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Angiotensin II type 1 receptors may not influence response of spinal autonomic neurons to axonal damage

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Abstract

Objectives—Angiotensin II can promote cell stress, and the expression of its AT₁ receptor is characteristic of neuronal populations that die off in multiple systems atrophy and Parkinson's disease. To explore the possible significance of these facts, we undertook to: (1) clarify the distribution of AT₁ in rat neurons; (2) use selective antagonists as a means of determining whether AT₁ activation predisposes stressed neurons to die.

Methods—AT₁-expression was examined by immunohistochemistry and by autoradiography for [¹²⁵I]-sarcosine¹-angiotensin II binding in sensory, motor and autonomic neurons. To induce cell loss in a specific neuronal population, rats were given systemic i.v. injection of anti-acetylcholinesterase antibodies, which cause a delayed death of pre-ganglionic sympathetic neurons in the intermediolateral nucleus (IML). As pharmacologic intervention, some immunolesioned rats were treated with the selective AT₁ antagonist, Candesartan.

Results—Immunohistochemistry and autoradiography revealed AT₁ expression in dorsal root ganglia, superior cervical ganglion. In the dorsal horn of the spinal cord, AT₁ immunostaining and angiotensin binding were both prominent. In ventral horn and IML, immunoreactivity for AT₁ and choline acetyltransferase co-localized in pre-ganglionic sympathetic and somatic motor neurons. Immunolesion caused over 50% loss of IML perikarya within 3 months. Concurrent treatment with the AT₁ antagonist, Candesartan, did not affect the outcome.

Discussion—AT₁ expression is surprisingly widespread in sensory, autonomic and somatic motor neurons of the rat. This expression may be important to the normal physiology of these systems. Present data, however, do not support the concept that AT₁ activation contributes to the loss of autonomic neurons after axonal damage.

Keywords

Candesartan; immunohistochemistry; sympathetic nervous system; neurodegeneration; primary sensory neurons

Introduction

The renin-angiotensin system regulates arterial blood pressure through the actions of angiotensin II (Ang II) at type one receptors (AT₁), which mediate vasoconstriction,

aldosterone release, renal sodium reabsorption and central osmoregulatory actions. The same receptors participate in inflammation by activating the transcription factor NF κ B¹. They also play a role in stress responses mediated through the hypothalamic-pituitary-adrenal axis and may mediate signals leading to cellular damage at various loci, including the nervous system^{2,3}.

AT₁ expression is high in hypothalamic and brainstem regions targeted by Parkinson's disease and multiple system atrophy. AT₁ immunoreactivity at those sites distinguishes the particular neuronal populations that appear vulnerable to the degenerative pathology⁴. Moreover, AT₁-like binding sites are decreased in the caudate-putamen and substantia nigra of Parkinson's patients⁵. These findings do not prove an involvement of AT₁ receptors in neurodegeneration, but they indirectly support such a possibility. Therefore, one should raise the question whether treatment with AT₁ antagonists might protect compromised neurons.

To address this question, we investigated the response of AT₁-positive neurons to peripheral nerve damage in an animal model involving immunolesion of pre-ganglionic sympathetic neurons in the intermediolateral nucleus (IML) of the rat spinal cord^{6,7}. These neurons are unusual in undergoing widespread death 2–3 months after mechanical or immunologic damage to their ganglionic terminals⁸.

The relative abundance of AT₁ in neurons outside the brain was not fully known at the outset of our study, though specific immunoreactivity had been demonstrated in the spinal cord⁹. Therefore, we used AT₁ immunohistochemistry and ligand-binding autoradiography to examine the ventral and dorsal horns of the spinal cord as well as the dorsal root and superior cervical ganglia. These works were followed by experiments in which rats were treated with the selective antagonist, Candesartan, before and after induction of nerve damage. Candesartan has high affinity for AT₁ and readily passes diffusion barriers to inhibit central as well as peripheral receptors^{10,11}, making it a good candidate for therapeutic intervention. Here we report on the survival of IML neurons, with or without Candesartan treatment, after immunologic lesion.

Methods and Procedures

Immunolesion

Adult male Sprague–Dawley rats (200–250 g Harlan, Madison, WI, USA) were handled in accord with the *NIH Guide for Care and Treatment of Laboratory Animals* under protocols approved by the Mayo Institutional Animal Care and Use Committee. For immunolesion, we used murine monoclonal ZR 2, 3, 4 and 6 antibodies¹², which target multiple epitopes of rat acetylcholinesterase (AChE). An equal-parts antibody mixture (1.6 mg protein in 2 ml 0.9% NaCl) was injected into the tail vein without anesthesia.

Drug treatment

Candesartan stock solution contained 100 mg drug mixed with vehicle: 10 ml polyethylene glycol 400 (Sigma-Aldrich), 5 ml ethanol, 2 ml cremophor-EL (Sigma), and water to a final volume of 100 ml. Every 2–3 days, a working quantity of stock (pH $\approx$ 9) was freshly diluted into drinking water by a factor of 10 or more, calculated to deliver average daily doses of 10 mg/kg based on measured water intake per cage. Rats started on Candesartan 1 week before antibody treatment and continued with *ad lib* access for 40 days. There were six experimental groups in the immunolesion study: (1) normal drinking water and no further treatment ($n=8$); (2) drug vehicle and no further treatment ($n=6$); (3) Candesartan but no other treatment ($n=5$); (4) AChE antibody ($n=11$); (5) drug vehicle followed by antibody ($n=6$); (6) Candesartan followed by antibody ($n=6$).

Western blotting

In preparation for immunohistochemistry, we tested the specificity of AT₁ antibodies by Western blot analysis on rat spinal cord tissues. Approximately 0.1 g samples of thoracic spinal cord were dissected and homogenized in 3 ml homogenization buffer: 10 mM EDTA, 300 mM sucrose, 1% SDS, 0.1 mM PMSF and 450 μ l protease inhibitor cocktail stock (Roche, 1836170). Samples were centrifuged at 12,000 rev/min for 10 minutes, supernatants were heated at 100°C for 5 minutes, and 20 μ l aliquots were separated on 10% Tris-HCl SDS-Ready gels (BioRad, 161-1155) at 100 V for 1 hour. Proteins were then transferred onto Immobilon-P Transfer Membrane (Millipore IPVH00010 from Fisher) in transfer buffer (Bio-Rad 161-0734) at 200 mA for 1.5 hour at 4°C. Following transfer, the membranes were blocked with 5% non-fat milk for 1 hour at room temperature, followed by incubation with AT₁ antibodies from Novus (Littleton Co., Ab 18801-50) and Santa Cruz (SC-1173) at 1 : 500 dilutions overnight at 4°C. After washing thoroughly with 0.1% Tween in PBS, membranes were incubated with AP conjugated goat-anti-rabbit IgG (Santa Cruz, SC-2007) at 1 : 3000 for 1.5 hours at room temperature. After another several washes with 0.1% Tween in PBS, immunoreactivity was visualized by development in Nitroblue Tetrazolium Chloride (Roche, 1087-479) and 5-Bromo-4-chloro-3-indolyl phosphate (Roche, 760-994).

Immunohistochemistry

Rats under pentobarbital anesthesia (45 mg/kg) were perfused through the heart with phosphate-buffered saline (PBS), 0.1M, pH 7.4, followed by 4% paraformaldehyde in PBS. Representative segments of thoracic spinal cord (T1, T2 and T8) were located by dorsal root entries. Tissues were post-fixed for 2 hours and immersed overnight in 20% sucrose-PBS at 4°C. From each segment examined, a total of 24 consecutive 30 mm cryostat sections were cut to span at least two clusters of sympathetic pre-ganglionic neurons. Alternating sections were taken for immunohistochemistry and cell counting.

Floating samples and slide-mounted sections of spinal cord and ganglia were incubated overnight at 4°C with primary antibodies (1 : 50 dilution unless otherwise indicated): rabbit anti-AT₁ (Novus 18801-50) at 1 : 800 for fluorescent staining and 1 : 400 for peroxidase staining; mouse anti-ChAT (from Dr B. K. Hartmann); anti-CGRP (SC-7448); anti-parvalbumin (SC-8856); or anti-VR1 (Ch 5566, Chemicon, Temecula, CA, USA) at 1 : 500. After thorough rinse, sections for 'ABC staining' were incubated with biotinylated secondary antibodies at 1 : 150 (Vector Labs, Burlingame, CA, USA) for 2 hours at room temperature. The sections were then processed with avidin-coupled horseradish peroxidase and were developed with diaminobenzidine. Second antibodies for fluorescence stains were fluorescein-conjugated goat anti-rabbit at 1 : 80, rhodamine goat anti-mouse at 1 : 50 and Cy3 donkey anti-goat at 1 : 50 (AP132F, AP124R and AP180C; Chemicon). Stained samples were mounted, coverslipped and examined with a standard Zeiss microscope or a confocal fluorescence imaging system.

Ang II receptor binding

To examine Ang II binding sites, consecutive 16 μ m sections of fresh frozen tissues were cut at -20°C, thaw-mounted on poly-L-lysine coated slides (Erie Scientific, Portsmouth, NH, USA), dried overnight in a desiccator at 4°C, and stored at -80°C until used. Binding works were performed as described previously¹³. Sections were pre-incubated for 15 minutes at room temperature in 10 mM sodium phosphate buffer, pH 7.4, containing 120 mM NaCl, 5 mM Na₂EDTA, 0.1 mM bacitracin (Sigma-Aldrich, St Louis, MO, USA) and 0.2% protease-free bovine serum albumin (Sigma). Slides were then incubated for 2 hours at room temperature in fresh buffer with 0.5 nM [¹²⁵I]-sarcosine¹-Ang II (Peninsula Laboratories, Belmont, CA, USA) iodinated to 2176 Ci/mmol by the Peptide Radioiodination Center, Department of Pharmacology, University of Mississippi, MS, USA. Adjacent sections were incubated as

above but in the presence of 5 μ M unlabeled Ang II (Peninsula Laboratories). Radioactivity displaced by the excess of unlabeled agonist was our measure of specific binding to Ang II receptors in general. To determine binding to receptor subtypes, additional sections were incubated with 0.5 nM [125 I]-sarcosine¹-Ang II in the presence of selective AT₁ or AT₂ antagonists (10 μ M, a concentration previously shown to yield maximal specific displacement¹³). Our standardized and previously validated procedure used Losartan (Dupont-Merck, Wilmington, DE, USA) as the selective AT₁-displacing agent and PD 123319 (Sigma-Aldrich) as the selective AT₂-displacing agent.

After incubation, slides were rinsed four times for 1 minute each in fresh ice-cold 50 mM Tris-HCl buffer (pH 7.5), dipped in ice-cold water and dried under a stream of air. Dried sections were exposed to Kodak BioMax MR film (Eastman Kodak, Rochester, NY, USA) together with [14 C] micro-scales (American Radiolabeled Chemicals, St Louis, MO, USA). Exposure times varied, depending on the observed binding, to obtain images within the linear portion of the standard curve. Films were developed for 4 minutes in ice-cold GBX developer (Eastman Kodak), fixed in Kodak GBX for 4 minutes at room temperature and rinsed in running water for 15 minutes. Finally, using a program based on 'NIH Image' (Scion Image, Scion Corporation), we normalized the [125 I] autoradiograms to the [14 C] standards and converted the optical densities to values of fmol/mg protein¹⁴.

Counting and statistical analysis

Total numbers of positively stained IML neurons were recorded for each whole cross-section of cord. From each rat, 12 alternating sections were analysed. In IML, all neuronal profiles of 25–30 μ m in one axis were scored for staining at least double the background. Group differences were evaluated with paired and unpaired *t*-tests, as noted; *p*<0.05 was considered statistically significant.

Results

Autoradiography for Ang II receptors

We detected Ang II receptors by autoradiography in multiple segments of the spinal cord and associated dorsal root ganglia (Figure 1) and in the superior cervical ganglion (Figure 2). The marked displacement of [125 I]-Ang II with the selective AT₁ antagonist (Figures 1B and 2B) indicated that most of the Ang II receptors in these tissues were of the AT₁ type, while AT₂ receptors were scarce in the samples under study. AT₁-binding in the superior cervical ganglia was localized in small clusters. AT₁ receptors were present throughout the gray and white matter of the cord and in the root-filled space around the sacral segments. The highest AT₁ concentrations at all spinal levels were found in the superficial dorsal horn. The selective AT₂ receptor ligand, PD 123319, caused little displacement of labeled Ang II at most sites (Figures 1C and 2C). In particular, AT₂ receptors seemed sparse or absent in the parenchyma of the spinal cord and ganglia, although they appeared to be highly expressed in the meninges.

Immunohistochemistry for AT₁ receptors

Polyclonal antibodies to AT₁ from two different vendors were pre-tested in Western blots (Figure 3). The blots indicated that Novus 18801-50 was far more specific than Santa Cruz SC-1173; hence it was the only antibody used for immunohistochemistry. The resulting stains showed AT₁ immunoreactivity in ventral horn, dorsal horn and the IML nucleus of the spinal cord (Figure 4). Immunopositive neurons exhibited this reactivity in the cytoplasm and also at or on the cell surface. In ventral horn, at L4 and L5, the average number of AT₁-positive neurons was 28 ± 0.9 and 25 ± 2.8 per section, respectively. These numbers represent at least 80% of the total cholinergic population as determined by immunohistochemistry for the selective marker, ChAT. As predicted by the autoradiography, strong AT₁ staining was also found in

the dorsal horn. In the thoracic IML, this antibody also revealed closely similar numbers of AT₁-positive and ChAT-positive neurons, 8–10 per section, and double fluorescence staining confirmed that these two populations were nearly identical (Figure 5). Therefore, we relied on ChAT staining to count cells and determine degrees of loss in the drug-intervention study, to be described later.

Neurons in the superior cervical ganglion contained substantial AT₁ immunoreactivity (Figure 6A). Dorsal root ganglia were an even richer site, with many neurons expressing specific immunoreactivity at high levels (Figure 6C). To explore this novel observation further, dual-label immunohistochemistry was conducted with antibodies to peptides characteristic of different neuronal subpopulations in the dorsal root ganglia. This experiment revealed substantial co-localization of AT₁ with parvalbumin, in large and intermediate sized neurons, and less co-localization with CGRP and the capsaicin receptor, VR1, in smaller neurons (Figure 7). A limited quantitative analysis (three sections per ganglion) confirmed that the overlap of expression was greatest with parvalbumin. Thus, 86% of all AT₁-positive neurons also expressed parvalbumin, while 80% of parvalbumin-positive neurons also expressed AT₁. In contrast, only 36% of AT₁-positive neurons expressed CGRP (though nearly all CGRP-positive neurons also expressed AT₁). The association was the poorest with VR1, since barely half the AT₁-positive neurons expressed this peptide and only 58% of the VR1-positive neurons expressed AT₁.

AT₁ blockade and neuronal survival

Neuronal survival was evaluated in pre-ganglionic sympathetic neurons after lesion of terminals with acetylcholinesterase antibody. In line with expectations and our results from many previous experiments^{7,8}, cell counting showed that immunolesion caused a 55% loss of ChAT-positive neurons in the IML (Figure 8). As will be discussed below, we have established that this effect reflects cell death rather than reduced enzyme expression.

To determine if AT₁ blockade might affect the neuronal loss after peripheral injury, the selective antagonist, Candesartan, was given to other groups of rats subjected to neuronal injury. Sustained delivery of substantial quantities (10 mg/kg/day) was accomplished by presenting drug continuously in the drinking water. The treatment began 1 week before antibody injection, in order to ensure adequate receptor blockade at the time of lesion. Drug delivery continued for the first month after lesion in order to span the period likely to be critical for neurons reacting to axonal damage. Neuronal survival was assessed after 3 months (Figure 8). At this time, the loss of ChAT-positive IML neurons was ~60% (to 3.4 ± 0.3 per section) and not significantly different from the 55% loss in unprotected rats (to 3.7 ± 0.3 per section). The data thus provided no evidence that Candesartan protected sympathetic neurons with damaged ganglionic terminals.

Discussion

Summary of main findings

This study extends previous information on the distribution of AT₁ in spinal cord and associated tissues. AT₁ receptors have a surprisingly wide distribution in motor, sensory and autonomic neurons of the spinal cord and dorsal root ganglia. Nearly all somatic motor neurons and IML sympathetic neurons were seen to express this receptor together with the acetylcholine-synthesizing enzyme, ChAT. Neurons of the DRG are especially well endowed with AT₁, often co-localized with parvalbumin. These receptors, however, may not participate in the chain of events leading to death of IML neurons after axonal lesion, because prolonged treatment with Candesartan, a selective antagonist, failed to prevent cell loss.

Strengths and limitations of the study

The study gained strength from its combined use of immunohistochemistry and ligand binding autoradiography to elucidate the distribution of AT₁ receptors and from its well-defined model of lesion-induced autonomic neuronal death. The study also included a controlled interventional trial with Candesartan, a therapeutically relevant, subtype specific receptor antagonist. We did not obtain direct evidence that agent reached spinal neurons in completely effective concentration, but two features counterbalance this limitation. First, Candesartan is known to penetrate the central nervous system and even when administered peripherally, moderate doses have been shown to reduce Ang II binding at receptors in the rat brain¹⁰. Second, the doses used here were at a high level that has been reported to block cardiovascular and behavioral responses to intracerebroventricular injection of Ang II¹¹. Therefore, the absence of a treatment effect in this study strongly suggests that activation of AT₁ receptors does not influence the neuropathologic outcome of axonal injury to central autonomic neurons.

Relation to existing literature

Neuronal location of AT₁ receptors—Early data on this topic came from radioligand binding and autoradiography on sheep spinal cord and rat superior cervical ganglia^{15,16}. Recently, however, specific antibodies were used to observe neurons with AT₁-like reactivity in multiple lamini of the cord, including the ventral horn, dorsal horn and IML⁹. In the IML, this immunoreactivity overlapped with Fluoro-gold label that was transported retrogradely from the superior cervical ganglion, indicating that the AT₁-expressing cells were pre-ganglionic sympathetic neurons. In support, our observations of co-localized ChAT immunoreactivity show that these cells, like the AT₁-positive neurons in ventral horn, are cholinergic. AT₁ receptors probably also account for the ganglionic Ang II-binding sites reported by Pinto *et al.*¹⁵.

The low resolution of ¹²⁵I autoradiography does not allow true cellular localization, and this well-recognized property accounts for the grainy appearance of the exposed films. Nonetheless, it is apparent that our binding data were consistent with the immunohistochemical stains. First, the displacement with selective ligands confirmed that Ang II receptors in the superior cervical and dorsal root ganglia, and the spinal cord were mostly of the AT₁ type. AT₂ receptors were few or absent at these locations, though abundant in the meninges. In ganglia, AT₁ binding was detected in clusters that immunohistochemistry resolved into individual neurons and groups of neurons. Because antibody staining for AT₁ in the dorsal root ganglia co-localized with parvalbumin, a marker of proprioceptive neurons¹⁷, one may hypothesize a functionally significant role for angiotensin in this sensory system.

Autoradiography and immunohistochemistry for AT₁ matched reasonably in the spinal cord as well. The AT₁-immunoreactive motor perikarya probably accounted for the clusters of Ang II binding observed in some parts of the ventral horn. We also noticed considerable immunostaining in lamina II, the substantia gelatinosa, although it was not as intense as the Ang II binding seen here, and earlier by others⁹. The slight difference may reflect preferential loss of immunoreactivity in receptors located on the surface of terminal axons during preparation for histochemistry with formalin fixative.

Overall, the morphologic picture is consistent with physiologic data showing that Ang II owes some of its pressor effect to excitation of central neurons in the sympathetic pathway^{18–20}. The structural data also fit well with the recent finding that Ang II can directly excite spinal cord motor neurons through AT₁ and a G-protein effector system²¹.

Receptor expression and neuronal survival—Our study began in light of observations that AT₁ expression characterizes the neuronal populations that degenerate in Parkinson's

disease and multiple system atrophy⁴. Those findings suggested that neurons expressing AT₁ receptors could be at elevated risk under stress. With nissl stains and retrograde fluorescence labeling works, we had previously shown that the loss of ChAT-positive IML neurons exposed to immunolesion by AChE antibody reflected cell death and not merely reduced enzyme expression^{7,8}. This cell death was accompanied by reactive and inflammatory changes including a proliferation of astrocytes expressing glial fibrillary acidic protein⁷. Once these IML neurons were shown to be AT₁-positive, it became reasonable to investigate whether pharmacologic intervention with a selective antagonist would reduce cell losses after axonal immunolesion.

Implications for future research and clinical practice

Besides being antihypertensive, AT₁ antagonists have been shown to protect cells and tissues from certain kinds of stress. For example, Candesartan reduced cerebrovascular events in stroke-prone, spontaneously hypertensive rats, even at a dose that hardly affected blood pressure²². Candesartan also decreased the size of cerebral infarcts in a rodent model of cerebral ischemia, again without changing blood pressure appreciably²³. Another AT₁ antagonist, Olmesartan, enhanced neurite outgrowth, increased ChAT activity and prevented death of axotomized motor neurons in primary cultures of ventral spinal cord²⁴. These beneficial effects may involve unmasked Ang II stimulation of the related receptor, AT₂. Thus, works of a stroke model showed that AT₁ blockade reduced an ischemia-induced up-regulation of c-Fos and c-Jun proteins, but not when an AT₂ antagonist was also given²⁵.

We designed our intervention experiments to determine whether a blockade of AT₁ could rescue pre-ganglionic sympathetic neurons from death after complement-mediated destruction of their peripheral terminals. Because nearly all ChAT-positive neurons in the IML were found to express AT₁ as well, we used the cholinergic marker enzyme for definitive cell counts. The AT₁ antagonist in these experiments was Candesartan. Among existing AT₁ antagonists, Candesartan has the highest binding affinity and the greatest ability to penetrate the blood–brain barrier¹⁰. The dosage in the present study was also large in relation to typical Candesartan doses used in clinical treatment of hypertension²⁶. A reasonable interpretation of Candesartan's disappointing failure to protect IML neurons is that AT₁ receptors have little impact on cell death and survival in our model of immunolesion. Therapeutic effects of antagonists are likely to be greater when Ang II promotes tissue inflammation and neuronal death by acting on the vascular endothelium. Stroke is an important example of such a case²⁷. Further research will provide us with a better understanding of AT₁ antagonists and their potential usefulness in limiting degenerative responses to different kinds of neuronal insults.

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Figure 1. Autoradiography for Ang-II receptors in cervical, thoracic, lumbar and sacral spinal cord. [125 I]-sarcosine¹-Ang II was applied to consecutive sections under four conditions: (A) no other ligand present; (B) Losartan added to occupy AT₁ receptors; (C) PD123319 added to occupy AT₂ receptors; (D) unlabeled Ang-II added to occupy AT₁ and AT₂ receptors. Filled (black) arrows point to superficial dorsal horn; arrowheads point to dorsal root ganglion; empty (white) arrows point to nerve roots; thin arrows point to meninges. Scale bar=1 mm

Figure 2.

Autoradiography for Ang II-receptors in the superior cervical ganglion. [125 I]-sarcosine¹-Ang II was applied to consecutive sections under four conditions: (A) no other ligand present (total binding); (B) Losartan added to occupy AT₁ receptors; (C) PD123319 added to occupy AT₂ receptors; (D) unlabeled Ang-II added to occupy AT₁ and AT₂ receptors. Scale bar=1 mm (approximate size of dissected ganglion)

Figure 3. Western blotting of AT₁ antibodies. When detected with SC-1173 AT₁ antibody from Santa Cruz, Western blots of rat spinal cord showed a faint band at 45 kDa, attributable to AT₁, and a strong band at 160 kDa that may represent a contaminant. Antibody 18801-50, on the other hand, showed a major band at 45 kDa and little cross-reaction with extraneous antigens

Figure 4.

AT₁ immunoreactivity in the rat spinal cord. (A) Thoracic segment T1–T2 (scale bar=200 μm). AT₁ immunoreactivity expresses widely in the gray matter, especially in the neurons of the ventral horn, in the dorsal horn, and in ependymal cells around the central canal. (B) IML region (scale bar=50 μm). (C) Dorsal horn (scale bar 50 μm). (D) Ventral horn (scale bar=50 μm). (E) Dorsal horn (scale bar=50 μm). (F) Negative control, primary antibody omitted (scale bar=200 μm)

Figure 5. Overlap of AT₁ and ChAT immunoreactivity in rat spinal cord (T1–T2 level). (**A**, **C** and **E**) Confocal images of a single field from IML region; (**B**, **D** and **F**) single field from ventral horn. **A** and **B**: AT₁ stain, FITC detection; **C** and **D**: ChAT stain, rhodamine detection; **E** and **F**: overlay; **G** and **H**: negative controls, no primary antibody. Overall, in most fields of IML and ventral horn, essentially all ChAT-positive neurons are AT₁-positive, and *vice versa*. Calibration bar=50 μ m

Figure 6.

AT₁ immunoreactivity in superior cervical and dorsal root ganglia. (A) Superior cervical ganglion. Specific AT₁ immunoreactivity occurred both in neurons and neuropil. (B) Adjacent section, no first antibody and higher magnification of A (inset). (C) Dorsal root ganglion. Many but not all large neurons showed intense AT₁ immunoreactivity. Immunoreactivity also occurred in intermediate and some smaller neurons. (D) Adjacent section, no first antibody and higher magnification of C (inset with arrow marking large positive neuron and arrowhead marking small positive neuron). Calibration bars 5100 mm (large fields) and 50 μ m (inset)

Figure 7. Peptide expression in AT₁-positive neurons of a thoracic dorsal root ganglion. Rhodamine-label immunohistochemistry was carried out with antibodies for CGRP (A), parvalbumin (C) and capsaicin receptor VR1 (E). On the same sections, fluorescein-label immunohistochemistry was carried out with Novus-18801 for AT₁ (B, D and F). Calibration bar=100 μ m. Most ganglionic neurons stained both for AT₁ and parvalbumin (compare C and D). Fewer AT₁-positive neurons stained for CGRP and VR1. Arrowheads in B and F point out cells in which peptides clearly did not co-localize

Figure 8.

Effect of AChE antibody and Candesartan on IML neurons. Rats were subjected to peripheral immunolesion by AChE antibodies, with or without concurrent Candesartan (see the section on 'Methods and procedures'). Drug-control rats received either normal drinking water (control) or water with the diluent used to solubilize Candesartan (vehicle). Three months after the immunologic challenge, neurons expressing ChAT were counted in the thoracic IML (average of 12 sections each at T1, T2 and T8 levels). Means $\pm$ SEM of surviving neurons are shown (6–11 rats/group). Antibody-induced neuronal losses in the IML were statistically significant by *t*-test (** $p < 0.01$), and neither Candesartan nor vehicle affected this outcome