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EXPRESSION OF NEUROKININ-1 RECEPTORS ON CULTURED DORSAL ROOT GANGLION NEURONS FROM THE ADULT RAT

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Abstract—The expression of neurokinin-1 receptors in isolated dorsal root ganglion neurons of adult rats was investigated using substance P covalently bound to a 1.4-nm gold particle. Binding of substance P–gold was determined in neurons after 0.8, 1.8 or 3.8 days under culture conditions. Substance P–gold binding sites were identified in 9.5 ± 1.8% of the neurons that were cultured for 0.8 days. The proportion of neurons with substance P–gold binding sites increased to 21.5 ± 3.6% after 1.8 days in culture and returned to the initial values (9.2 ± 2.1%) after 3.8 days in culture. Binding of substance P–gold was suppressed by co-administration of [Sar^9, $Met(O_2)^{11}$] substance P, a specific agonist at the neurokinin-1 receptor, but not by co-administration of [β-Ala^8] Neurokinin A (4–10), an agonist at the neurokinin-2 receptor, and senktide, an agonist at the neurokinin-3 receptor. This indicates that substance P–gold was bound specifically to neurokinin-1 receptors. Double-labelling with RT97, an antibody that selectively labels somata of A-fibres revealed that substance P binding sites were present in small neurons with myelinated and unmyelinated axons.

These data show that a proportion of dorsal root ganglion neurons of adult rat in culture exhibit neurokinin-1 receptors. A transient increase in the proportion of neurons expressing neurokinin-1 receptors after 1.8 days in culture suggests that the expression of neurokinin-1 receptors is subjected to regulation. © 1999 IBRO. Published by Elsevier Science Ltd.

Key words: substance P, substance P–gold, substance P binding sites, A-fibres, C-fibres, nanogold.

The neuropeptide substance P (SP) is produced in a proportion of neurons of the peripheral and central nervous system. In the peripheral nervous system, substance P-like immunoreactivity is contained in a proportion (up to 20%) of dorsal root ganglion (DRG) neurons with unmyelinated axons (C-fibres).[17,56] SP can be released from peripheral and central terminals of primary afferents by electrical stimulation of peripheral nerves,[3,12] by application of noxious stimuli to the tissue[10,23] and during inflammation.[11,39,46] The release of SP from peripheral terminals of primary afferent neurons is involved in neurogenic inflammation.[18,24,26,27] SP released from central terminals is thought to act as a mediator that is involved in the responses of spinal cord neurons to noxious stimuli to peripheral tissue[7,15,33,36,42] and in the generation and maintenance of hyperexcitability of spinal cord neurons evoked by intradermal injection of capsaicin[9] and peripheral inflammation or injury.[37,53] During inflammation in peripheral tissue, the synthesis of SP in DRG neurons is increased.[8,14,32,38,50,51]

SP acts through the G-protein-coupled neurokinin-1 (NK-1) receptor.[29,34,43] Neurons expressing NK-1 receptors have been identified in numerous areas of the CNS including the spinal cord.[1,5,28,30,35,57] Some evidence has been provided that NK-1 receptors may also be expressed in the peripheral nervous system. Low levels of NK-1 receptor mRNA were identified in DRG of the mouse.[2] NK-1 receptors have also been demonstrated in a proportion of unmyelinated axons of rat glabrous skin by immunohistochemical staining.[4] Further evidence for NK-1 receptors in the peripheral nervous system was provided by application of SP to skin[21] or joint[16] which activated or sensitized a proportion of primary afferent neurons. However, other studies were unable to show an effect of SP on the discharge properties of primary afferent neurons.[6,22] Recently, Hu *et al.*[19] reported that 89.5% of freshly isolated DRG neurons responded with an inward current to application of SP, suggesting that a large majority of DRG neurons exhibit SP receptors.

In the present study we investigated the expression of SP binding sites on cultured DRG neurons of adult rats using SP covalently linked to a single gold particle (SP–gold). In order to examine whether

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Abbreviations: BSA, bovine serum albumin; BSA-C, bovine serum albumin-C; DMEM, Dulbecco's modified Eagle's medium; DRG, dorsal root ganglion; FITC, fluorescein isothiocyanate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; NGF, nerve growth factor; NK-1, neurokinin-1; PBS, phosphate-buffered saline; NHS-Nanogold, *N*-hydroxy-succinimido-Nanogold; SP, substance P.

SP–gold is specifically bound to the NK-1 receptor, suppression of binding of SP–gold to neurons by co-administration of specific agonists at the NK-1, NK-2 and NK-3 receptor was assessed. Furthermore, double-labelling with RT97 (a marker for myelinated neurons) was used to determine whether SP binding sites were located on myelinated or unmyelinated neurons. Preliminary data have been published as abstract.[49]

EXPERIMENTAL PROCEDURES

Primary culture

Sprague–Dawley rats (Charles River, Sulzfeld, Germany; male, body weight 200–460 g) were killed by an i.p. injection of a lethal dose of sodium pentobarbital (Narcoren^{R}). DRGs were dissected from all spinal segments. As described previously,[40] the ganglia were incubated at 37°C in 0.28 U/ml collagenase type A dissolved in Dulbecco's modified Eagle's medium (DMEM) for 110–120 min, depending on the body weight of the rat. After washing with phosphate-buffered saline (PBS) (20 mM, pH 7.4), the ganglia were incubated in PBS containing 25,000 U/ml trypsin for 11 min at 37°C. Then the ganglia were dissociated into single cells by gentle agitation and by trituration through a fire-polished Pasteur pipette. The cells were washed three times in DMEM by centrifugation (500 *g*, 5 min). The final cell pellet was suspended in Ham's F-12 medium containing 10% heat-inactivated horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 100 ng/ml nerve growth factor.

Cells were plated on poly-L-lysine (200 μg/ml) coated 12 mm glass coverslips and kept for 0.8, 1.8 or 3.8 days at 37°C in a humidified incubator gassed with 3.5% CO_2 and air. Cells were fed every day with supplemented Ham's F-12 medium (see above).

Preparation of the substance P–gold conjugate

The SP–gold conjugate was prepared as described earlier.[47] Briefly, 1 μmol SP was dissolved in 500 μl HEPES (20 mmol, pH 7.5). This solution was added to 6 nmol sulfo-*N*-hydroxy-succinimido Nanogold (NHS-Nanogold) reagent dissolved in 500 μl double-distilled H_2O, and incubated for 1 h at room temperature. To separate SP–gold conjugate from unbound SP, a membrane centrifugation (Amicon microcon-10 system) was used. The SP–gold conjugate was dissolved in PBS containing 0.1% bovine serum albumin (BSA), 0.2 M sucrose, 4 μg/ml leupeptin and 10 mM sodium azide. This solution was aliquoted and stored at −20°C for a maximum of three months.

Substance P–gold binding to cultured neurons. The cells were prefixed with 2% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 30 min. After washing with PBS (20 mM, pH 7.4), the cells were pre-treated with 50 mM glycin in PBS and thereafter with 5% BSA and 0.1% gelatine in PBS for 30 min. Then the cells were washed with 0.1% BSA-C and incubated overnight with 0.6 nmol/ml SP–gold in PBS containing 0.1% BSA-C, bacitracin (40 μg/ml), leupeptin (4 μg/ml) and chymostatin (2 μg/ml) at 4°C in a moist chamber. Following washing with PBS plus 0.1% BSA-C and thereafter with PBS to remove unbound SP–gold, cells were postfixed with 2% glutaraldehyde in PBS for 10 min. After extensive washing with PBS and double-distilled H_2O, the gold particles were intensified with silver enhancer (R-Gent, pH 5.5) for 20 to 25 min depending on room temperature. The reaction was stopped by washing in double-distilled H_2O. The preparations were dehydrated and embedded in DePeX.

In order to examine whether SP–gold is bound specifically to NK-1 receptors (and not to NK-2 and NK-3 receptors), 0.6 nmol/ml SP–gold was incubated in the presence of either 1 μmol/ml [Sar^9,$Met(O_2)^{11}$] substance P, a specific agonist at the NK-1 receptor, or 1 μmol/ml [β-Ala^8] Neurokinin A (4–10), a specific agonist at the NK-2 receptor, or 1 μmol/ml senktide, a specific agonist at the NK-3 receptor, respectively.

Control incubations

Two different control experiments were performed: (i) to determine the specificity of the SP–gold complex used in the binding studies, a displacement control was performed. Neurons were incubated in 1 μmol/ml unlabelled SP that was added to 0.6 nmol/ml SP–gold; (ii) to determine any non-specific binding of the NHS–Nanogold particles, neurons were incubated with 0.6 nmol/ml NHS–Nanogold only. In neither case did the gold particles alone produce staining above background.

Double labelling of the dorsal root ganglion neurons

In order to discriminate neurons with myelinated or unmyelinated axons, the mouse monoclonal antibody RT97 was used in four additional cultures (1.8 days). RT97 recognizes the 200,000 mol. wt neurofilament in its phosphorylated form and in the DRG of the rat it selectively labels the somata of sensory A-fibres.[25,44] In the first step the procedure of SP–gold binding was performed as described above except that methanol was used for fixation instead of paraformaldehyde and glutaraldehyde. After the gold particles were intensified with silver enhancer, the cells were washed with double-distilled H_2O and PBS. In a second step the neurons were incubated for 3 h with the antibody RT97 diluted 1:20 in PBS. Then the coverslips were rinsed with PBS and incubated for 2 h with an anti-mouse antibody (diluted 1:50 in PBS) labelled with fluorescein isothiocyanate (FITC). Finally, the coverslips were rinsed in PBS, mounted on glass slides with Vectashield antifade mounting medium and stored at 4°C protected from light. To visualize the fluorescence signal, standard filters were used. In control experiments performed without the first antibody no fluorescence signal was detectable. To determine the subpopulation of RT97-positive cells, immunolabelling with the antibody RT97 was also done without previous incubation with SP–gold.

Data analysis

At each time-point (0.8, 1.8 and 3.8 days in culture), six to eight DRG preparations were used. The data analysis was done as described earlier.[48] Briefly, from every coverslip 100 structurally intact neurons were examined with a light microscope (Zeiss, Axiophot) coupled to a CCD colour video camera (Sony) and an image analysing system (Optimas). On each coverslip neurons were randomly selected; neurons obstructed by other neurons or by tissue were not included. In total 600–800 neurons were sampled at each time-point (0.8, 1.8 and 3.8 days in culture). The relative grey value (grey value of the soma/grey value of the substrate background) was determined for each soma. From each individual binding experiment one coverslip was used to perform a displacement control incubation (see above). This procedure allowed us to determine the grey value range of neurons with no SP–gold binding in each experiment (see Fig. 2A, white bars). In all other coverslips of the particular experiment, neurons were considered as positive for SP-binding sites if they had a relative grey value above that of neurons from the control incubation. For the final analysis, data from all experiments were pooled. Proportions of neurons are expressed as the mean ± S.D. To evaluate the cell size, the cross-sectional area was taken from each selected neuron.

Materials

Sulfo-*N*-hydroxy-succinimido Nanogold, R-Gent and acetylated BSA were purchased from BioTrend (D-50876

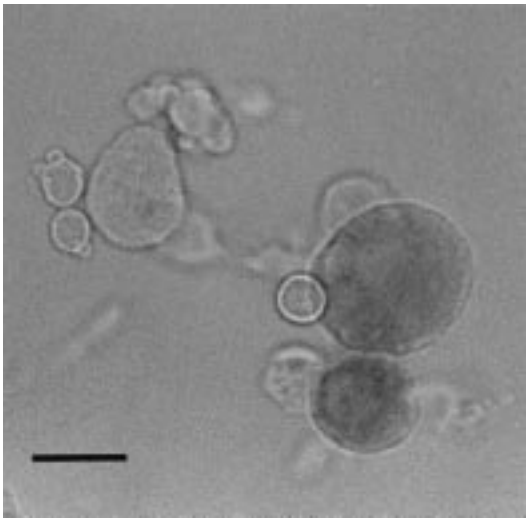

Fig. 1. Isolated DRG neurons of the adult rat after an incubation with SP–gold and subsequent silver enhancement. The neurons were in culture for 1.8 days. The black staining indicates binding of SP–gold. Scale bar = 20 μm.

Köln, Germany), substance P from Sigma (D-82039 Deisenhofen, Germany). [Sar^{9} Met(O$_2$)^{11}] substance P, [β-Ala^{8}] Neurokinin A (410) and succinyl-[Asp6, N-Me-Phe^{8}] substance P (6–11) (=senktide) were purchased from Bachem (D-69126 Heidelberg, Germany). Ham's F-12 medium, DMEM, heat-inactivated horse serum, penicillin and streptomycin were purchased from Gibco BRL (D-76339 Eggenstein, Germany). Sodium pentobarbital (Narcoren^{R}) was purchased from Rhone Merieux GmbH (D-88464 Laupheim, Germany). Collagenase type A and nerve growth factor (7S, recombinant human) were purchased from Boehringer Mannheim GmbH (D-68298 Mannheim, Germany). The monoclonal antibody to antineurofilament 200 (clone NE14) and the anti-mouse antibody labelled with FITC were ordered from Sigma. Vectashield was purchased from Camon (D-65205 Wiesbaden, Germany). All other reagents were purchased from Fluka (D-89231 Neu-Ulm, Germany) or Sigma.

RESULTS

After treatment with SP–gold and silver enhancer, the neurons exhibited different grey staining. Typically, the grey staining was evenly distributed on the soma membrane. Figure 1 shows single DRG neurons after 1.8 days under culture conditions. After one day in culture neurons developed processes. For analysis of the grey value of a neuron only the soma membrane was analysed.

Proportion of neurons with substance P binding sites after different times under culture conditions

Figure 2A displays the distribution of the grey values in neurons that were 0.8, 1.8 or 3.8 days in culture. Each histogram shows the proportion of neurons with particular binned relative grey values. Pooled neurons from control incubations that were treated with SP–gold plus an excess of unlabelled SP to prevent binding of SP–gold are shown in Fig. 2A4. These neurons showed relative grey values in the range of 0.00–0.17, and therefore grey values <0.17 are not due to binding of SP–gold. By contrast, Fig. 2A1–3 display the distribution of grey values of neurons that were in culture for 0.8 days (Fig. 2A1), 1.8 days (Fig. 2A2) and 3.8 days (Fig. 2A3) and then treated only with SP–gold. A proportion of these neurons (Fig. 2A1–3) exhibited grey values >0.17 indicating binding of SP–gold. With this criterion, 9.5 ± 1.8% of the neurons showed SP–gold binding sites after 0.8 days in culture, 21.5 ± 3.6% of the neurons after 1.8 days in culture and 9.2 ± 2.1% of the neurons after 3.8 days in culture. The higher proportion of neurons with SP–gold binding sites after 1.8 days in culture resulted from an increased number of neurons that exhibited grey values between 0.2 and 0.3.

Figure 2B1–3 shows the distribution of the cross-sectional areas of all neurons (white bars) and of the subgroup of neurons with SP–gold binding sites (black bars) after 0.8, 1.8 and 3.8 days in culture, respectively. The distribution of the neurons with respect to their cross-sectional areas was similar after the different times in culture, ranging from 50 to 2000 μm^2. Obviously, the increase in the number of neurons with SP–gold binding sites after 1.8 days in culture is restricted to neurons with a small cross-sectional area (less than 500 μm^2).

Discrimination between binding of substance P–gold to neurokinin-1, neurokinin-2 and neurokinin-3 receptors

In order to examine whether binding of SP–gold was specific to NK-1 receptors, neurons of three DRG cultures were incubated with SP–gold solution plus one of the agonists at NK-1, NK-2 or NK-3 receptors, respectively. The experiments were performed on neurons cultured for 0.8, 1.8 or 3.8 days. Figure 3 displays the proportion of neurons with SP–gold binding sites after the different incubations. The white bars show the proportions of neurons with SP–gold binding sites (relative grey value >0.17) after incubation with SP–gold only. When [Sar^{9},Met(O$_2$)^{11}] substance P, a specific agonist at the NK-1 receptor, was co-administered with the SP–gold solution, the proportion of neurons with SP–gold binding sites was significantly smaller than in the sample of neurons that were only treated with SP–gold (Fig. 3, black bars). After co-administration of [β-Ala^{8}] Neurokinin A (4–10), the agonist at NK-2 receptors, a small and not significant reduction of the SP-binding was seen at 1.8 days but not at 0.8 and 3.8 days (Fig. 3, light grey bars). Co-administration of the agonist at the NK-3 receptor, senktide, did not reduce the proportion of neurons with SP–gold binding sites (Fig. 3, dark grey bars). Thus, only the agonist at NK-1 receptors reduced the binding of SP–gold, indicating that binding of SP–gold was specific to NK-1 receptors.

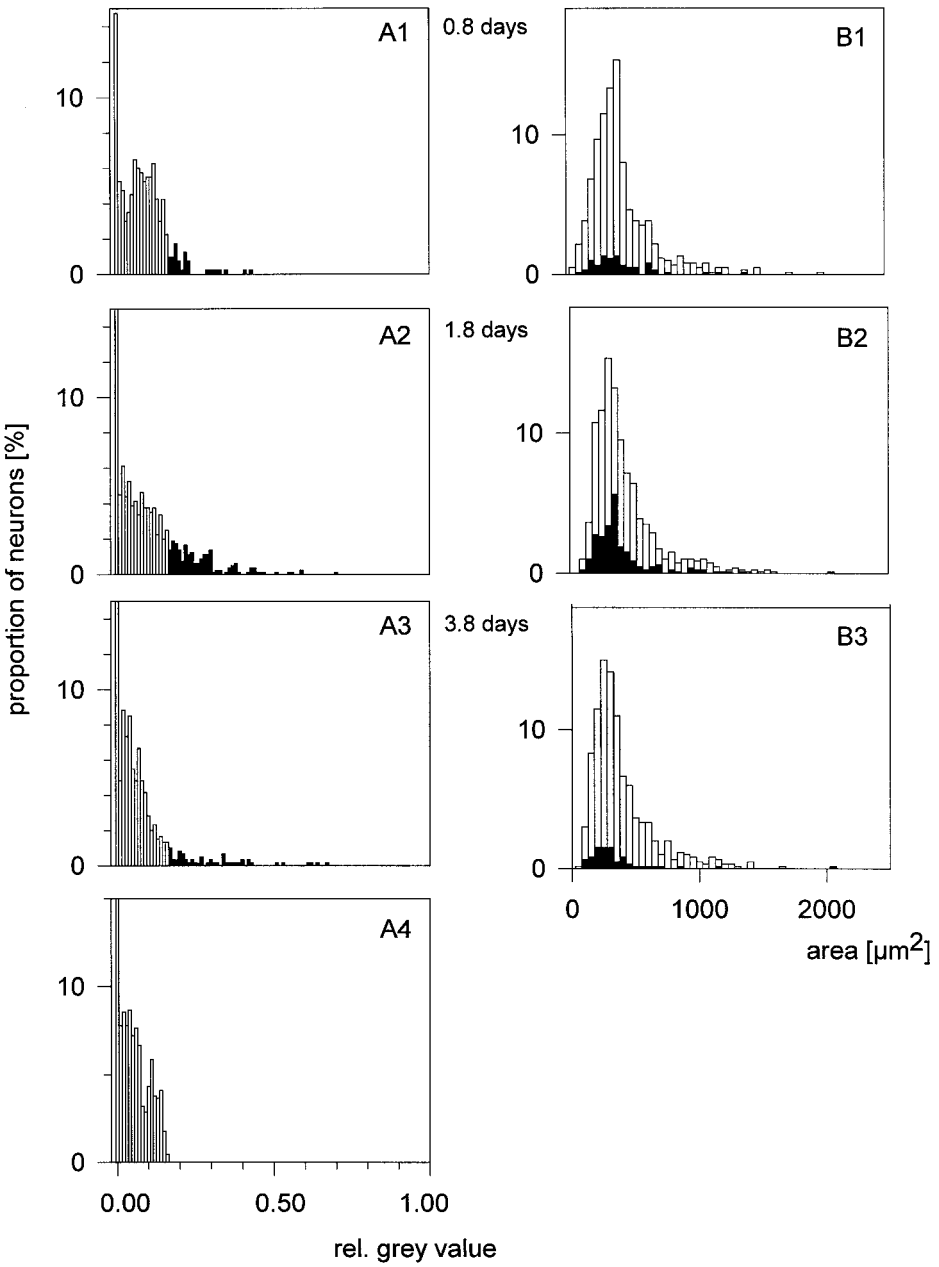

Fig. 2. (A) Proportions of neurons with particular relative grey value. (A1) Neurons (n=600 from six cultures) treated with SP–gold after 0.8 days in culture. (A2) Neurons (n=800 from eight cultures) treated with SP–gold after 1.8 days in culture. (A3) Neurons (n=600 from six cultures) treated with SP–gold after 3.8 days in culture. (A4) Neurons (n=1000 from 10 cultures) treated with SP–gold and SP after 0.8, 1.8 and 3.8 days in culture (control incubations). White bars: neurons exhibiting grey densities that were in the range of those observed in neurons from control incubations. Black bars: neurons exhibiting grey densities that were higher than those observed in the neurons from control incubations. (B) Histograms of the soma size distribution. The open columns show the cross-sectional areas of all sampled neurons at 0.8 days (B1), 1.8 days (B2) and 3.8 days (B3), the black bars show the neurons with SP–gold binding (grey densities >0.17).

Discrimination between neurons with myelinated and unmyelinated axons

The antibody RT97 labelled 47% of all neurons cultured for 1.8 days indicating that these were somata of DRG neurons with A-fibres (three experiments). To identify the proportion of SP–gold binding neurons within the groups of RT97-positive and -negative neurons, double labelling was performed in another four experiments. The result of the latter experiments is shown in Table 1. In both RT97-positive and RT97-negative neurons, a subgroup of

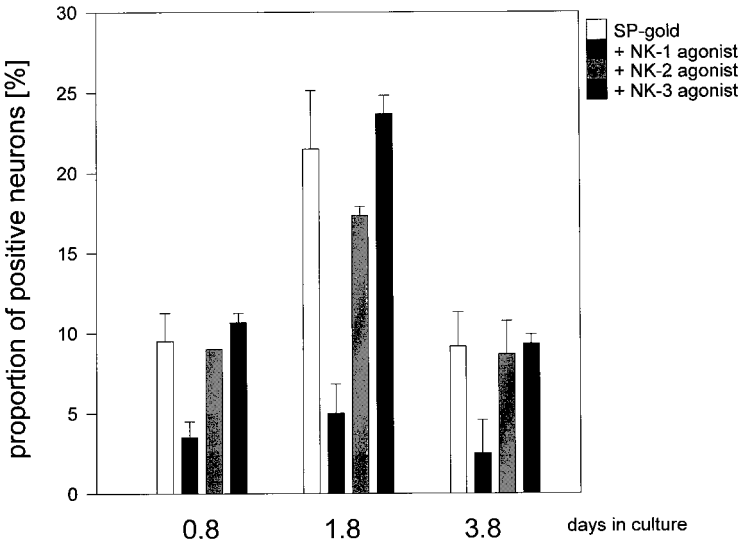

Fig. 3. Proportion of the neurons with SP–gold binding after 0.8, 1.8 and 3.8 days in culture when the neurons were treated only with SP–gold and when SP–gold was co-administered with either an agonist at NK-1 receptors, [Sar^9, Met(O$_2$)^{11}] substance P (1 μmol/ml), or at NK-2 receptors, [β-Ala^8] Neurokinin-(4–10) (1 μmol/ml), or at NK-3 receptors, senktide (1 μmol/ml). In total 1500 neurons were analysed.

Table 1. Proportion of RT97-positive neurons and of RT97-negative neurons and subsets of RT97-positive and -negative neurons that had substance P–gold binding sites

	Proportion of neurons [%]
RT97-positive	47 ± 3.4
RT97-positive plus SP–gold binding sites	8 ± 1.6
RT97-negative	53 ± 3.4
RT97-negative but with SP–gold binding sites	14 ± 1.6

Values are expressed as mean ± S.D.
Total sample: 400 neurons from four experiments.

the neurons exhibited binding of SP–gold. Thus, neurons with SP–gold binding sites were identified on somata of neurons with A- and C-fibres but the ratio was higher in neurons with C-fibres.

DISCUSSION

This study shows that a proportion of the somata of cultured DRG neurons of the adult rat exhibit binding sites for SP–gold. The binding of SP–gold to neurons was suppressed by the co-administration of an excess of SP or a specific agonist of the NK-1 receptor, but not by the co-administration of agonists acting specifically at NK-2 and NK-3 receptors. The data indicate, therefore, that a proportion of DRG neurons of the adult rat express NK-1 receptors. NK-1 receptors were found in somata of primary afferent neurons with myelinated and unmyelinated axons (A- and C-fibres). There was a transient increase in the proportion of neurons with SP binding sites after 1.8 days in culture, suggesting that expression of NK-1 receptors is subjected to regulation.

SP labelled with Nanogold is suitable to identify NK-1 receptors on DRG neurons. The gold particle is covalently linked to the primary amino group of SP whereas the binding of SP to the NK-1 receptor is largely determined by the C-terminal sequence.[54] The suppression of binding of SP–gold to the neurons by unlabelled SP and by [Sar^9, Met(O$_2$)^{11}] substance P, a specific agonist at the NK-1 receptor, but not by [β-Ala^8] Neurokinin A (4–10), a specific agonist at the NK-2 receptor, or by senktide, a specific agonist at the NK-3 receptor, directly shows the specific binding of SP–gold to NK-1 receptors. This pattern was found at 0.8, 1.8 and 3.8 days in culture showing that SP–gold was specifically bound to NK-1 receptors at all time-points.

In order to analyse whether NK-1 receptors are expressed in neurons with myelinated and/or unmyelinated axons, we used the antibody RT97. This antibody selectively labels the soma of DRG neurons with myelinated A-fibres[25,44] and is therefore a marker to discriminate between neurons with A- and C-fibres. In the sample of neurons studied, about two-thirds of the somata with SP binding sites were RT97-negative, thus belonging to the population of DRG neurons with C-fibres. The somata of neurons with C-fibres are small in size, and indeed most neurons with positive SP–gold staining were found to be of small size. Since many DRG neurons with C-fibres and Aδ-fibres are nociceptors[56] it is likely that at least a proportion of neurons with NK-1 receptors are nociceptive primary afferent neurons.

While NK-1 receptors have been localized on 32% of the peripheral endings of unmyelinated primary

afferent neurons supplying the skin of the rat hindpaw,[4] the present study shows that NK-1 receptors are also expressed in the somata of DRG neurons. Support for the existence of NK-1 receptors on the somata of DRG neurons was also provided by an electrophysiological study by Hu *et al.*[19] who could elicit an inward current by the application of SP in isolated DRG neurons. However, while Hu *et al.*[19] found an effect of SP in 90% of the DRG neurons, the present study revealed NK-1 receptors in a much smaller proportion of neurons. Hu *et al.*[19] discussed the possibility of a bias in their sampling towards neurons with NK-1 receptors since they did not choose neurons at random. The proportion of neurons with NK-1 receptors in the present study is more close to the data of Carlton *et al.*[4] with the difference that some of the neurons in the present study had A-fibres as judged from positive labelling with RT97. However, a direct comparison between the proportions of axons and somata with NK-1 receptors is of limited validity since the nerves supplying different tissue may contain different proportions of neurons with NK-1 receptors. Finally, one cannot totally exclude the possibility that the processing of the neurons, in particular the fixation, could have reduced the density of binding sites leading to an underestimation of the proportion of neurons exhibiting SP–gold binding.

The proportion of neurons expressing NK-1 receptors showed a transient increase after 1.8 days in culture. This increase could result from an up-regulation of the expression of NK-1 receptors in neurons that do not (or only very weakly) express the NK-1 receptor after 0.8 and 3.8 days. However, such an effect may also result from a loss of neurons in the cell culture with a selective survival of neurons expressing NK-1 receptors. In our opinion, the latter explanation is unlikely as the distribution of the sizes of the neurons was quite similar after 0.8, 1.8. and 3.8 days in culture, suggesting that the population of neurons was similar at the three time-points. Furthermore, the up-regulation was only observed after 1.8 days in culture whereas the proportion of neurons with SP–gold binding sites was similar after 0.8 and 3.8 days. This rather suggests that the increase of the proportion of neurons with SP–gold binding sites is due to a transient up-regulation. *In vivo*, up-regulation of NK-1 receptors in the spinal cord has been observed during inflammation of peripheral tissue.[20,31,45,52]

At present, the reason for the up-regulation of the expression of NK-1 receptors in DRG neurons after 1.8 days under culture conditions is not known. In a previous study we found an up-regulation in the expression of bradykinin binding sites on isolated DRG neurons of mice[41] and rats.[48] Interestingly, the up-regulation of the expression of the bradykinin receptor in mice-DRG neurons did not occur in the presence of an antibody to nerve growth factor (NGF) suggesting that the up-regulation is dependent on NGF.[41] The up-regulation of the bradykinin receptor was also absent in p75 knock-out mice suggesting that this particular regulatory effect is mediated via interaction of NGF with the p75 receptor.[41]

It is assumed that the expression of receptors in the somata of DRG neurons reflects the expression of receptors and ion channels in the peripheral terminations in the periphery (e.g., Ref. 13). SP, the endogenous ligand of NK-1 receptors, can be released from cultured DRG neurons.[55] Whether this occurs *in vivo* is unknown. Alternatively, SP could reach DRG neurons via the blood. However, the functional significance of NK-1 receptors in the soma of DRG neurons has to be determined. NK-1 receptors on peripheral terminals of primary sensory neurons could provide the basis for the reported effects of SP on these neurons, namely the sensitization of joint afferents for mechanical stimuli,[16] the sensitization of cutaneous afferents for the effect of inflammatory mediators[21] and the induction of mechanical hyperalgesia in the paw.[4] Whether NK-1 receptors are present on central endings of DRG neurons has not been determined to our knowledge.

CONCLUSION

The present study shows that a proportion of somata of primary sensory neurons exhibit NK-1 receptors. Neurons expressing NK-1 receptors are unmyelinated or myelinated. The transient up-regulation of NK-1 receptors after 1.8 days in culture suggests that NK-1 receptors on DRG neurons are subjected to regulation.

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