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Research article

A combined acetylcholinesterase and immunohistochemical method for precise anatomical analysis of intrinsic cardiac neural structures

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ABSTRACT

A significant challenge when investigating autonomic neuroanatomy is being able to reliably obtain tissue that contains neuronal structures of interest. Currently, histochemical staining for acetylcholinesterase (AChE) remains the most feasible and reliable method to visualize intrinsic nerves and ganglia in whole organs. In order to precisely visualize and sample intrinsic cardiac nerves and ganglia for subsequent immunofluorescent labeling, we developed a modified histochemical AChE method using material from pig and sheep hearts. The method involves: (1) chemical prefixation of the whole heart, (2) short-term and weak histochemical staining for AChE in situ, (3) visual examination and extirpation of the stained neural structures from the whole heart, (4) freezing, embedding and cryostat sectioning of the tissue of interest, and (5) immunofluorescent labeling and microscopic analysis of neural structures. Firstly, our data demonstrate that this modified AChE protocol labeled intrinsic cardiac nerves as convincingly as our previously published data. Secondly, there was the added advantage that adrenergic, cholinergic and peptidergic neuropeptides, namely protein gene product 9.5 (PGP 9.5), neurofilament (NF), tyrosine hydroxylase (TH), vesicular monoamine transporter (VMAT2), neuronal nitric oxide synthase (nNOS), choline acetyltransferase (ChAT), calcitonin gene related peptide (CGRP), and substance P may be identified. Our method allows the precise sampling of neural structures including autonomic ganglia, intrinsic nerves and bundles of nerve fibers and even single neurons from the whole heart. This method saves time, effort and a substantial amount of antisera. Nonetheless, the proof of specific staining for many other autonomic neuronal markers has to be provided in subsequent studies.

1. Introduction

Considerable progress has been achieved in neuroscience since the advent of histological and immunohistochemical techniques which underpin anatomical research. New accurate methods for the detection of neuronal structures from whole organs are still in demand because of extraneous tissues surrounding the sparse distribution and the small amount of neuronal material present within organs and the cost of the necessary substances used. This is compounded by inherent difficulties of antibody penetration that restricts whole mount immunohistochemistry. Previously, methylene blue (MB) was used to visualize neurons (Müller, 1989). However, MB is susceptible to bleaching and is often unstable following routine chemical fixation (Müller, 1990). Currently, histochemical staining for acetylcholinesterase (AChE), as initially described by Karnovsky and Roots (1964), remains a popular method to visualize intrinsic nerves and ganglia in whole organs. In the heart, this method allows a distinct and permanent signal that has facilitated uncovering the entire epicardial intrinsic cardiac nervous system from a wide range of species (Pauza et al., 1999). For a complete profile of neurotransmitter content of the epicardial nerve network, detailed immunohistochemistry is essential.

Abbreviations: AChE, acetylcholinesterase; Ao, aorta; CGRP, calcitonin gene related peptide; ChAT, choline acetyltransferase; IR, immunoreactivity; MB, methylene blue; nNOS, neuronal Nitric Oxide Synthase; NF, neurofilament; RA, uright auricle; TH, tyrosine hydroxylase; PGP 9.5p, rotein gene product 9.5; PT, pulmonary trunk; SP, substance P; VMAT2, vesicular monoamine transporter 2.

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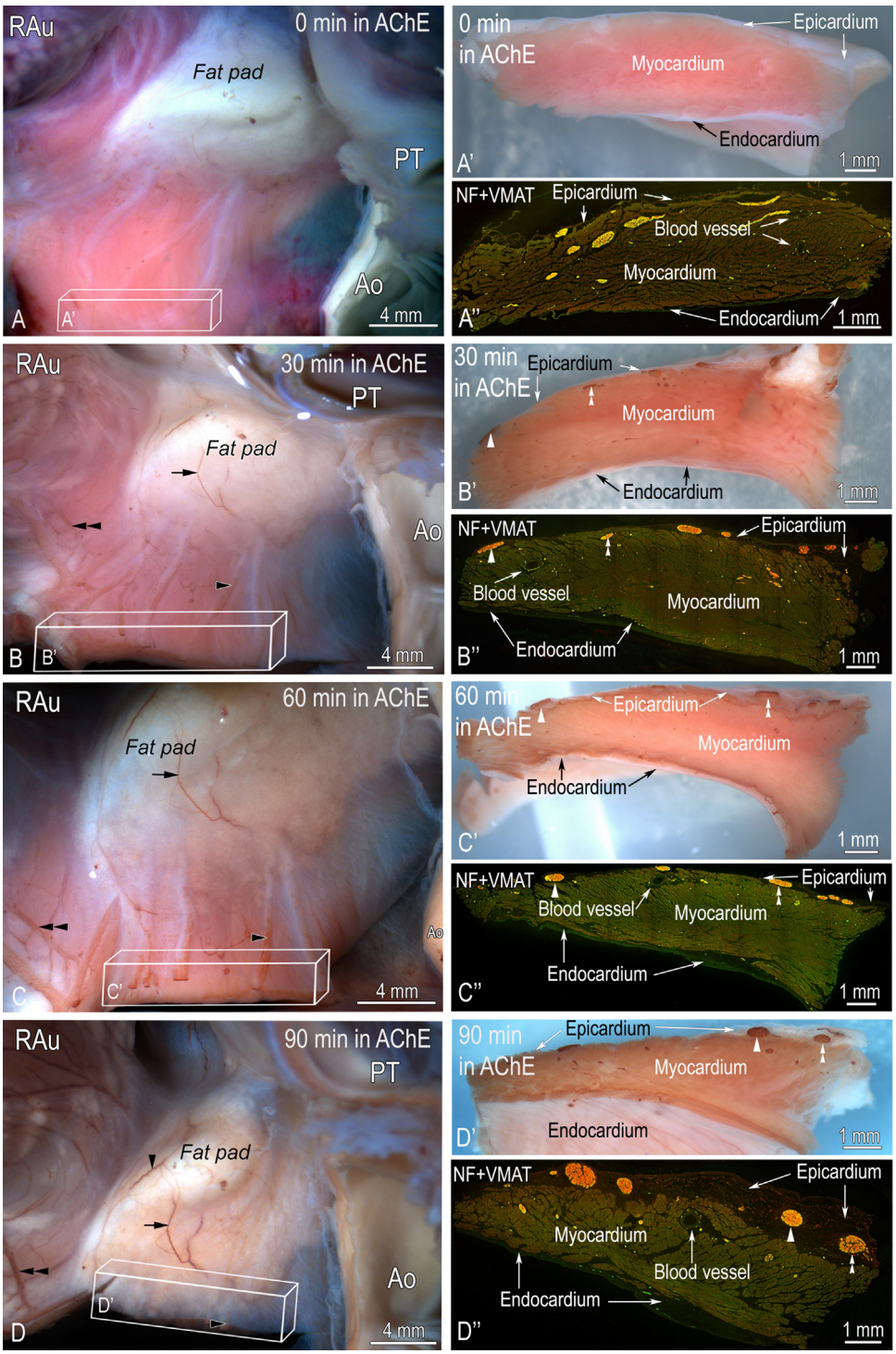

Fig. 1. Photographs illustrate the consecutive stages of the method used on the pig atrial tissue. Panel A shows the anterior wall of the right atrium from the total heart preparation before the control sample of the tissue was taken. Boxed area A' in panel A is the erected vertically and the enlarged sample of the anterior wall of the right atrium with the invisible epicardial nerves that are well seen in the panel A" following the double fluorescent immunohistochemistry for neurofilament (NF, green color) and vesicular monoamine transporter 2 (VMAT2, red color). Note, the immunohistochemical reaction for VMAT2 on the pig atrial tissues is shown in detail in Fig. 6B, D and F. Panel B demonstrates the anterior wall of the right atrium following 30 min incubation in Karnovsky–Roots solution. Only weakly stained profiles of epicardial nerves are visible in the vertically erected sample, as illustrated in the panel B'. Panel B" demonstrates the well visible epicardial nerves of this section processed for double immunohistochemistry for neurofilament (NF, green color) and vesicular monoamine transporter 2 (VMAT2, red color). Panels C and D as well as C', C", D' and D" illustrate the same heart, tissue samples and sections with double immunohistochemistry for NF and VMAT2 following the incubation in Karnovsky–Roots solution for 60 and 90 min, respectively. After each interval, the epicardial nerves were better stained for AChE and clearly seen both on the atrial wall and tissue samples. Note, the immunoreactivity for NF and VMAT2 (C" and D") is equally high following 60 and 90 min of incubation in AChE solution. Black arrows, arrowheads and double arrowheads indicate profiles of the same epicardial nerves on the total heart in the panels B–D. White arrowheads and double arrowheads indicate the same nerves in the stained for AChE tissue sample and in the cryosectioned and immunohistochemically stained sections in the panels B, B'; C', C" and D', D". Abbreviations: Ao, orifice of aorta; PT, orifice of pulmonary trunk; RAu, right auricle. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)