayed sequence-specific binding to vasoactive intestinal polypeptide with only 5-fold lower affinity than the complete parent antibody (Sun et al., 1994). These findings indicate that the variable region of IgLC to antigen-specific binding in an antibody contributes to antigen recognition. Recently in our laboratory a functional role for IgLC was described by demonstrating that IgLC mediates cutaneous

hypersensitivity responses (Redegeld et al., 2002). Passive immunization of mice with antigen-specific IgLC followed by topical skin application of the antigen resulted in plasma extravasation, cutaneous swelling and mast cell degranulation (Redegeld et al., 2002). Further evidence for IgLC-induced hypersensitivity-like responses and mast cell degranulation was provided by studies in a hapten-induced murine model for non-atopic asthma (Kraneveld et al., 2005) and a similar model for colitis (Rijnierse et al., 2005). The clinical relevance of a role of IgLC in diseases, such as asthma and inflammatory bowel disease (IBD), was suggested by demonstrating significantly increased levels of IgLC in sera of asthmatic patients (Kraneveld et al., 2005) and in sera and intestinal biopsies of IBD patients (submitted manuscript) compared to healthy controls. These experiments demonstrate that IgLC-mediated hypersensitivity may be relevant to mast cell-associated non-IgE-mediated disorders like non-atopic asthma, rheumatoid arthritis, multiple sclerosis, IBD and cutaneous hypersensitivity.

The close proximity of mast cells to nerve endings, mostly of sensory origin, in various tissues like skin (Alving et al., 1991; Donelan et al., 2006), dura mater (Dimitriadou et al., 1997), intestine (Stead et al., 1989, 1987) and airways (Alving et al., 1991) suggests a direct bi-directional (Suzuki et al., 1999, 2001) communication between mast cells and the nervous system. This neuro-immune interaction is well acknowledged and has been described extensively (Bauer and Razin, 2000; McKay and Bienenstock, 1994; Rijnierse et al., 2007). Increased numbers of mast cells and mast cell–nerve contacts are particularly

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found in inflamed and infected tissues (Barbara et al., 2004) although an anatomical relationship between mast cells and nerves has also been demonstrated in non-inflamed areas (Dimitriadou et al., 1997). In addition to the key role attributed to mast cells in hapten-induced hypersensitivity reactions, it has been shown that sensory neurons are also important for the initiation of the response (Pastore et al., 1996). Lack of the NK₁ receptor or treatment with a NK₁ receptor antagonist in different hapten-induced rodent models for pulmonary hypersensitivity abolished airway hyperresponsiveness and decreased tracheal vascular permeability (Kraneveld and Nijkamp, 2001). In hapten-induced hypersensitivity responses in the gastrointestinal tract similar inhibitory effects of treatment with the NK₁ receptor antagonist on leukocyte accumulation and development of diarrhea were observed (Kraneveld et al., 1995; Rijnerse et al., 2006). Mast cell NK₁ receptors are thought to be activated by substance P released from sensory neurons (Furuno et al., 2004). Most interestingly, it has been suggested that such release can be triggered by immunoglobulin–antigen complexes, since the specific receptors for IgG and IgE, FcγRI and FcεRI respectively, are present on murine dorsal root ganglion (DRG) neurons (Andoh and Kuraishi, 2004a,b) and IgG–antigen complexes can specifically induce calcium responses in these neurons (Andoh and Kuraishi, 2004a).

Based on the role of neurons in hypersensitivity responses and the observation that IgLC plays a crucial role in the development of hapten-induced hypersensitivity responses, we propose a role for IgLC in direct antigen-specific activation of sensory neurons in relation to hypersensitivity reactions. We examined this using immunohistochemistry and measurements of intracellular Ca²⁺ on cultured murine adult DRG neurons. We report specific binding of IgLC and IgE to these neurons. Subsequently, we were able to show that significant increases in intracellular Ca²⁺ concentrations are induced by activation of IgLC- or IgE-sensitized DRG neurons by antigen. We therefore conclude that IgLC and IgE not only mediate direct mast cell activation, but also may induce antigen-specific neuronal activation that can contribute to hypersensitivity responses associated with inflammatory conditions.

2. Materials and methods

2.1. Animals

Male BALB/c mice were obtained from Charles River (Maastricht, The Netherlands). Mice were 6–8 weeks of age and weighed 20–25 g at the time of use. The animals were housed in groups not exceeding 8 mice per cage. Tap water and chow food were allowed *ad libitum* and a 12/12 h day–night cycle was maintained. All experiments were conducted in accordance with The Animal Care Committee of Utrecht University (Utrecht, The Netherlands).

2.2. DRG neuron cultures

Single neuronal cells originating from DRG from the cervical to the lumbar segments were cultured as described previously (De Jonge et al., 2004) with small modifications. Animals were sacrificed by i.p. injection of overdoses of sodium pentobarbital and pinned down dorsally. All organs were removed and the spinal cord was revealed. DRG were dissected one-by-one by carefully picking up the spinal cord exposing the DRG. Spinal roots were cut as close to the ganglion as possible. DRG were enzymatically digested with 10 mg/ml collagenase in L-15 medium at 37 °C, 5% CO₂ for 30 min. The reaction was stopped by adding an equal volume of FCS for 2 min. The ganglia were suspended in DRG culture medium (DMEM high glucose without glutamine and DMEM F-12 medium at a 1:1 ratio enriched with 1% N2 human serum supplement, 1% glutamine, 50 mg/ml gentamicin, 2 μM cytosine arabinoside) by mechanically dissociating with pipettes with decreasing diameters. The cell suspension was placed on serum-

coated petri dishes to allow non-neuronal cells to adhere for 90 min at 37 °C, 5% CO₂. After separation neuronal cells were collected from the dishes and placed onto poly-L-lysine-coated petri dishes with glass bottoms. Cultures were maintained at 37 °C, 5% CO₂ and studied at days 2–5 of the culture; medium was refreshed at day 3.

2.3. Isolation of antigen-specific IgLC and IgE

Mouse TNP-IgG₁ was purified from culture supernatant of mouse hybridoma (1B7-11; ATCC) with affinity chromatography with protein G sepharose. The IgG fraction was dialyzed against 150 mM NaCl, 50 mM Tris pH 8.0 and concentrated to the desired concentration using a Vivaspin 20 (MWCO 10.000) spin filter. Subsequently, TNP-IgG₁ was reduced in 200 mM 2-mercaptoethanol for 60 min at 37 °C, followed by alkylation with 300 mM iodoacetamide for 30 min on ice. Heavy and light chains were separated with size exclusion chromatography in 6 M guanidine HCl, pH 6.5 on a HiLoad Superdex200 column (Amersham). Fractions with immunoglobulin light chains were immediately dialyzed against PBS for several days with multiple buffer exchange. For antigen-specific immunoglobulin-free light chains (TNP)-specific IgG (1B7-11) was reduced, alkylated and purified as described previously (Redegeld et al., 2002). Mouse TNP-specific IgE (clone H1DNP ε26.82, kindly provided by J. Rivera, NIH, USA) was isolated with a hapten-affinity column containing TNP-BSA coupled to sepharose beads. The column was washed with PBS and subsequently eluted with 0.1 M glycine, pH 2.5. The eluate was dialyzed against PBS. Both immunoglobulin free light chains and IgE were concentrated with a spin filter and checked for purity with (non-reducing) SDS-PAGE/ Coomassie staining.

2.4. Immunohistochemistry

Cultured primary DRG neurons were fixed in 4% paraformaldehyde for 24 h at 4 °C on the glass bottom of poly-L-lysine-coated petri dishes. Subsequently, they were washed with and kept in PBS until further use.

To visualize binding affinity of IgLC and IgE to DRG neurons the following staining was performed. Cells were incubated with 25 μg/ml FITC-labeled mouse IgLC and FITC-labeled mouse IgE diluted in TBS with 1% BSA and 0.1% Triton X-100 for 90 min. The binding of IgLC and IgE to DRG neurons was dose-dependent and 25 μg/ml per DRG neuronal culture was found to be the optimal concentration. Aspecific binding of the secondary antibody was blocked with 10% normal goat serum (NGS) in TBS with 0.1% Triton X-100. Subsequently, rabbit-anti-FITC (1/1000) was incubated for 1 h. The third antibody, HRP-conjugated goat-anti-rabbit, was incubated for 1 h. The staining was developed with DAB enriched with 0.025% nickel ammonium sulphate to expose an intense and sharply outlined dark-brown/black color at the surface of the neurons. To expose the morphological structure of the cells an eosin background staining of the cytoplasm was performed subsequently. All incubations were performed at room temperature in a darkened incubation chamber. The secondary and tertiary antibodies were diluted in TBS containing 1% NGS and 0.1% Triton X-100. After each incubation step, the cells were washed with TBS with 0.1% Triton X-100. DRG neurons with a crisp dark brown to black color sharply outlining the surface were considered to be positive for either IgLC or IgE, DRG neurons with diffuse eosin background staining without distinct borders were regarded as negative. The number of positive and negative stained DRG neurons were in a blinded manner manually counted in a randomly selected region of each culture dish (view field 575 μm by 425 μm), containing on average 67 neurons. Criteria to distinguish DRG neurons from fibroblasts (the two cell types in culture) used were shape, diameter, and thickness of the soma, presence of neurites, and neurite size. Fibroblasts appear as thin long-stretched cells with no distinct neuritis and were excluded from further analysis.

To investigate specific binding affinity of IgLC and IgE, competition binding studies were performed with cold (i.e. unlabeled) ligand. In these studies the same staining protocol as described above was carried out in the presence of 250 µg/ml unlabeled IgLC or unlabeled IgE diluted in TBS with 1% BSA and 0.1% Triton X-100 for 15 min. Without washing the cells, 25 µg/ml of FITC-labeled IgLC or IgE was added. The staining was further performed as described above.

2.5. Sensitization with IgLC and IgE

Primary cultures of DRG neurons were used at days 2–5 of the culture. Cells were washed twice with DRG medium and incubated with 25 µg/ml IgLC, IgE or IgG at 37 °C, 5% CO₂ for 90 min. After this incubation cells were washed twice with DRG medium and used for optical imaging of cytosolic [Ca²⁺]_i.

2.6. Solutions

The buffer used in the optical Ca²⁺ recordings was artificial cerebrospinal fluid (ACSF) of the following composition (in mM): 122 NaCl, 2.1 KCl, 25 NaHCO₃, 2.5CaCl₂, 1.3 MgSO₄, 1.2 KH₂PO₄, and 11 glucose (pH 7.4; oxygenated with 95%CO₂–5%O₂). In the ACSF used to evoke brief (10 s) membrane depolarization the concentration of KCl was increased to 70 mM, while the NaCl concentration was reduced to maintain osmolality. To induce antigen-specific activation dinitrophenyl coupled to human serum albumin (DNP-HSA) was used (10 ng/ml in ACSF). A solution of 10 ng/ml BSA in ACSF was used as a control solution.

2.7. Optical recording of cytosolic [Ca²⁺]_i

Intracellular calcium mobilization was assessed by confocal laser scanning microscopy (CLSM) and used as an index of neural activation. Dishes with DRG neurons were incubated with the Ca²⁺ indicator dye Fluo-4 AM (1 µM) and 0.005% of the detergent pluronic F-127 in ACSF buffer for 40 min at RT. Approximately 20–30 min after stopping dye loading by refreshing the buffer, the dishes were transferred to an inverted microscope (Axiovert 100 M; Zeiss) equipped with a ×16 water immersion objective (Zeiss; Plan-neofluar; numerical aperture 0.8) in a CLSM system (Zeiss; LSM-510). Digital images (size 368.5×368.5 µm) were recorded (usually for a period of 40 min) at RT with a spatial resolution of 256×256 pixels and a temporal resolution of one image per 6 s. The 488-nm argon laser line (200 mW) was used to excite Fluo-4 fluorescence in the cells, which was measured using a long-pass 505-nm filter. Laser illumination intensity was kept to a minimum (max 1% of laser output) to avoid phototoxicity and photobleaching. The calcium signals of the cultured DRG neurons were recorded in the culture dishes. All fluorescence measurements were made from subconfluent areas of the dishes, enabling identification of individual cells. To study intracellular calcium changes in response to application of KCl, DNP-HSA and BSA, these compounds were dissolved in ACSF buffer and perfused through the bath in a constant flow rate (4 ml/min) by using a 4-channel perfusion setup. Because the culture dishes with DRG neurons also contained some fibroblasts all neurons within the image were identified by visual inspection. Criteria used were shape, diameter, and thickness of the soma, presence of neurites, and neurite size. Fibroblasts appear as thin long-stretched cells with no distinct neurites and were excluded from analysis.

Image data were analyzed off-line using the Zeiss LSM510 analyzing software V2.53. A selected image in each image set was used as a template for designating each neuronal soma as a region of interest. The Ca²⁺ response to depolarization with 70 mM KCl was used to localize a subconfluent area with vital neurons. At the end of each experiment depolarization with KCl was again performed to check cell viability. Neurons lacking a Ca²⁺ response of the same order of magnitude as the response to the first KCl depolarization were

excluded from further analysis. Because Fluo-4 is a single-wavelength indicator, it was not possible to apply the ratiometric method for quantitative determination of [Ca²⁺]_i. Therefore, data were normalized with respect to the mean fluorescence intensity (F₀) during approximately the first 3 min of the recording. The temporal fluorescence intensity of the dye during the 40-min recording was divided by F₀. The relative fluorescence (RF) obtained in this way represents integrated [Ca²⁺]_i. The RF values of each region of interest were plotted as a function of time. The amplitude of the responses of neurons to application of DNP-HSA was quantified as the RF level reached at the end of the 40 min measuring period using the last 15 images of the recording.

Neurons were identified as being responsive on the basis of the following criteria: 1) an initial and transient response to application of DNP-HSA (see Results) followed by 2) a clear and gradual rise in RF values as a function of time and 3) a RF value at the end of the DNP-HSA application exceeding 1.2. The percentage of responsive neurons was determined by dividing their number by the total amount of vital neurons in that particular recording. The lag time of the neural response to application of specific compounds was determined by measuring the time between compound application (as recorded by computer) and the first noticeable rise in RF.

2.8. Western blotting

Cultured DRG neurons of 3 days old were lysed in RIPA buffer and lysates were frozen (–70 °C) until use. An amount of 200 µg protein of DRG neuron lysate and 40 µg protein of bone marrow-derived mast cell lysate (control) of boiled non-reduced samples were separated electrophoretically (SDS-PAGE 12%) and transferred onto polyvinylidene difluoride membranes (BIO-RAD Laboratories). The membrane was blocked with Tween-PBS containing 2% milk proteins. FcεRI α-chain was detected with hamster anti-mouse FcεRIα (1/500) by incubation for 4 h at room temperature. HRP-labeled goat anti-hamster (1/2000) was applied for 1 h at room temperature as secondary antibody. Blots were washed in Tween-PBS three times for 10 min, incubated in commercial ECL reagent and exposed to photographic film. Films were scanned on a GS710 Calibrated Imaging Densitometer (Bio-Rad, Veenendaal, The Netherlands).

2.9. Materials

L-Glutamine, cytosine β-D-arabinofuranoside (ara-C), diaminobenzidine (DAB), DNP-HSA, pluronic F-127 and BSA were all purchased from Sigma Chemical Co., St. Louis, MO, USA. Collagenase A was purchased from Roche Diagnostics, Almere, The Netherlands. The following

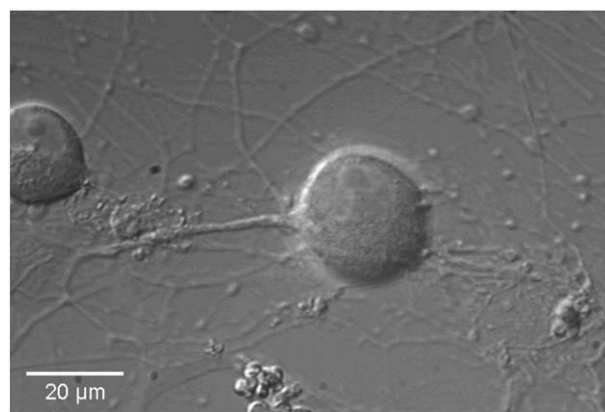


Fig. 1. Differential-Interference-Contrast photomicrograph of a DRG neuron 3 days in culture exhibiting one axon (left of the cell soma) and numeral newly grown neurites making contacts with other neurites and cell bodies over long distances within the culture dish.

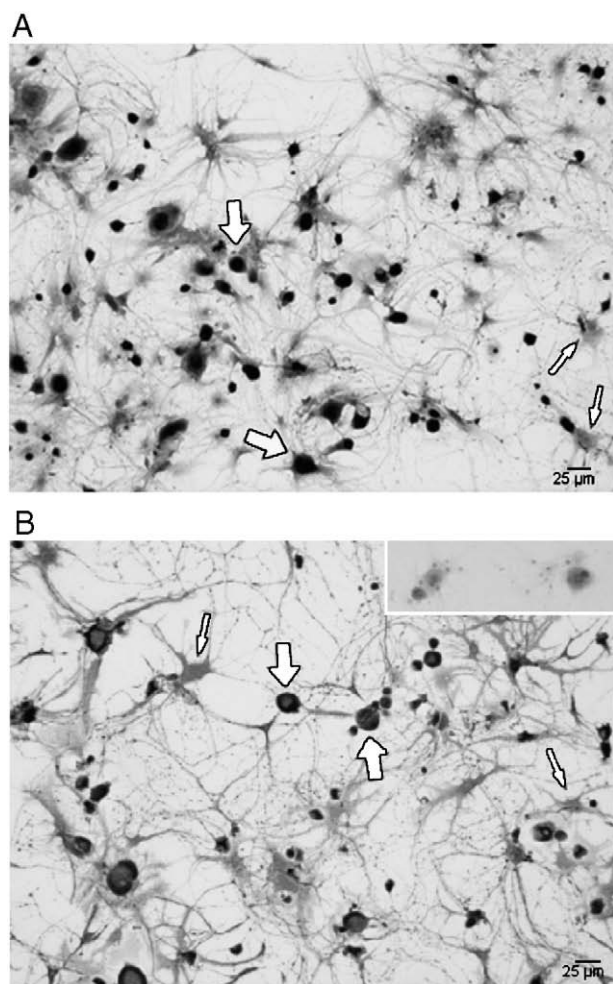


Fig. 2. Representative pictures of IgLC (A) and IgE (B) binding to primary cultured DRG neurons. The designated FITC-labeled primary antibody was incubated for 90 min after which the FITC label was detected with a secondary and a tertiary antibody, visualized with DAB/nickel staining resulting in black coloring. The morphological structure was visualized with an eosin background staining revealing that most primary cultured DRG neurons show binding affinity for IgLC (74% of total) and IgE (81%) on both cell bodies and neurites. The insert of figure (B) shows an idiotypic negative control for IgG. DRG neurons with a black crisp staining pattern sharply outlining the surface of the cultured neurons were considered to be positive, while negatively stained neurons were recognized by a diffuse staining of the cytoplasm without distinct borders. Examples of positively stained DRG neurons are indicated with a thick white arrow whereas examples of negatively stained DRG neurons are pointed out by a thin white arrow. The images are representative of 4 separate binding experiments performed in duplicate.

materials were obtained from Invitrogen, Breda, The Netherlands: L15 medium, DMEM F12, DMEM high glucose without glutamine, gentamicine and N2 supplement. FCS came from Perbio, Ettenleur, The Netherlands. Glass bottom culture dishes were bought at MatTek Cultureware, Ashland, MA, USA. The Cy-3-conjugated donkey anti-rabbit antibody was from Jackson ImmunoResearch Europe, Soham, UK. Hamster anti-mouse Fc ϵ R1 α was purchased from eBioscience. Rabbit anti-FITC, HRP-conjugated goat anti-rabbit IgG, and HRP-labeled goat anti-hamster were from DakoCytomation, Heverlee, Belgium. Molecular Probes, Eugene, OR, USA supplied the Fluo-4 AM.

2.10. Statistics

The responses to stimulation of IgLC- and IgE-sensitized DRG neurons with DNP-HSA were subjected to a two-way analysis of variance (ANOVA) to test whether the responses on the different days of the culture could be pooled for further analysis. These statistical tests revealed no difference between the different days of culture

within experimental conditions. Consequently, results obtained at different days were pooled, which resulted in mean values for the response amplitude for every experimental group. Responses in control groups and non-responsive neurons from experimental groups were compared in a one-way ANOVA followed by a Dunn's multiple comparison test. In experimental groups (IgLC- and IgE-sensitized) responsive neurons were distinguished from non-responsive neurons (see criteria above) and comparisons were made by an unpaired *t*-test. The results are expressed as mean $\pm$ sd. The results in Fig. 4 are presented in a box-and-whisker plot indicating the median (horizontal line in the box), distribution of the data by 25th and 75th percentiles (boxes) and lower and upper extremes (whiskers). The dot indicates an outlier of the dataset. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. DRG neuron culture

The culture of mouse DRG neurons consisted of a heterogeneous population with respect to neural size and shape. After 2 days, the DRG neurons exhibited an axon and full-size neurite outgrowths contacting with other neurites and neuronal cell bodies. This is in agreement with previous studies in which DRG neurons were cultured in a similar way (De Jonge et al., 2004). Neurite growth continued up to day 5 of culture, after which degradation of the neurons started to take place. In Fig. 1, a representative picture of a DRG neuron (3 days in culture) is shown.

3.2. IgLC and IgE bind specifically to DRG neurons

To determine binding affinity of IgLC and IgE to DRG neurons, binding studies were performed via immunohistochemical staining. Incubation of the DRG neurons in the culture dishes with 25 μ g/ml IgLC and IgE demonstrated that 74% $\pm$ 7 of IgLC (total 666 neurons in 9 culture dishes from 4 independent cultures) and 81% $\pm$ 5 of IgE (total 338 neurons in 6 culture dishes from 3 cultures) were able to bind to neuronal cell bodies. Fig. 2 shows representative pictures of IgLC (Fig. 2A) and IgE (Fig. 2B). Although quantification was only performed on neuronal cell bodies it can be seen from Fig. 2 that both IgLC and IgE were also able to bind to the neurites.

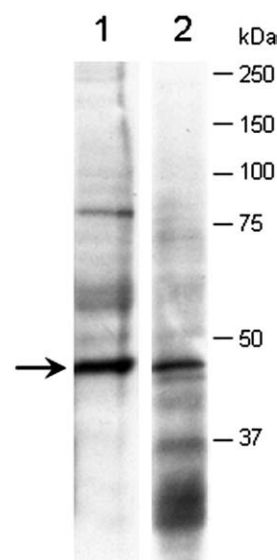


Fig. 3. Immunoblot for Fc ϵ R1 α on cultured DRG neuron lysates. Lane 1 demonstrates the presence of Fc ϵ R1 α on DRG membranes (protein loading 200 μ g). Cultured mouse bone marrow-derived mast cells were used as a positive control (lane 2; protein loading 40 μ g). The arrow indicates the band specific for Fc ϵ R1 α at 45 kDa.

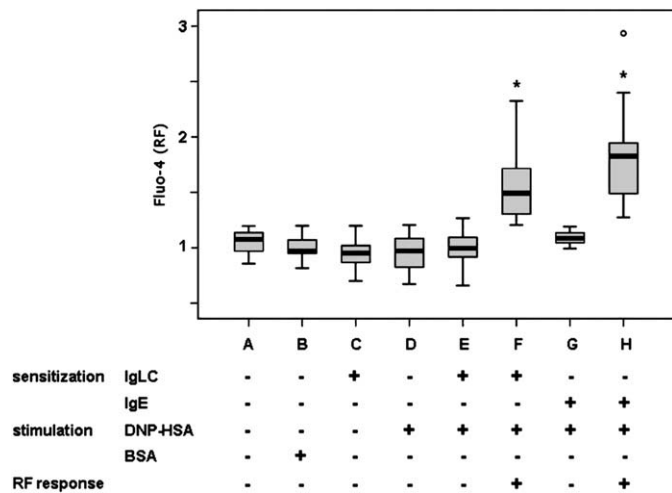


Fig. 4. RF values of non-sensitized, IgLC-sensitized and IgE-sensitized DRG neurons after 40 min of recording. The boxes represent the following applications: (A) non-sensitized DRG neurons, no DNP-HSA application ($n=19$); (B) non-sensitized DRG neurons, application of 10 ng/ml BSA ($n=18$); (C) IgLC-sensitized DRG neurons, no application of DNP-HSA ($n=21$); (D) non-sensitized DRG neurons, application of 10 ng/ml DNP-HSA ($n=94$); (E) IgLC-sensitized DRG neurons, non-responsive to application of 10 ng/ml DNP-HSA ($n=66$); (F) IgLC-sensitized DRG neurons, responsive to application of 10 ng/ml DNP-HSA ($n=35$); (G) IgE-sensitized DRG neurons, non-responsive to application of 10 ng/ml DNP-HSA ($n=14$); (H) IgE-sensitized DRG neurons, responsive to application of 10 ng/ml DNP-HSA ($n=19$). The n represents the number of DRG neurons. $*P<0.001$ compared to non-responding neurons subjected to the same sensitization and stimulation protocol.

Pre-incubation with unlabeled IgLC and IgE prior to incubation with FITC-labeled IgLC and IgE resulted in reduced staining intensity (data not shown) demonstrating competitive binding sites for both

IgLC and IgE and suggesting specificity of the binding to the neuronal cell bodies. Binding of IgE to DRG neurons is likely to be mediated via the high affinity receptor for IgE, FcεRI (Prussin and Metcalfe, 2006) since this receptor was demonstrated to be expressed by DRG neurons by western blotting (Fig. 3).

3.3. Antigen-specific activation of IgLC and IgE increases $[Ca^{2+}]_i$ in DRG neurons

The $[Ca^{2+}]_i$ of individual DRG neurons (day 2 to 5 of culture) loaded with Fluo-4 was recorded in culture dishes using a multi-channel perfusion system (ACSF; 4 ml/min). Recordings were made from fields (size $368.5 \times 368.5 \mu m$; one per dish) which displayed a representative number of DRG neurons (on average 20). The viability of these neurons was assured by recording their rapid and reversible Ca^{2+} response to a 10 s application of 70 mM KCl (RF 1.7 ± 0.3 ; $n=37$). Continuous perfusion of the dishes with ACSF for 40 min did not induce any change in neural $[Ca^{2+}]_i$ levels (RF 1.1 ± 0.1 ; $n=19$) (Fig. 4A). A few neurons and neurites showed spontaneous activity consisting of rapid rises in $[Ca^{2+}]_i$, which quickly returned to baseline values.

The neurons used to measure Ca^{2+} responses to antigen-specific activation of IgLC and IgE, were sensitized by incubation with IgLC or IgE (25 $\mu g/ml$) for 90 min just prior to loading with Fluo-4 and a 40 min Ca^{2+} -recording. The duration of this sensitization was based on the optimal incubation time determined in the binding studies. After about 3 min of baseline Ca^{2+} recording the perfusion was switched to ACSF containing DNP-HSA (10 ng/ml), which induced within about 10 s a relatively small and transient rise in neural $[Ca^{2+}]_i$ in all viable neurons, sensitized as well as non-sensitized (asterisks in Figs. 5B and 6B). This Ca^{2+} response had a duration of about 3 min and an amplitude smaller than 1.2 RF. Since in control experiments application of BSA (10 ng/ml) induced a similar response in all neurons, most likely this transient Ca^{2+} -response can be ascribed to a non-specific

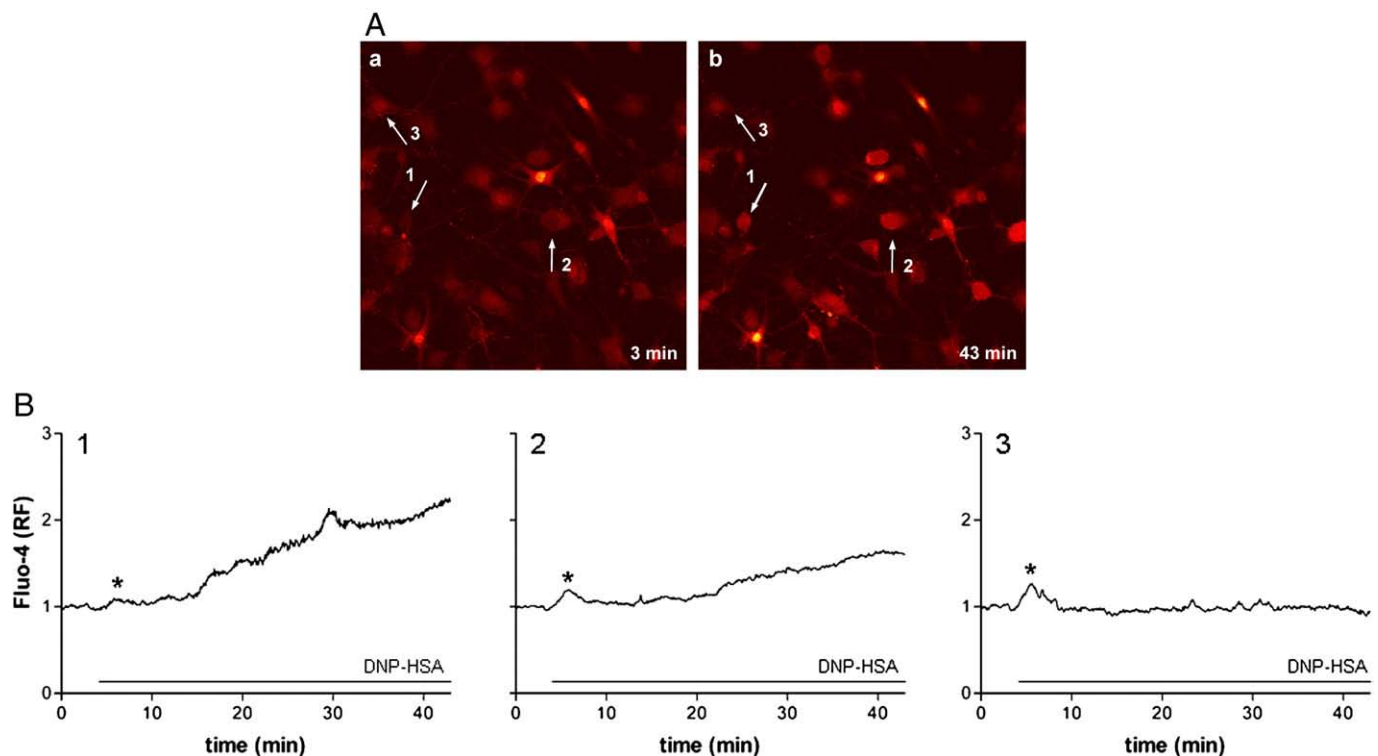


Fig. 5. Typical examples of the $[Ca^{2+}]_i$ response of IgLC-sensitized DRG neurons to application of DNP-HSA. (A) Time-lapse Fluo-4 images of DRG neurons. DNP-HSA was applied to the bath at 3 min (Aa) and continuously perfused up to 43 min (Ab). In figure A three representative neurons are indicated with arrows and numbered to correspond with the activation patterns depicted in figure B. (B) RF as function of time of IgLC-sensitized DRG neurons. Neurons 1 and 2 are examples of neurons responding to DNP-HSA application whereas neuron 3 is representative of a non-responsive DRG neuron. The bar indicates continuous application of DNP-HSA. In this particular recording 10 out of 16 vital neurons were responsive to the application of DNP-HSA. Note the small transient response shortly after application of DNP-HSA as indicated by the asterisk.

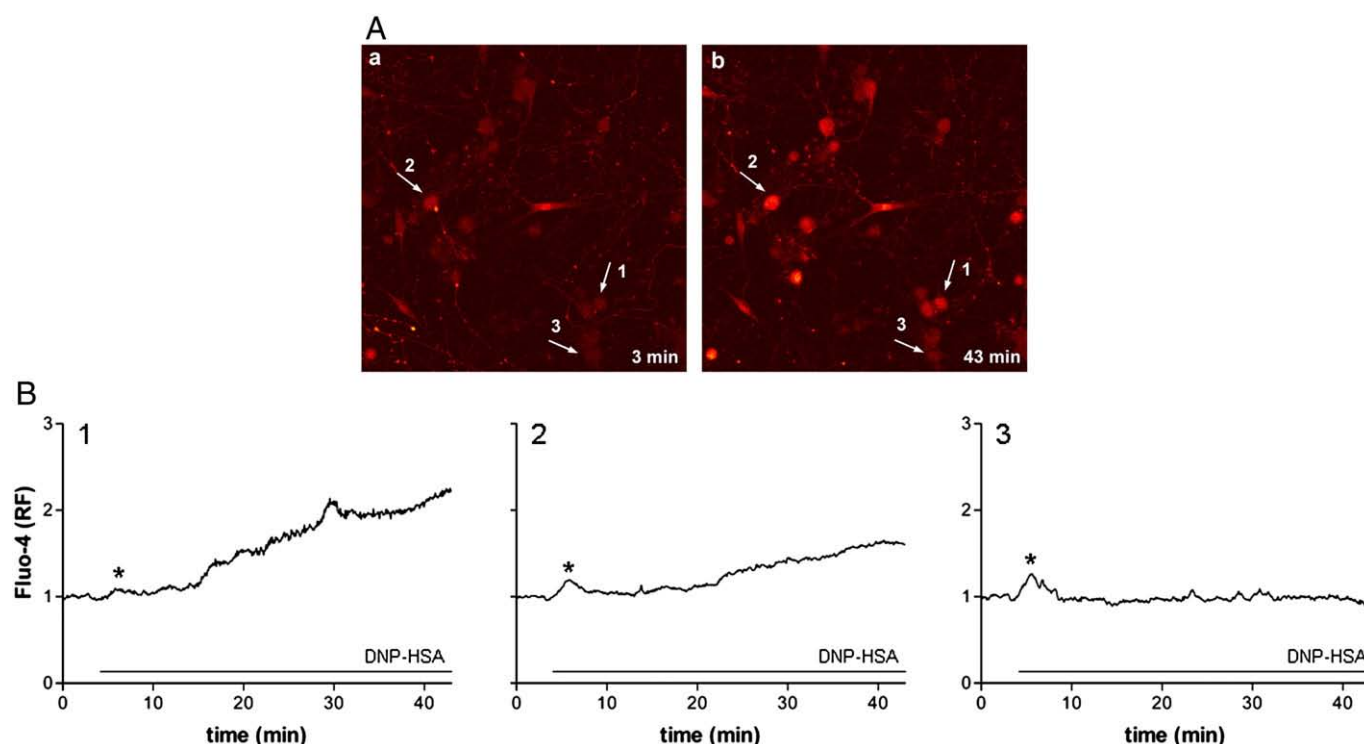


Fig. 6. Typical examples of the $[Ca^{2+}]_i$ response of IgE-sensitized DRG neurons to application of DNP-HSA. (A) Time-lapse series of Fluo-4 images of DRG neurons. DNP-HSA was applied to the bath at 3 min (Aa) and continuously perfused up to 43 min (Ab). In figure A three representative neurons are indicated with arrows and numbered to correspond with the activation pattern in figure B. (B) RF as function of time of IgE-sensitized DRG neurons. Neurons 1 and 2 are examples of neurons responding to DNP-HSA application whereas neuron 3 is representative of a non-responsive DRG neuron. The bar indicates continuous application of DNP-HSA. In this particular recording 10 out of 15 vital neurons were responsive to the application of DNP-HSA. Note the small transient response shortly after application of DNP-HSA as indicated by the asterisk.

effect of the protein coupled to DNP on the neuronal membrane. This suggestion is supported by the observation that the transient responses for all neurons had an identical lag time (10.2 ± 3.6 s ($n=59$)).

Perfusion of the dishes with DNP-HSA, however, also led to a much slower and more gradual rise in $[Ca^{2+}]_i$ in about 35% (35/101; 9 cultures) of the IgLC-sensitized DRG neurons. A representative example is shown in Fig. 5. This Ca^{2+} rise started after a lag time of about 11 ± 8 min ($n=35$) and persisted up to the end of the 40-min DNP-HSA perfusion. At the end of the recording period, the RF of the responding neurons (RF 1.6 ± 0.3 ; $n=35$) was higher than that of the non-responding neurons (RF 1.0 ± 0.1 ; $n=66$) (Fig. 4E/F; $P < 0.001$). Control experiments showed that application of 10 ng/ml BSA (RF 1.0 ± 0.1 ; $n=18$) or DNP-HSA (RF 0.9 ± 0.1 ; $n=94$) to non-sensitized DRG neurons during the 40-min period did not alter the $[Ca^{2+}]_i$ concentration (Fig. 4B and D respectively). In addition, sensitization of DRG neurons with IgLC alone did not result in changes in intracellular Ca^{2+} concentrations during the subsequent 40 min period with ACSF (RF 1.0 ± 0.1 ; $n=21$) (Fig. 4C).

Activation of IgE-sensitized DRG neurons with DNP-HSA resulted in similar responses as seen in IgLC-sensitized DRG neurons, as illustrated in Fig. 6. About 58% (19/33; 3 cultures) of the IgE sensitized neurons showed a gradual rise in $[Ca^{2+}]_i$ which started after a lag time of about 10 ± 7 min ($n=19$). At the end of the 40-min recording period, the RF of the responding IgE-sensitized neurons (RF 1.8 ± 0.4 ; $n=19$) was higher than that of the non-responding neurons (RF 1.1 ± 0.1 ; $n=14$) (Fig. 4G/H; $P < 0.001$).

The data obtained in this study indicate that an antigen-induced increase in $[Ca^{2+}]_i$ of DRG neurons can be mediated via crosslinking of IgLC and IgE bound to the neuronal cell membrane.

4. Discussion

It has been described that IgLC is able to mediate hypersensitivity responses in skin and airways by provoking the activation of mast cells

(Kranefeld et al., 2005; Redegeld et al., 2002). The present study demonstrates that IgLC and IgE can also specifically bind to cultured DRG neurons and induce an increase in $[Ca^{2+}]_i$ in these neurons after antigen-specific stimulation.

The binding studies demonstrated that IgLC and IgE are capable of binding to most of the neurons in the culture. Furthermore, only a subpopulation of IgLC- and IgE-sensitized neurons showed a Ca^{2+} response to the application of DNP-HSA (35% and 58% of total, respectively). This can most likely be ascribed to the fact that cultured DRG neurons exhibit different phenotypes with different sensory function (Lawson, 1992; Perl, 1992) and may therefore not be all susceptible to immunoglobulin-binding and antigen stimulation. Phenotyping the sensitized DRG neurons that are responsive to antigen application remains to be performed in future studies.

Since the kinetics of IgLC- and IgE-mediated activation of DRG neurons have not yet been described, we compare the time course of the neural Ca^{2+} responses to that of IgE-triggered mast cells. The lag time of the Ca^{2+} response of the neurons (about 10 min) was an order of magnitude larger than that reported for mast cells (generally < 1 min (Millard et al., 1989; Scharenberg and Kinet, 1994)). Moreover, like previously reported for the Ca^{2+} increase induced by IgG-antigen complexes (Andoh and Kuraishi, 2004a), the IgLC- and IgE-mediated responses of DRG neurons were slow and gradual, thereby sharply contrasting with the fast and sometimes oscillatory increase of Ca^{2+} in mast cells (Millard et al., 1989; Scharenberg and Kinet, 1994). Hitherto, it is not known if the difference in rate of the Ca^{2+} response to antigen-triggered activation between DRG neurons and mast cells is related to differences in kinetics of antigen binding (which is fast (< 1 min) in mast cells (Scharenberg and Kinet, 1994; Xu et al., 1998)) or in kinetics of the intracellular pathways leading to elevated Ca^{2+} . The prominent difference in response rate suggests that where activation by antigen results in rapid degranulation of mast cells, such activation of DRG neurons leads to a more gradual and perhaps modulatory response.

The demonstrated expression of the high affinity receptor for IgE, FcεRI, on the DRG neurons confirms its presence previously reported by Andoh and Kuraishi (2004b). In this study, we extended this by demonstrating the binding specificity of IgE to the DRG neurons. Our finding that IgE–antigen complexes could induce increased levels of intracellular Ca²⁺ is in accordance with the observation by Andoh and Kuraishi for IgG (Andoh and Kuraishi, 2004a). Taken together, the results suggest that the Ca²⁺ responses observed on IgE sensitized neurons are mediated via this specific receptor and that IgE can act directly on sensory neurons in allergic responses.

Furthermore, the results obtained in this study show specific binding of IgLC to DRG neurons and demonstrate that IgLC–antigen complexes induce an increase in intracellular Ca²⁺ of DRG neurons. Although there are several lines of evidence suggesting the presence of a putative receptor for IgLC, it has not been identified yet (Redegeld et al., 2002). Studies using knockout mice for γ-chain-associated receptors, such as FcεRI, FcγRIII and PIR-A demonstrated that these receptors are not involved in IgLC-mediated mast cell activation (Redegeld et al., 2002). Currently, the nature of a mast cell membrane-associated protein that interacts with IgLC is further analyzed in our laboratory. Preliminary research suggests that potential IgLC-binding proteins may also be present in cultured DRG neurons (our unpublished observations).

A substantial part of DRG neurons exhibit unmyelinated C-fibers which are mainly of the nociceptive type (Markenson, 1996). C fibers have an important afferent function in transducing pain (Djouhri et al., 2006; Tai and Baraniuk, 2003), itch (Handwerker et al., 1991; Tai and Baraniuk, 2003) and flare signals (Izumi and Karita, 1992), but also an efferent function in inducing local neurogenic inflammation in hypersensitivity responses (increased vascular permeability, vasodilatation and induction of infiltration of inflammatory cells) (Richardson and Vasko, 2002). The observation that IgLC and IgE can bind to DRG neurons and subsequently increase intracellular Ca²⁺ suggests that neurons may become activated by an antigen-specific activation pathway. Complexes of IgLC and Ig molecules and antigens can affect primary sensory neurons and therefore may contribute to local neurogenic inflammation and centrally-mediated sensations such as pain.

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