

Low-threshold heat response antagonized by capsazepine in chick sensory neurons, which are capsaicin-insensitive

Antonia Marín-Burgin, Stephan Reppenhagen, Andreas Klusch, Jens R. Wendland and Marlen Petersen

Department of Physiology, University of Würzburg, Röntgenring 9, D-97070 Würzburg, Germany

Keywords: cobalt uptake, dorsal root ganglion, heat receptors, nociception, vanilloid receptor

Abstract

The heat-transducing receptor VR1 cloned from rat sensory neurons can be activated by both noxious heat and capsaicin. As the response of sensory neurons to capsaicin is species dependent, it is conceivable that the responses to noxious heat and to capsaicin are transduced by distinct receptors across different species. Therefore, we investigated responses to noxious heat from a capsaicin-insensitive (chick) and a capsaicin-sensitive (rat) species. In chick, whole-cell patch-clamp experiments in isolated dorsal root ganglion neurons revealed two populations of neurons with different thresholds to noxious heat, activated at $\approx 43^\circ\text{C}$ and $\approx 53^\circ\text{C}$. In cobalt uptake experiments, the proportion of neurons showing a heat-induced response increased with increasing heat stimuli. Application of capsaicin ($1\text{--}10\ \mu\text{M}$) did not result in inward currents or cobalt uptake. Rat neurons yielded comparable results in heat experiments, but were capsaicin-sensitive. Although chick neurons are insensitive to capsaicin, the competitive capsaicin antagonist capsazepine ($1\text{--}10\ \mu\text{M}$) was effective in blocking heat-induced responses, verified by patch-clamp and cobalt uptake methods. The noncompetitive capsaicin antagonist ruthenium red ($10\ \mu\text{M}$) reduced to almost nil the proportion of heat-responsive neurons identified with the cobalt uptake method. These findings suggest that chick DRG neurons express a low-threshold heat-transducing receptor with a pharmacological profile distinct from the low-threshold heat receptor VR1 cloned from rat DRG neurons. The data support the idea that there might be heat receptor subtypes with differences in the capsaicin binding site.

Introduction

Noxious thermal stimuli are detected at the peripheral terminals of specialized primary sensory neurons called nociceptors. Activation of these neurons causes nocifensive reactions in animals and man and the sensation of pain in man (Fields, 1987). Recently, a heat-transducing receptor has been cloned from rat dorsal root ganglion (DRG) neurons; it was named vanilloid receptor subtype 1 (VR1), as it has a binding site for vanilloid compounds like capsaicin (Caterina *et al.*, 1997). In HEK293 cells and oocytes, both transfected with the cloned VR1 receptor, temperatures $>43^\circ\text{C}$ and capsaicin cause an inward current (Caterina *et al.*, 1997; Tominaga *et al.*, 1998). These data suggest that VR1 may be a heat-transducing receptor in nociceptors.

In cultured rat DRG neurons, at least two populations have been described with respect to their sensitivity to noxious heat. One population has a low threshold ($\approx 45^\circ\text{C}$) and is sensitive to capsaicin, whereas the other has a high threshold ($\approx 52^\circ\text{C}$) and is capsaicin-insensitive (Nagy & Rang, 1999a). Another heat receptor homologous to VR1 has been cloned and was called vanilloid-receptor-like protein 1 (VRL-1). VRL-1 might be expressed in mechano-heat nociceptors with A δ fibers and this receptor has a high threshold for heat but does not respond to capsaicin, as shown in HEK293 cells and oocytes transfected with the receptor (Caterina *et al.*, 1999). In addition to the different thresholds to heat, both

receptor types have distinct pharmacological profiles. VR1 can be blocked by the competitive capsaicin antagonist capsazepine as well as by the noncompetitive antagonist ruthenium red (RR) (Caterina *et al.*, 1997), whilst VRL-1 can be blocked only by RR (Caterina *et al.*, 1999).

One striking feature of nociceptors in different species is their different susceptibility to capsaicin (for reviews, see Holzer, 1991; Szallasi, 1994). In behavioural studies, mammals respond to capsaicin with strong nocifensive reactions (for review, see Szolcsányi, 1982) whereas birds are insensitive even at very high concentrations (Szolcsányi *et al.*, 1986; Sann *et al.*, 1987). In accordance, there is no $^{45}\text{Ca}^{2+}$ uptake in chick DRG cells in the presence of capsaicin (Wood *et al.*, 1988) and no specific binding of capsaicin (Szallasi & Blumberg, 1990). However, unlike capsaicin, noxious thermal stimuli in birds do cause behavioural responses as well as activation of primary sensory neurons, as shown by single-fibre recordings (Necker & Reiner, 1980; Sann *et al.*, 1987; Koltzenburg & Lewin, 1997).

Therefore, the insensitivity of birds to capsaicin but their responsiveness to noxious heat suggest that there might be heat-transducing receptors in these animals different from the known VR1 in mammalian DRG neurons.

Here, physiological and pharmacological properties of the responses to noxious heat and capsaicin in chick and rat DRG neurons were investigated. We found that chick neurons respond to noxious heat with thresholds of $\approx 43^\circ\text{C}$ or $\approx 53^\circ\text{C}$, similar to rat neurons. Surprisingly, the low-threshold noxious heat response could

be reversibly blocked by the competitive capsaicin antagonist capsazepine although chick neurons do not respond to capsaicin.

Materials and methods

Animals

DRG neurons from chicks (40–90 g, cobalt uptake and patch-clamp) and male Sprague-Dawley rats (180–250 g, cobalt uptake; 35–70 g, patch-clamp) were used. Before excision of ganglia, animals were killed with an i.p. lethal dose of sodium pentobarbital.

Cell dissociation

DRGs from all segments of the spinal cord were excised. The ganglia were incubated in Dulbecco's modified Eagle's medium (D-MEM) (Gibco, Eggenstein, Germany) containing collagenase type CLS IV (260 U/mg) (Biochrom, Berlin, Germany) at 37°C for 50–116 min (depending on the weight of the animals) followed by an incubation for 11 min in D-MEM containing trypsin (9820 U/mL) (Sigma, Neu-Ulm, Germany). The ganglia were suspended in D-MEM containing gentamicin (25 µg/mL) (Gibco) and mechanically dissociated. Cells were centrifuged and suspended in TNB-100 medium supplemented with TNB-100 lipid protein complex (Gibco), penicillin (100 U/mL) (Gibco), streptomycin (100 µg/mL) (Gibco) and nerve growth factor 7S (100 ng/mL) (Calbiochem-Novabiochem, Bad Soden, Germany) isolated from mouse submaxillary glands. Cells were plated on sterile 10-mm glass coverslips coated with poly-L-lysine (200 µg/mL; Sigma) and maintained in supplemented TNB-100 medium at 37°C and 3.5% CO₂. Half of the medium was replaced daily. The protocol for cell dissociation and culture was the same for both species. For patch-clamp experiments, neurons had been in culture for 1–3 days; for cobalt uptake experiments, for 2–3 days.

Whole-cell patch-clamp experiments

Experiments were performed similarly as described previously (Petersen *et al.*, 1996). Briefly, coverslips were continuously superfused with external solution (1.5 mL/min). Neurons were voltage-clamped at –80 mV using an Axopatch 200A amplifier (Axon Instruments). Series resistance and cell capacitance were compensated by nulling circuitry. The clamp command signals were generated via the amplifier and a PC with a DigiData 1200 interface and pClamp 6.0 software (Axon Instruments). Electrodes had a resistance between 2 and 6 MΩ. They were filled with (in mM): 140 KCl, 1 CaCl₂, 11 EGTA, 10 HEPES, 2 Mg-ATP; pH 7.3. The external solution consisted of (in mM): 140 NaCl, 3.5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES; pH 7.3.

Test solutions were applied via a fast multichannel superfusion system (Dittert *et al.*, 1998). The orifice was placed at a distance of <100 µm from the neuron. Solutions were changed by a microprocessor controlling the activation of valves. Temperature stimuli were recorded together with the evoked inward current using Clampex 6.0 software. Ramp-shaped heat stimuli starting from ambient temperature were used with 5 s maximum temperature duration (see Fig. 1A). In a subgroup of neurons, response to 1 µM capsaicin (Sigma) was tested by superfusion for 6–10 s before heat stimuli.

For antagonist experiments, we chose a 2-s maximum temperature stimulus to avoid desensitization. Neurons were first subjected to a heat stimulus in normal external solution followed by a 2-min preincubation with capsazepine (ICN Biomedicals, Eschwege, Germany) and a heat stimulus in the presence of capsazepine

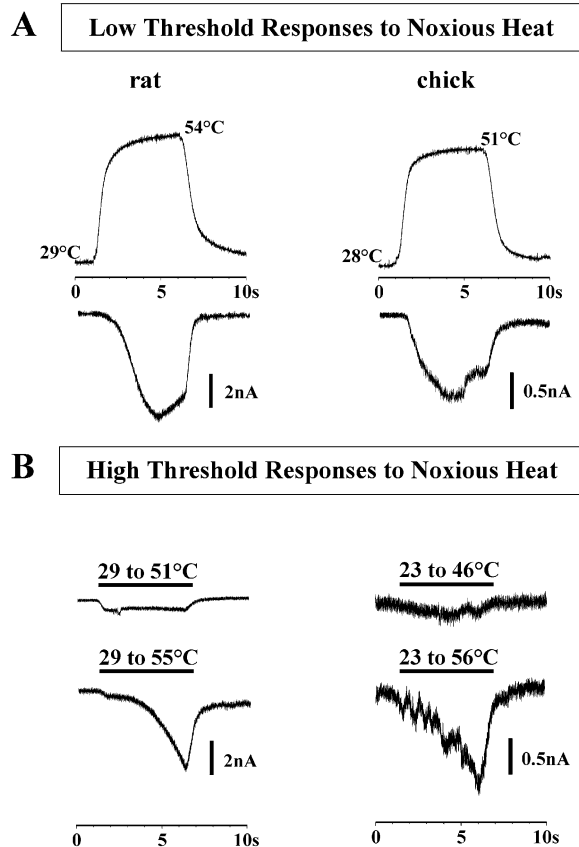


FIG. 1. Whole-cell voltage-clamp recordings in rat (A and B, left) and chick (A and B, right) neurons stimulated with heat. (A) Low-threshold heat responses. Upper traces, temperature stimuli; base and maximum temperatures as indicated. Lower traces, current responses. (B) High-threshold heat responses. Upper traces, no responses to subthreshold stimuli. Lower traces, subsequent high-threshold responses of the same neuron. Stimulus durations are indicated by the bars above the recordings. Baseline and peak temperatures as indicated, shape of stimuli similar to the ones shown in A. The time between heat stimuli was 3 min. Current amplitudes and time scale as indicated. The membrane potential of rat neurons was –56 and –57 mV, of chick neurons, –75 and –55 mV.

(10 µM). After a 2-min washout, neurons were again stimulated with heat. In all experiments, only one neuron on each coverslip was tested.

Data analysis of whole-cell patch-clamp experiments

The threshold temperature of the response was determined as the temperature of the stimulus at the onset of the evoked current. Due to the steep increase of the stimulus temperature in some experiments the exact threshold was hard to define, but it was possible to assign the responses as either belonging to the low- or high-threshold type. Some high-threshold responses were only elicited after consecutive increasing stimuli, making it possible to clearly recognize the earlier stimuli as subthreshold. Only neurons with pronounced voltage-dependent inward and outward currents were investigated. In some neurons voltage-dependent currents were recorded after application of high temperature (up to 56°C) to verify the viability of the neuron.

Cobalt uptake experiments

To determine the proportion of responsive neurons with increasing capsaicin or heat stimuli, the cobalt uptake method was performed

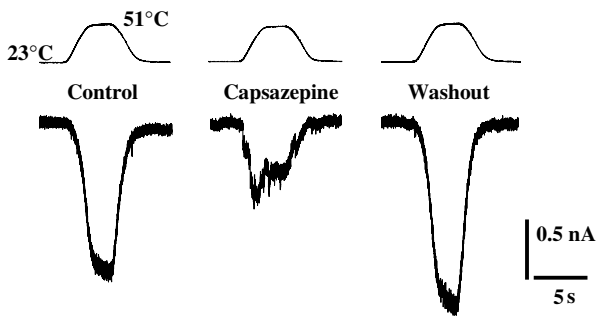


FIG. 2. Whole-cell voltage-clamp recording of a chick neuron stimulated with heat in the presence of capsazepine. Upper traces show the respective heat stimuli with a baseline of 23 °C and a peak temperature of 51 °C. Lower row: left, heat-evoked inward current with normal external solution; middle, response of the same neuron to heat in the presence of capsazepine (10 μ M) after 2 min preincubation; right, response after 2 min washout with normal external solution. The membrane potential was -68 mV.

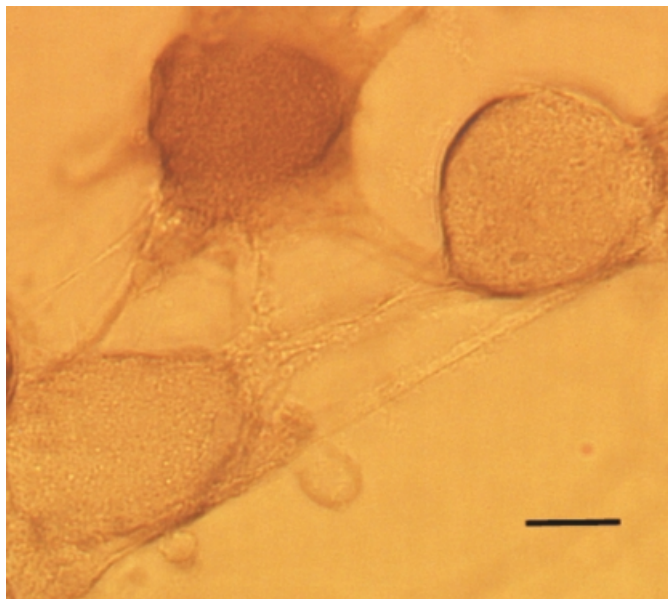


FIG. 3. Microphotograph of heat-sensitive (brown) and heat-insensitive (light) chick neurons after stimulation with heat of 44 °C. Scale bar, 10 μ m.

(Hogan, 1983; Winter, 1987; Reichling *et al.*, 1997). Briefly, neurons were washed for 2 min in assay buffer containing (in mM): 5.8 NaCl, 5 KCl, 2 MgCl₂, 0.75 CaCl₂, 12 glucose, 10 HEPES; osmolarity was adjusted to 290 mOsm, pH 7.4. For stimulation with capsaicin, coverslips were incubated in assay buffer containing 5 mM CoCl₂ (Co²⁺-assay buffer) and capsaicin (0.01–10 μ M) for 8 min at room temperature. For thermal stimulation (39–46 °C), cells were placed into Co²⁺-assay buffer preheated to the stimulus temperature and kept in a temperature-controlled chamber for 8 min. The cells were then washed for 2 min in assay buffer at room temperature and placed into 1.25% ammonium sulphide (diluted in assay buffer, 298 mOsm) (Fluka, Neu-Ulm, Germany) for 2 min to precipitate the cobalt to cobalt sulphide. In order to detect dead cells, coverslips were incubated in 0.1% Trypan Blue (Fluka) for 10 min. Cells were washed, fixed in 4% paraformaldehyde and mounted in DABCO (Sigma). For antagonist experiments, coverslips were preincubated for 2 min at 37 °C in assay buffer containing the antagonists RR

(10 μ M; Sigma) or capsazepine (0.1–10 μ M). Cells were stimulated with heat in Co²⁺-assay buffer containing the antagonist and then processed as described above. To rule out heat-activated cobalt influx via voltage-dependent calcium channels, control experiments were performed in the presence of lanthanum (10 and 100 μ M) (Reichling & MacDermott, 1991). To determine background cobalt staining, experiments were performed at room temperature. In chick and rat the proportion was $\approx 2\%$.

Data analysis of cobalt uptake experiments

Neurons were analysed with a Zeiss microscope (Axiophot) coupled to a CCD colour video camera (Sony) and an OPTIMAS image analysing system (Optimas Corp.). The neurons were selected by systematic scanning of the coverslip. All neurons unobstructed by other neurons or by tissue debris and not stained blue due to the Trypan Blue were included in the data set. The criterion for a responsive neuron was a distinct brown colour due to the cobalt sulphide precipitate (see Fig. 3), which was determined by visual inspection. Measurement of the soma cross-sectional area was achieved by tracing the border of the somata on the video screen with a pointing device for computation by the image analysing program. From every coverslip, 100–200 neurons were analysed. Results are expressed as mean \pm SEM.

Solutions

Capsaicin solution was prepared from a stock solution of 5.45 mM ethanol and stored at -20 °C. Capsazepine solution was prepared just prior to use as a stock solution of 10 mM in dimethyl sulfoxide which was further diluted in normal external solution. Lanthanum (Sigma) stock solution (2.0 mM) was diluted in assay buffer adjusted to pH 7.0 (Reichling & MacDermott, 1991).

Results

Patch-clamp experiments

Heat-evoked currents

Inward currents in response to low-threshold noxious heat have been shown in mouse (Stucky & Lewin, 1999) and rat (Cesare & McNaughton, 1996; Kirschstein *et al.*, 1999; Nagy & Rang, 1999a) DRG neurons. In rat neurons two types of heat-evoked currents have been demonstrated by whole-cell patch-clamp method, a low-threshold current that activates at ≈ 45 °C and a high-threshold current that activates at ≈ 52 °C (Nagy & Rang, 1999a). We performed experiments with the patch-clamp method in neurons from chick and rat in order to compare heat-evoked responses in a capsaicin-insensitive and a capsaicin-sensitive species.

Our results in rat DRG neurons confirm those of Nagy & Rang (1999a). An example of a low-threshold heat response is shown in Fig. 1A, left. The heat stimulus with an increase from 29 to 54 °C (Fig. 1A, left, upper trace) caused an inward current. It was activated at ≈ 43 °C and inactivation occurred before the maximum stimulus temperature was reached (Fig. 1A, left, lower trace). A previous application of 1 μ M capsaicin for 5 s evoked an inward current of 5.3 nA (not shown). Out of 24 neurons tested, 9 (37.5%) responded with an inward current (2.7 ± 0.9 nA) activated at low temperatures (44.3 ± 1.8 °C). Seven of these nine neurons were also tested to capsaicin and all responded. Because VR1 is sensitive to capsaicin and to temperatures above ≈ 43 °C, these data suggest that the responses are due to activation of the VR1 (Caterina *et al.*, 1997). An example of a high-threshold heat response in rat neurons is given in Fig. 1B, left. This neuron did not respond to 1 μ M

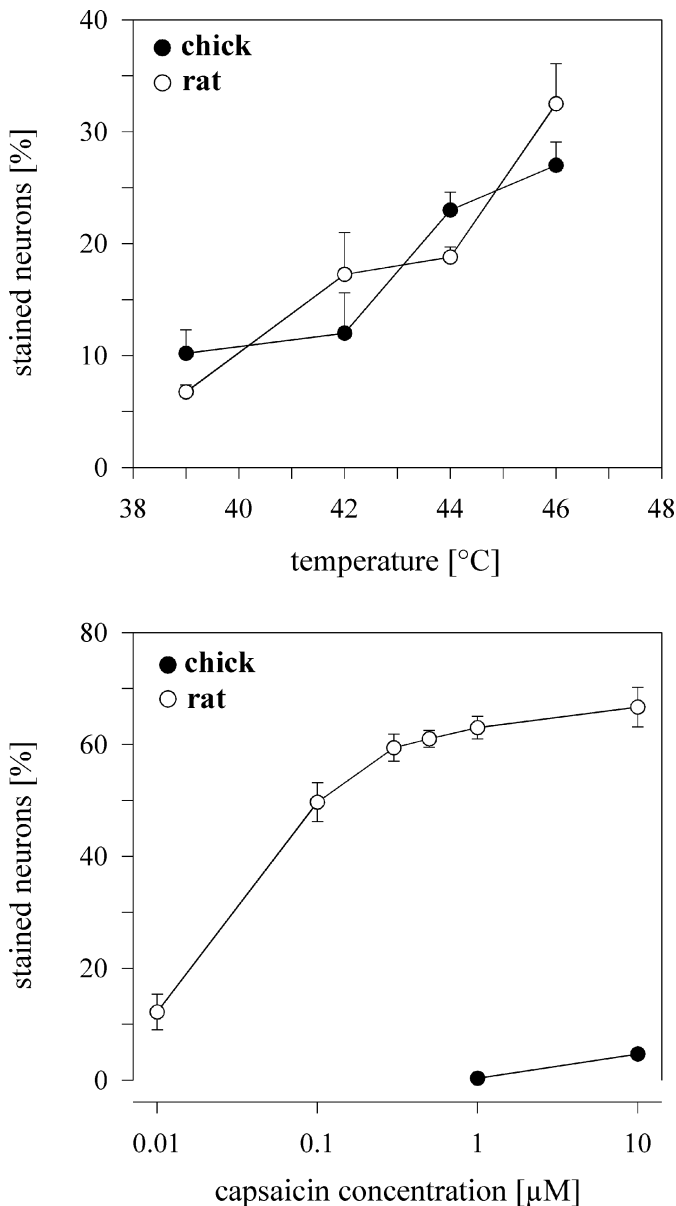


FIG. 4. (Top) Proportion of neurons with cobalt staining induced by increasing temperature in chick (●) and rat (○). Values are expressed as the mean \pm SEM of 3–8 independent cultures with a total of 300–800 neurons, except for 44 °C in chick, where 19 independent cultures with a total of 2369 neurons were investigated. (Bottom) Concentration-dependent proportion of neurons with cobalt staining induced by capsaicin in chick (●) and rat (○). Values are expressed as the mean \pm SEM of 3–6 independent cultures with a total of 300–600 neurons.

capsaicin (not shown) or to temperatures <51 °C (Fig. 1B, left, upper trace). However, a higher stimulus of up to 55 °C elicited an inward current with a threshold of ≈ 53 °C (Fig. 1B, left, lower trace). Out of the 24 neurons tested, six (25%) responded with an inward current (5.7 ± 1.5 nA) activated at high temperatures (53.0 ± 0.4 °C). Five of these neurons were tested to capsaicin and none responded.

Chick neurons responded to noxious heat with an inward current but not to capsaicin. Figure 1A, right, shows the response of a neuron to low-threshold noxious heat. The threshold temperature of the response was ≈ 42 °C. The current started to inactivate during the stimulus duration. Out of 109 neurons tested, 47 (43%)

responded with an inward current elicited by temperatures <50 °C. The mean threshold temperature was 43.2 ± 0.8 °C ($n=25$). In 22 neurons the exact threshold could not be determined due to the steep temperature gradient of the stimulus but was clearly in the low noxious heat range. The mean current amplitude within stimulus time was 2.3 ± 0.2 nA ($n=47$). In addition to neurons responding to low noxious heat there was also a population responding only to high noxious heat. The recording in Fig. 1B, right, upper trace, shows a neuron not responding to a stimulus of up to 46 °C. In the lower trace, the same neuron was subsequently stimulated from 24 to 56 °C and responded at ≈ 52 °C. Out of the 109 neurons tested, 17 (16%) responded only to high temperatures with an inward current. The mean threshold was 52.6 ± 0.5 °C ($n=15$). In two neurons the exact threshold could not be determined due to the steep temperature gradient of the stimulus but it was >54 °C. The mean amplitude of the current reached during the stimulus duration was 2.2 ± 0.4 nA ($n=17$).

Only small to medium sized neurons (400 – 1250 μm^2) were selected. The mean of the resting membrane potential of all responsive neurons was -54.6 ± 0.8 mV ($n=64$) and was not significantly different (Student's *t*-test, $P>0.1$) from the mean resting membrane potential of the nonresponsive neurons of -53.1 ± 0.9 mV ($n=45$).

Before heat applications, some neurons were tested for response to capsaicin (1 μM); none of the chick neurons tested responded ($n=42$).

These patch-clamp experiments indicate that there are two populations of chick DRG neurons responding to noxious heat, one that responds to low-threshold heat (≈ 43 °C) and one to high-threshold heat (≈ 53 °C), with inward currents. Both types of neurons are completely insensitive to capsaicin.

Effects of the competitive capsaicin antagonist capsazepine on heat-induced inward currents

In VR1-expressing HEK293 cells as well as in rat DRG neurons, heat-activated currents can be blocked by the competitive capsaicin antagonist capsazepine (Caterina *et al.*, 1997; Nagy & Rang, 1999b). We investigated in patch-clamp experiments whether in chick DRG neurons, which are capsaicin insensitive, capsazepine blocks low-threshold heat-activated currents. Figure 2 shows a neuron responding to a heat stimulus from 23 to 51 °C with an inward current of 1.3 nA in normal external solution, while a stimulus in the presence of 10 μM capsazepine with 2 min preincubation resulted in a clearly reduced current amplitude of 0.5 nA. After a washout of 2 min, the current evoked was comparable to the response under control conditions.

Cobalt uptake experiments

Responses to heat and capsaicin

In order to investigate the proportion of chick DRG neurons that respond to low-threshold heat, we performed experiments using the cobalt uptake method (Hogan, 1983; Winter, 1987; Reichling *et al.*, 1997). The stimulus-evoked cobalt uptake is visualized by precipitating the cobalt with a sulphide to yield a brown staining of the neurons. Figure 3 shows one stained and two unstained chick neurons after stimulation with heat of 44 °C.

First, we investigated the response to increasing heat stimuli in chick and rat neurons (Fig. 4, top). In both cases the proportion of heat-induced cobalt-stained neurons increased with increasing heat stimuli, in chick from $10.2 \pm 2.1\%$ at 39 °C to $27.0 \pm 2.1\%$ at 46 °C and in rat from $6.8 \pm 0.6\%$ at 39 °C to $32.5 \pm 3.6\%$ at 46 °C.

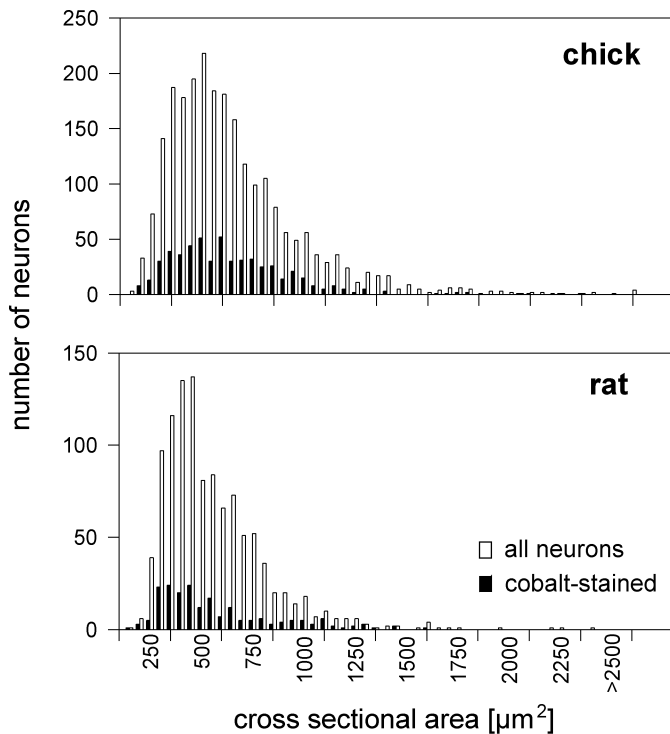


FIG. 5. Size distribution of chick (top) and rat (bottom) neurons. White columns represent all neurons; black columns represent cobalt-stained neurons at 44 °C. Chick, 19 animals, $n=2369$ neurons; rat, eight animals, $n=1101$ neurons.

It is known that in rat VR1 is expressed in small to medium sized DRG neurons, which was determined by *in situ* hybridization of the transcripts (Caterina *et al.*, 1997) and by labelling with antibodies (Guo *et al.*, 1999). We measured the soma sizes in cobalt uptake experiments. With the temperature stimulus of 44 °C the size of the stained neurons, in both chick and rat, was small to medium (Fig. 5).

Secondly, we investigated the responsiveness of neurons to capsaicin. Chick neurons were stimulated with concentrations of 1 and 10 μM and rat neurons with concentrations between 0.01 and 10 μM capsaicin (Fig. 4, bottom). In agreement with our patch-clamp experiments, neurons of chick were virtually insensitive to capsaicin, whereas those of rats responded in a concentration-dependent manner. In chick neurons, capsaicin induced almost no cobalt staining; at a concentration of 10 μM , only 4% of neurons were positive. In rat neurons, there was an increase in cobalt staining from 12% with 0.01 μM capsaicin to 60% with 1 μM capsaicin.

To exclude the possibility that cobalt influx was mediated via voltage-dependent calcium channels, we performed control experiments in the presence of lanthanum in concentrations of 10 and 100 μM . The proportion of neurons responding to heat, tested at 44 °C, was not affected by lanthanum (data not shown).

Effects of capsazepine and ruthenium red on heat-induced cobalt uptake

Responses to heat of the low-threshold VR1 are antagonized not only by capsazepine but also by the noncompetitive antagonist RR (Caterina *et al.*, 1997). To compare chick and rat neurons, both were stimulated with 44 °C in the presence of capsazepine (0.1–10 μM) or RR (10 μM). The effect of capsazepine is shown in Fig. 6. Whilst in chick 1 μM capsazepine caused a significant reduction to 46% with respect to control (χ^2 , $P<0.01$) in the proportion of neurons

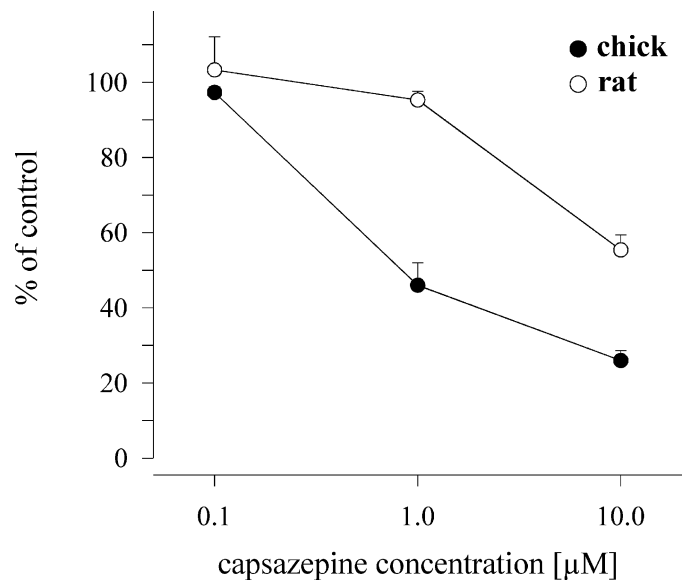


FIG. 6. Effect of the capsaicin antagonist capsazepine on heat-induced cobalt uptake in neurons of chick (●) and rat (○). Neurons were stimulated with 44 °C (control) and with 44 °C in the presence of 0.1, 1 and 10 μM capsazepine. Data are represented as the fraction (%) of stained neurons with respect to that of control (100%). Values are expressed as the mean \pm SEM of 3–5 independent cultures with a total of 300–500 neurons.

responding to heat, in rat a reduction to 52% was only obtained by a tenfold higher concentration. In both species, the proportion of neurons responding to heat was strongly reduced by RR. In chick neurons it caused a $93 \pm 2.5\%$ decrease of neurons stained by cobalt; in rat, the reduction was $94 \pm 2.4\%$.

Discussion

This study provides the first indication of a low-threshold noxious-heat-transducing receptor expressed in chick DRG neurons, which is similar to the VR1 with respect to the threshold temperature for activation, but distinct in its pharmacological profile. The putative heat receptor in chick neurons is insensitive to capsaicin, but nevertheless the heat response can be blocked by the capsaicin receptor antagonist capsazepine.

Patch-clamp experiments revealed a subpopulation of chick neurons responding to low-threshold heat stimuli with inward currents activated above ≈ 43 °C. The threshold was similar to the one we found in a population of rat DRG neurons, which confirms data of Nagy & Rang (1999a); however, in rats the same neurons also responded to capsaicin, whereas in chick they did not. The proportion of responding chick neurons was 43%, which is somewhat higher than the 23% found in our cobalt uptake experiments at 44 °C. This could be explained by a deliberately biased selection of neurons with respect to size and appearance to maximize the number of responding neurons in the electrophysiological experiments. In addition to the current evoked by low-threshold heat we found a high-threshold heat-activated current in chick neurons. The threshold temperature was similar to the one we found in rat neurons, which is in agreement with Nagy & Rang (1999a); in both species these neurons were insensitive to capsaicin. Single-unit recordings from afferent fibers of a cutaneous nerve of pigeons revealed three different thresholds for heat responses: 43, 47 and 52 °C, proposing subtypes of heat nociceptors (Necker & Reiner, 1980). The lowest and the highest

threshold correspond well to the heat thresholds we found in patch-clamp experiments in chick neurons.

In cobalt uptake experiments, the proportion of cobalt-stained neurons in chick and rat increased similarly with increasing temperature. The percentage of stained rat neurons at each temperature corresponded to the cobalt uptake data from rats reported by Reichling *et al.* (1997). At a temperature of 46 °C, ≈30% of the neurons responded with cobalt uptake. As the response–stimulus correlation did not reach a plateau it is possible that this proportion does not reflect the maximum of responsive neurons. Assuming that VR1 is the heat-transducing receptor in rat DRG neurons, one would expect a similar proportion as the one responding to 1 μM capsaicin (≈60%). A study using a calcium imaging method showed that only about half of the rat neurons excited by capsaicin also responded to heat up to 50 °C (Greffrath *et al.*, 1999). The discrepancy in the proportion of neurons responding to heat or capsaicin could indicate that there are different molecular entities that account for the membrane responses to both stimuli. Single-channel recordings on rat DRG neurons showed that distinct channels respond to heat or capsaicin, respectively, but only a few show dual sensitivity (Nagy & Rang, 1999b). This could indicate that there are subtypes of receptors, some responding only to heat, some only to capsaicin and some to both. It could be speculated that the heat receptor found in chick neurons is the same as the putative receptor responding only to low threshold heat in rat. Recently, two independent groups created mice with a disrupted VR1 gene (Caterina *et al.* 2000; Davis *et al.* 2000). These animals lack the capsaicin, acid and many of the heat responses. However, both groups observed residual heat evoked responses suggesting additional receptors for noxious heat transduction.

The size distribution of heat-induced cobalt-stained neurons in chick showed that the responsive neurons are of small to medium size. In rat neurons we observed a similar size distribution and our findings compare to the size distribution of VR1-expressing rat DRG neurons determined by labelling with specific antibodies (Guo *et al.*, 1999).

In spite of the fact that chick DRG neurons do not respond to capsaicin, the response to low-threshold noxious heat stimuli was antagonized by the competitive capsaicin antagonist capsazepine. In contrast to capsazepine (Bevan *et al.*, 1992), RR is a noncompetitive capsaicin antagonist (Maggi *et al.*, 1993). Therefore, it was not surprising that also in chick neurons low-threshold heat responses were antagonized by RR. The higher efficacy of RR compared to capsazepine in blocking the heat response was also reported in patch-clamp experiments in rat DRG neurons (Nagy & Rang, 1999b). The previously reported failure of RR to block the heat-induced cobalt uptake in rat neurons could have been due to the 10-fold lower concentration used with no preincubation with RR (Reichling *et al.*, 1997).

The disparity of capsaicin- and heat-sensitivity between chick and rat neurons indicates that the low-threshold heat receptor in chick neurons is not identical to VR1. However, the finding that capsazepine blocks heat responses in chick neurons suggests that the heat receptor may be a homologue to VR1 with a slightly different sequence of amino acids. This putative change in sequence could on the one hand cause the failure of capsaicin to open the heat-activated channel and on the other hand explain the significantly higher efficacy of capsazepine in chick neurons compared to that in rat neurons.

These results suggest that the differences in capsaicin susceptibility between species could be due to differences in the capsaicin binding site of noxious-heat-transducing receptors. Whilst vanilloid-receptor subtypes have been proposed we suggest the possibility of heat receptor subtypes which differ in their capsaicin binding site.

Acknowledgements

The authors are especially indebted to Dr C. Stucky for critical reading of the manuscript and helpful hints, to Drs K. Schmidt and B. Roth for supportive work, and to T. Martini for expert technical assistance. This work was supported by Deutsche Forschungsgemeinschaft PE 299/3–2 and SFB 353 to M.P.

Abbreviations

D-MEM, Dulbecco's modified Eagle's medium; DRG, dorsal root ganglion; RR, ruthenium red; VR1, vanilloid receptor 1; VRL-1, vanilloid-receptor-like protein 1.

References

- Bevan, S., Hothi, S., Hughes, G., James, I.F., Rang, H.P., Shah, K., Walpole, C.S. & Yeats, J.C. (1992) Capsazepine: a competitive antagonist of the sensory neurone excitant capsaicin. *Br. J. Pharmacol.*, **107**, 544–552.
- Caterina, M.J., Leffler, A., Malmberg, A.B., Martin, W.J., Trafton, J., Petersen-Zeit, K.R., Koltzenburg, M., Basbaum, A.I. & Julius, D. (2000) Impaired nociception and pain in mice lacking the capsaicin receptor. *Science*, **288**, 306–313.
- Caterina, M.J., Rosen, T.A., Tominaga, M., Brake, A.J. & Julius, D. (1999) A capsaicin-receptor homologue with a high threshold for noxious heat. *Nature*, **398**, 436–441.
- Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D. & Julius, D. (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature*, **389**, 816–824.
- Cesare, P. & McNaughton, P. (1996) A novel heat-activated current in nociceptive neurons and its sensitization by bradykinin. *Proc. Natl Acad. Sci. USA*, **93**, 15435–15439.
- Davis, J.B., Gray, J., Gunthorpe, M.J., Hatcher, J.P., Davey, P.T., Overend, P., Harries, M.H., Latcham, J., Clapham, C., Atkinson, K., Hughes, S.A., Rance, K., Grau, E., Harper, A.J., Pugh, P.L., Rogers, D.C., Bingham, S., Randall, A. & Sheardown, S.A. (2000) Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. *Nature*, **405**, 183–187.
- Dittert, I., Vlachova, V., Knotkova, H., Vitaskova, Z., Vyklicky, L., Kress, M. & Reeh, P.W. (1998) A technique for fast application of heated solutions of different composition to cultured neurones. *J. Neurosci. Meth.*, **82**, 195–201.
- Fields, H.L. (1987). *Pain*. McGraw-Hill, New York.
- Greffrath, W., Kirschstein, T., Nawrath, H. & Treede, R.-D. (1999) Noxious heat increases cytosolic calcium in a subpopulation of rat dorsal root ganglion neurones. In Elsner, N. & Eysel, U. (eds), *Proceedings of the 1st Göttingen Conference of the German Neuroscience Society 1999*, Vol. II, 398. Georg Thieme-Verlag, Stuttgart, New York.
- Guo, A., Vulchanova, L., Wang, J., Li, X. & Elde, R. (1999) Immunocytochemical localization of the vanilloid receptor 1 (VR1): relationship to neuropeptides, the P2X3 purinoceptor and IB4 binding sites. *Eur. J. Neurosci.*, **11**, 946–958.
- Hogan, P.G. (1983) Expression of markers for pain sensory neurons in cell culture. *PhD dissertation*, Harvard University, Cambridge, MA, USA.
- Holzer, P. (1991) Capsaicin: cellular targets, mechanisms of action, and selectivity for thin sensory neurons. *Pharmacol. Rev.*, **43**, 143–201.
- Kirschstein, T., Greffrath, W., Büsselberg, D. & Treede, R.D. (1999) Inhibition of rapid heat responses in nociceptive primary sensory neurons of rats by vanilloid receptor antagonist. *J. Neurophysiol.*, **82**, 2853–2860.
- Koltzenburg, M. & Lewin, G.R. (1997) Receptive properties of embryonic chick sensory neurons innervating skin. *J. Neurophysiol.*, **78**, 2560–2568.
- Maggi, C.A., Bevan, S., Walpole, C.S., Rang, H.P. & Giuliani, S. (1993) A comparison of capsazepine and ruthenium red as capsaicin antagonists in the rat isolated urinary bladder and vas deferens. *Br. J. Pharmacol.*, **108**, 801–805.
- Nagy, I. & Rang, H. (1999a) Noxious heat activates all capsaicin-sensitive and also a subpopulation of capsaicin-insensitive dorsal root ganglion neurons. *Neuroscience*, **88**, 995–997.
- Nagy, I. & Rang, H. (1999b) Similarities and differences between the responses of rat sensory neurons to noxious heat and capsaicin. *J. Neurosci.*, **19**, 10647–10655.
- Necker, R. & Reiner, B. (1980) Temperature-sensitive mechanoreceptors, thermoreceptors and heat nociceptors in the feathered skin of pigeons. *J. Comp. Physiol.*, **135**, 201–207.
- Petersen, M., LaMotte, R.H., Klusch, A. & Kniffki, K.D. (1996) Multiple

- capsaicin-evoked currents in isolated rat sensory neurons. *Neuroscience*, **75**, 495–505.
- Reichling, D.B., Barratt, L. & Levine, J.D. (1997) Heat-induced cobalt entry: an assay for heat transduction in cultured rat dorsal root ganglion neurons. *Neuroscience*, **77**, 291–294.
- Reichling, D.B. & MacDermott, A.B. (1991) Lanthanum actions on excitatory amino acid-gated currents and voltage-gated calcium currents in rat dorsal horn neurons. *J. Physiol. (Lond.)*, **441**, 199–218.
- Sann, H., Harti, G., Pierau, F.K. & Simon, E. (1987) Effect of capsaicin upon afferent and efferent mechanisms of nociception and temperature regulation in birds. *Can. J. Physiol. Pharmacol.*, **65**, 1347–1354.
- Stucky, C.L. & Lewin, G.R. (1999) Isolectin B4-positive and -negative nociceptors are functionally distinct. *J. Neurosci.*, **19**, 6497–6505.
- Szallasi, A. (1994) The vanilloid (capsaicin) receptor: receptor types and species differences. *Gen. Pharmacol.*, **25**, 223–243.
- Szallasi, A. & Blumberg, P.M. (1990) Specific binding of resiniferatoxin, an ultrapotent capsaicin analog, by dorsal root ganglion membranes. *Brain Res.*, **524**, 106–111.
- Szolcsányi, J. (1982) Capsaicin type pungent agents producing pyrexia. In Milton, A.S. (ed.), *Handbook of Experimental Pharmacology*. Springer, Berlin, pp. 437–478.
- Szolcsányi, J., Sann, H. & Pierau, F.K. (1986) Nociception in pigeons is not impaired by capsaicin. *Pain*, **27**, 247–260.
- Tominaga, M., Caterina, M.J., Malmberg, A.B., Rosen, T.A., Gilbert, H., Skinner, K., Raumann, B.E., Basbaum, A.I. & Julius, D. (1998) The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron*, **21**, 531–543.
- Winter, J. (1987) Characterization of capsaicin-sensitive neurones in adult rat dorsal root ganglion cultures. *Neurosci. Lett.*, **80**, 134–140.
- Wood, J.N., Winter, J., James, I.F., Rang, H.P., Yeats, J. & Bevan, S. (1988) Capsaicin-induced ion fluxes in dorsal root ganglion cells in culture. *J. Neurosci.*, **8**, 3208–3220.