SNS/PN3 and SNS2/NaN sodium channel-like immunoreactivity in human adult and neonate injured sensory nerves

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Received 29 December 1999

Edited by Philip Randle

Abstract Two tetrodotoxin-resistant voltage-gated sodium channels, SNS/PN3 and SNS2/NaN, have been described recently in small-diameter sensory neurones of the rat, and play a key role in neuropathic pain. Using region-specific antibodies raised against different peptide sequences of their α subunits, we show by Western blot evidence for the presence of these channels in human nerves and sensory ganglia. The expected fully mature 260 kDa component of SNS/PN3 was noted in all injured nerve tissues obtained from adults; however, for SNS2/NaN, smaller bands were found, most likely arising from protein degradation. There was increased intensity of the SNS/PN3 260 kDa band in nerves proximal to the site of injury, whereas it was decreased distally, suggesting accumulation at sites of injury; all adult patients had a positive Tinel's sign at the site of nerve injury, indicating mechanical hypersensitivity. Injured nerves from human neonates showed similar results for both channels, but neonate neuromas lacked the SNS2/NaN 180 kDa molecular form, which was strongly present in adult neuromas. The distribution of SNS/PN3 and SNS2/NaN sodium channels in injured human nerves indicates that they represent targets for novel analgesics, and could account for some differences in the development of neuropathic pain in infants.

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Key words: Sodium channel; Peripheral nerve; Western blotting; Pain; SNS

1. Introduction

Subsets of sensory neurones express different sodium channels, which affect their electrophysiological properties, and may relate to their function in normal and pathophysiological states [1,2]. Sodium currents, including tetrodotoxin-sensitive (TTX-S) and TTX-resistant (TTX-R) currents, may contribute to ectopic generation of action potentials following nerve injury [3]. As ectopic impulse activity may be related to symptoms, including pain phenomena, these sodium channels form potential targets for novel analgesics in neuropathic pain.

The sodium channels are heterodimers composed of an α subunit (260 kDa) and two smaller subunits, β1 and β2, which can regulate the properties of the α subunit [4]. The sensory neurone-specific SNS/PN3 α subunit was initially identified as a 6.5 kb transcript expressed selectively in rat dorsal root ganglia (DRG) with a full-length cDNA clone encoding a 1957 amino acid protein that has 65% identity with the amino acid sequence of rat cardiac TTX-insensitive sodium channel [5,6]. In an in vitro transcription/translation system, a 220 kDa protein was generated [5].

There is increasing evidence that SNS/PN3 may play a key role in nociception. A recent study has shown that SNS-null mutant mice demonstrate pronounced analgesia to mechanical noxious stimuli [7]. Antisense mRNA experiments have suggested that SNS/PN3 but not SNS2/NaN [8,9], another TTX-R sodium channel that is distributed within the DRG, is critical in animal models of neuropathic pain [10]. This evidence is supported by our recent immunohistochemical studies of the distribution of these two channels in injured human sensory neurones [11]. However, little is known about the molecular forms of these channels in humans. The present study therefore examined the presence and molecular forms of αSNS/PN3 and αSNS2/NaN in normal and damaged human nerve preparations, using antibodies derived from rat peptide sequences of these channels. Since accumulation of these channels at sites of nerve injury may lead to ectopic impulse activity and chronic pain phenomena [11], which we have observed not to occur after nerve injury in the neonatal human and rat [12], we have also studied, for the first time, injured nerves from human neonates.

2. Materials and methods

2.1. Tissue specimens

Nerves and DRG were collected from adult patients ($n = 16$, age range 18–67 years) with spinal cord root avulsion injury, and damage to the brachial plexus. These included three neuromas, five nerves proximal to the site of injury, five nerves distal to the site of injury and five avulsed DRG, all collected within a week after injury. Six normal nerves were obtained from surgical limb amputations for non-neurological conditions. Injured nerves were also obtained from babies with obstetric brachial plexus palsy undergoing nerve repair ($n = 5$, age range: 3–4 months); the specimens included two neuromas, three nerves proximal and three distal to site of injury. Surgical trimmings of three normal nerves used for grafting were available. The tissues were snap frozen and stored at −70°C. The removal of all tissues was a necessary part of the surgical repair procedure, and their use for the study was approved by the local Ethics Committee, and the patients or parents.

2.2. Antisera

Two different αSNS/PN3 channel antibodies were used in this study, an antibody, SNS poly 11, raised against amino acid residues 424–591 of the intracellular linker between domain I and domain II (gift from Professor J. Wood, UCL), and antibody PN3 #20073, raised against amino acid residues 1059–1077 of the intracellular loop between domain II and domain III, obtained from Roche Biosciences, Palo Alto, CA, USA. An SNS2/NaN antibody (#33948) was

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PII: S0014-5793(00)01166-2

obtained from Roche Biosciences, Palo Alto, CA, USA. A monoclonal antibody that reacts with the phosphorylated form of the 200 kDa component of human neurofilaments was also used (Dako, Cambridge, UK, No. M 0762).

2.3. Extraction

Frozen tissue was first pulverised using a mortar and pestle precooled by liquid nitrogen. It was then homogenised with 20 strokes of a mechanical Dounce homogeniser in ice-cold extraction buffer (phosphate-buffered saline (PBS) containing 1% NP40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate (SDS)) containing freshly added protease inhibitors (5 mM PMSF, 2 mM benzamidine, 1 mM iodoacetamide and 50 mg/ml aprotinin) and incubated on ice for 30 min. Homogenates were then spun at $5000 \times g$ for 15 min. The pellet was discarded and the supernatant was either used immediately or stored at −70°C. An aliquot of the supernatant was taken and total protein measured using a Bradford dye-binding protein assay according to the manufacturer's instructions (Bio-Rad laboratories, Hertfordshire, UK). Homogenates containing equal amounts of protein (approximately 20 μg) were combined with SDS sample buffer and incubated at 100°C for 10 min before loading on a 6% SDS–polyacrylamide gel [13]. After electrophoresis, proteins were transferred to nitrocellulose membranes (Hybond Super, Amersham Life Science, Buckinghamshire, UK) using a semi-dry transblotter at a constant 200 mA for 16 h at 4°C. Protein bands were visualised by staining membranes with 0.1% (w/v) Ponceau S in 1% (v/v) acetic acid, and if necessary, strips were cut out. Excess dye was removed by several washes with PBS wash buffer.

2.4. Immunoblotting

Non-specific binding sites were blocked by incubating the strips in 5% (w/v) non-fat dry milk in a solution of PBS containing 0.1% (v/v) Tween 20 for 1 h. Strips were then incubated for a further 2 h with primary antibody. For competition studies, antibodies was pre-incubated by the addition of 10 μg of corresponding immunogen for 1 h at room temperature. After washing, the blots were incubated with secondary antibody-biotin complexes (Vector Laboratories, Peterborough, UK) for a further 45 min. A pre-formed complex between avidin and biotinylated horseradish peroxidase (Vector Laboratories, Peterborough, UK) was finally added for a further 45 min. Immunoreactive bands were visualised on Hyperfilm after treatment of the blots with ECL-plus Western blotting detection system (Amersham Life Science, Buckinghamshire, UK).

3. Results

3.1. SNS/PN3

A 260 kDa band, with an additional 220 kDa band in some preparations, was found to react with the antibody SNS poly 11. Fig. 1 shows the Western blot profile using the antibody SNS poly 11 in avulsed cervical DRG and normal nerve preparations. The predicted 260 kDa form is clearly detectable,

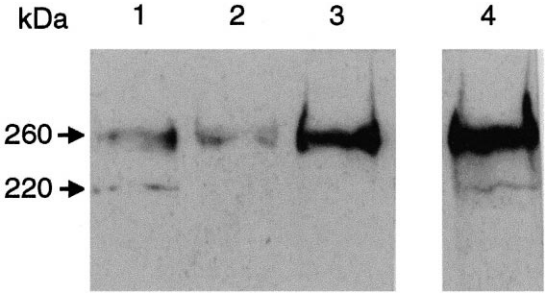

Fig. 1. Western blot analysis of adult human nerve homogenates. Blots were probed using the anti-SNS/PN3 antibody SNS poly 11 and visualised by enhanced chemiluminescence. Lanes 1–3, avulsed sensory ganglia from three different patients; lane 4, normal nerve. The positions of the SNS-immunoreactive bands are indicated by arrow heads.

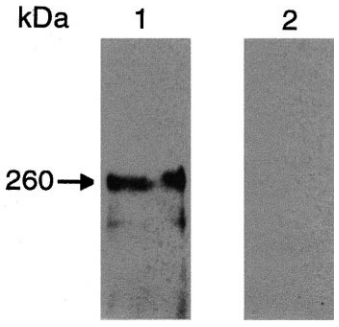

Fig. 2. Specificity of Western blot with anti-SNS antibody SNS poly 11. Blots were probed using the anti-SNS antibody SNS poly 11 in the absence (lane 1) or presence of 10 mg of SNS/PN3 fusion protein (lane 2), and visualised by enhanced chemiluminescence. The positions of the SNS/PN3-immunoreactive bands are indicated by arrow heads.

and a smaller 220 kDa band was seen in some preparations, similar to previous reports in the rat [5]. The intensity of both of these SNS/PN3-like bands was virtually abolished when primary antibody was pre-incubated with excess SNS/PN3 fusion protein (Fig. 2). With antibody PN3 #20073, intense SNS/PN3 immunoreactivity was detected at 220 kDa but not 260 kDa in all the preparations studied. This immunoreactivity was significantly diminished by pre-incubation with cognate antigen (data not shown).

SNS/PN3-like immunoreactivity was detected in all the avulsed DRG, and nerves proximal to the injury site. There was no 260 kDa band in two out of five distal to injury adult nerve extracts, and this was reduced where detected. The 260 kDa SNS/PN3-like band was also found in most of the neonatal extracts examined, which in some specimens was also accompanied by a 220 kDa band (Fig. 3a). There was a similar decrease in the 260 kDa band in damaged infant nerve preparations distal to the site of injury. In these distal preparations, neurofilament 200 kDa protein was also reduced, as expected (Fig. 3b). The 260 kDa band was not detected in either normal human muscle or spinal cord extracts that were examined (data not shown).

3.2. SNS2/NaN

In all three adult neuroma preparations, a strong 180 kDa band was detected with the SNS2/NaN antibody, and some-

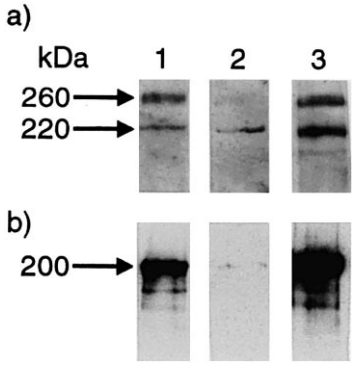

Fig. 3. Western blot analysis of neonate human nerve homogenates. Blots were probed using the anti-SNS antibody SNS poly 11 (a), or probed for neurofilaments (b), and visualised by enhanced chemiluminescence: lane 1, neuroma; lane 2, damaged nerve distal to site of injury; lane 3, damaged nerve proximal to site of injury.

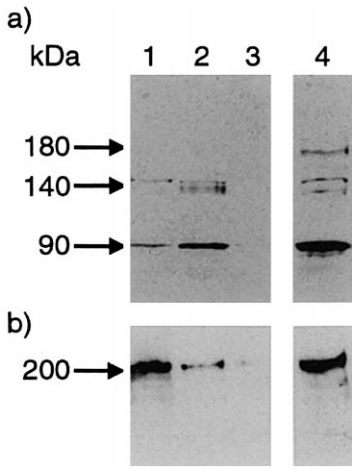

Fig. 4. Western blot of SNS2/NaN-like immunoreactivity in neonate and adult tissues. Blots were probed using the anti-SNS2/NaN antibody (a), or probed for neurofilaments (b), and visualised by enhanced chemiluminescence: lane 1, neonate normal nerve; lane 2, neonate damaged nerve proximal to site of injury; lane 3, neonate damaged nerve distal to site of injury; lane 4, adult neuroma.

times seen with a fainter 190 kDa band (Fig. 4, lane 4). These molecular weight forms of this channel were also detected, but as fainter bands, in 2/4 proximal nerve preparations, but were not detected in undamaged nerve preparations (including normal nerves from the same patient), or in five avulsed DRG. The 180 kDa band was not detected in any of the baby nerve preparations studied (Fig. 4, lanes 1–3). This was confirmed on five other separate experiments, even when more antigen was loaded on the gels (data not shown). However, smaller 140 kDa and 90 kDa were found in nearly all the nerve preparations studied. After competition with cold peptide, the main bands disappeared (180, 140 and 90 kDa, data not shown). The three major PN5-reacting proteins mentioned above were not detected in one human spinal cord and one human skeletal muscle extract preparation that were also studied.

4. Discussion

We have demonstrated, for the first time, the presence of different protein molecular forms of SNS/PN3 and SNS2/NaN in human nerve extracts. The expected 260 kDa glycoprotein representing the fully mature α subunit of SNS/PN3 was easily detectable in all the avulsed sensory ganglia, neuromas and nerves proximal to injury. It was also present in most but not all normal human nerve specimens that were studied. The protein was not detected in human muscle or spinal cord extracts, in accord with the tissue specificity of this channel [5,6].

The smaller 220 kDa SNS/PN3-like band in our study may correspond to the smallest αSNS/PN3 form of similar size that was described in an in vitro translation expression system by others [5]. Alternatively, it is possible that the 220 kDa SNS/PN3-like form represents a product of an alternate spliced form. The larger 260 kDa and 240 kDa bands may correspond to different post-translated glycosylated products, as has been described for the developmental forebrain sodium channels [14].

In specimens in which both proximal and distal nerve had been resected from the site of injury, the intensity of the 260 kDa SNS/PN3-like band always appeared higher in the proximal stump compared to the distal stump. As the patients from whom injured peripheral nerves were collected also had spinal cord root avulsions and pain arising from de-afferentation of the spinal cord, it is not possible to correlate changes in sodium channels with pain phenomena in any simple manner; however, they all had a positive Tinel's sign at the site of nerve injury, indicating mechanical hypersensitivity. SNS/PN3 is known to be synthesised in the DRG and transported to the site of nerve injury, as demonstrated in an animal model [15], where its increased density may contribute to ectopic impulse generation and pain phenomena, including hypersensitivity. After axotomy, rat DRG neurones down-regulate SNS/PN3 transcripts, but there is, nevertheless, accumulation of the sodium channels at the point of nerve injury, indicating translocation of sodium channels from the cell body to the axon [15]. Peripheral inflammatory processes may enhance such accumulation, as inflammatory modulators up-regulate TTX-R current [16–18] and αSNS/PN3 transcripts [18] in small-sized DRG neurones.

We failed to detect the fully glycosylated 260 kDa α unit of SNS2/NaN, which we could readily demonstrate for SNS/PN3. Instead, smaller immunoreactive bands were consistently detected in all specimens studied. This might suggest that the former is less abundant, or may be more rapidly broken down, either by endogenous proteases or during our extraction process. Proteolytic proteins of 90 kDa and 140 kDa have been described for sodium channels present in skeletal muscle [19] and in cultured foetal neurones [20]. The presence of the SNS2/NaN 180 kDa band in adult but not neonate neuromas is of interest, as we have previously observed that neonatal human and rats fail to develop the classical pain syndromes after peripheral nerve injury [12]. It may be hypothesised that full expression and clustering of sodium channels is necessary for the development of pain phenomena; in support, nerve conduction velocity, which is dependent on clustering of sodium channels at nodes of Ranvier, achieves adult values at the age of 2–3 years in humans, when nerve injury does lead to chronic pain syndromes.

In summary, antibodies raised against rat αSNS/PN3 and SNS2/NaN reacted specifically with these sodium channel-like proteins in peripheral nerve specimens in man. Only the mature αSNS/PN3 260 kDa band was found in abundance in all avulsed DRGs, nerves proximal to injury, and neuromas. Although further studies are thus clearly required to elucidate the function of this channel in human pain states, our work indicates that it may be a useful target for novel analgesics in neuropathic pain.

Acknowledgements: We thank Prof. J. Wood and Dr K. Okuse for the generous gift of SNS antibody and peptide.

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