Cytochrome b558 (p22phox) in the Guinea-pig Adrenal Medulla

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ABSTRACT Paraganglionic cells are sensitive to hypoxia, and the involvement of a plasmalemmal cytochrome b558-like protein in oxygen sensing by these cells has been suggested, but neither the identity of the immunoreactive protein detected by immunohistochemistry nor its anticipated subcellular (i.e., plasmalemmal) localization were directly proven. Thus, we extended these studies to the largest paraganglion, i.e., the adrenal medulla, in the guinea-pig, which, due to its size and accessibility, allowed us to address both of these issues utilizing antisera raised against synthetic peptides of the small (22 kD) subunit of cytochrome b558, p22phox. Cytochrome b558 was originally identified in granulocytes and macrophages, and antisera against this phagocyte p22phox were utilized. Immunoreactivity to p22phox was observed in all adrenal medullary endocrine cells, and the identity of the immunoreactive protein to the small cytochrome b558-subunit was confirmed by Western blotting. Immuno-electron microscopy of ultrathin cryosections and of resin-embedded tissue demonstrated its subcellular localization in the dense core vesicles of endocrine A-cells but not in the plasma membrane. In conclusion, the present study documents the presence of the small subunit of cytochrome b558 in guinea-pig adrenal medullary cells, but its subcellular vesicular localization does not support the initial interpretation of cytochrome b558 serving as a plasmalemmal oxygen sensor. *Microsc. Res. Tech. 47:215–220, 1999.*

INTRODUCTION

The adrenal medulla is the largest paraganglion of the body. A general feature of most paraganglionic cells is their sensitivity to hypoxia: The carotid body and structurally similar paraganglia in the aortic arch region serve as monitors of arterial oxygen tension (for review see Acker, 1989), SIF (small intensely fluorescent) cells of the rat sympathetic ganglia respond to hypoxia with an increased turnover of dopamine (Borghini et al., 1994; Dalmaz et al., 1993), and hypoxia causes release of catecholamines from retroperitoneal paraganglia (Brundin, 1966; Fried et al., 1988; Hervonen and Korkala, 1972). The hypoxia-dependent systemic release of catecholamines from retroperitoneal paraganglia and from the adrenal medulla appears to be crucial in late fetal life and during birth to control fetal circulation, and to prepare the lung for ventilation (Jones et al., 1988; Slotkin and Seidler, 1988; Cheung,1989).

The molecular mechanisms of oxygen sensing by paraganglionic cells are not fully understood. Three models (reviewed by Gonzalez et al.,1995) have been proposed: The *metabolic hypothesis* focusses upon mitochondrial functions, the *membrane model* is centered around oxygen-sensitive K^+-channels in the plasma membrane, and the *NAD(P)H oxidase model* postulates an oxygen-dependent continuous production of oxyradicals that serve as intracellular messengers. All models have in common that the primary oxygen sensor molecule shall be a heme protein. Originally, involvement of a cytochrome aa3 had been suggested (Mills and Jöbsis, 1970, 1972; Wilson et al., 1994) but with refined photometric techniques, a cytochrome b558 emerged as an additional candidate (Acker et al., 1989, 1992; Cross et al., 1990). A cytochrome with corresponding spectral characteristics (absorption peak at 558 nm) is known from the NADPH oxidase complex of phagocytes that is responsible for the burst-like generation of superoxide anion and reactive oxygen species in response to bacterial stimulation (Babior, 1992). This cytochrome b558 is a heterodimer consisting of a small (p22phox) and large subunit (gp91phox). These subunits, together with other components of the neutrophil NADPH oxidase complex, have been immunohistochemically demonstrated in paraganglionic cells of the carotid body of guinea-pig, rat, and man (Kummer and Acker, 1995; Youngson et al., 1997) and in SIF cells of guinea-pig sympathetic ganglia (Kummer and Acker, 1997). These photometric and immunohistochemical data are consistent with the hypothesis that plasmalemmal cytochrome b558 serves as an oxygen sensor in paraganglionic cells, but two important pieces of information are lacking: First, the identity of the immunoreactive protein detected by immunohistochemistry has not yet been confirmed by Western blotting since it was impossible to collect a sufficient amount of material from small aggregates of paraganglionic cells building up the carotid body and SIF cell clusters. Second, the model

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implies a plasmalemmal localization of the cytochrome, but its subcellular localization in paraganglionic cells has not yet been investigated. Thus, we extended these studies to the guinea-pig adrenal medulla that allowed us due to its size and accessibility to address both of these issues utilizing antisera raised against synthetic peptides of the small (22 kD) subunit of cytochrome b558, p22phox.

MATERIALS AND METHODS

Immunofluorescence

Twelve adult female Hartley-Dunkin guinea-pigs (Charles River GmbH; Kisslegg, Germany) were sacrificed by CO_2 inhalation, transcardiacly perfused with rinsing solution (Forssmann et al., 1977) followed by 4% paraformaldehyde in 0.1 M phosphate buffer, and adrenal glands were dissected. Frozen sections (6–14 µm) were cut with a cryostat (Leica, Bensheim, Germany) and subjected to routine immunofluorescence using two polyclonal rabbit antisera (code R5553, kindly provided by Dr. M. T. Quinn, Bozeman, MT, and code p22/16b by Dres J. T. Curnutte and R. Erickson, La Jolla, CA) against different synthetic peptides of the small subunit of cytochrome b558, p22phox (characterized in Kummer and Acker, 1995, 1997; Quinn et al., 1989, 1992). Primary antisera were applied overnight at room temperature (R5553 at 1:600; p22/16b at 1:1,000), and subsequently detected by incubations of 1 hour each with biotinylated donkey anti-rabbit IgG and streptavidin-conjugated Texas Red (1:50 and 1:100, respectively, both from Amersham Buchler, Braunschweig, Germany). Sections were coverslipped in carbonate-buffered glycerol at pH 8.4 and analyzed with an epifluorescence microscope (BX 60, Olympus, Hamburg, Germany). Preabsorption of the polyclonal antisera with their corresponding synthetic peptides at a concentration of 20 µg peptide per milliliter of antiserum diluted to working concentration resulted in absence of immunolabelling.

Immunogold Labelling

Three guinea-pigs were perfusion-fixed with buffered 4% paraformaldehyde and small pieces of adrenal medulla were embedded in LR White resin (Polyscience, Eppelheim, Germany) without osmication. Ultrathin sections were placed for 10 minutes each in 50 mM glycine in phosphate-buffered saline (PBS; 0.05 M phosphate buffer, 0.5% NaCl, pH 7.4) and 1% defatted milk powder in PBS, incubated for 1.5 hours with R5553-antiserum (1:200) and for another hour with goat-anti rabbit Ig conjugated to 5 nm colloidal gold (EM grade, 1:25; W. Plannet, Wetzlar, Germany), fixed for 2 minutes in 2% glutaraldehyde in PBS, and routinely contrasted with uranylacetate and lead citrate.

Cryoultramicrotomy was performed on adrenal medullae obtained from animals perfused with 2% paraformaldehyde, 15% saturated picric acid, and 0.5% glutaraldehyde in 0.1 M phosphate buffer. Specimens were cryoprotected with 2.3 M sucrose, frozen, and ultrathin sections were cut (Ultracut S equipped with FCR unit; Leica, Bensheim, Germany) with glass knifes (Tokuyasu, 1973). Sections were transferred onto grids in 2.1 M sucrose, 1% methylcellulose in distilled water (modified from Liou et al., 1996), and rinsed 10 minutes each in 0.1 M PBS, 1% glycine + 0.01% NaCN in PBS, and 4.5% fish skin gelatine (Sigma, Deisenhofen, Germany) + 1% acetylated bovine serum albumin in PBS ("blocking solution"), followed by a 1-hour incubation with antiserum R5553 (1:400 in blocking solution), PBS wash (15 minutes), and a 45-minute incubation with goat-anti-rabbit IgG conjugated to 5 nm colloidal gold particles (EM grade, W. Plannet, Wetzlar, Germany) diluted 1:25 in blocking solution. Sections were washed (PBS, 25 minutes), postfixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, washed for 5 minutes in distilled water, and stained/embedded in 2% methylcellulose, 3% uranylacetate in water (Griffiths et al., 1982).

Specificity of immunolabelling was tested by preabsorption of the antiserum with its corresponding synthetic peptide (20 µg/ml). Sections were evaluated with a EM 902 (Zeiss, Jena, Germany) both in conventional and elastic brightfield imaging mode.

Western Blotting

Protein extracts of freshly dissected guinea-pig adrenal medulla were prepared and analysed by electrophoresis on 12% SDS-polyacrylamide gels and blotted onto nitrocellulose (for details see Höhler et al., 1995). The membrane was reacted overnight with a polyclonal anti-p22phox antibody (code p22/16b, dilution 1:200) followed by incubation with a biotinylated anti-rabbit IgG (dilution 1:1,000, Amersham) and streptavidin-alkaline phosphatase (dilution 1:5,000, Dianova, Hamburg, Germany) for 1 hour each. Immunoreactive bands were visualized using development with 450 µM nitrobluetetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (both from Boehringer, Mannheim, Germany), 0.05 M $MgCl_2$, 0.1 M NaCl in 0.1 M Tris buffer, pH 9.5.

RESULTS

Intense p22phox-immunofluorescence was observed with both p22phox-antisera (codes R5553 and p22/16b) in all adrenal medullary endocrine cells of the guinea-pig while cortical cells were not labelled (Fig. 1a,b).

Ultrastructurally, the formaldehyde-fixed and non-osmicated tissue allowed clear identification of the numerous endocrine adrenaline-cells (A-cells), containing round dense core vesicles measuring 120–250 nm, few sustentacular cells with denser cytoplasm and lacking dense core vesicles, capillary endothelial cells, and bundles of nerve fibers (Fig. 2). Classical noradrenaline-cells (NA-cells) with secretory vesicles containing eccentrically located, crescent dark cores were not observed. Immunolabelling to p22phox was observed on the numerous dense core vesicles of endocrine A-cells in both resin-embedded and cyrosectioned medullae, with an overall higher labelling intensity in cyrosections (Figs. 2–4). In general, there was an inverse relationship between density of immunolabelling and electron density of secretory vesicles in resin-embedded specimens, and in individual cases labelling of such vesicles was undetectable (Fig. 2, inset). In cryosections, however, densities of immunolabelling and electron density of secretory vesicles were much less variable (Figs. 3, 4). Other organelles and the plasma membrane of adrenal medullary A-cells were not labelled (Fig. 3).

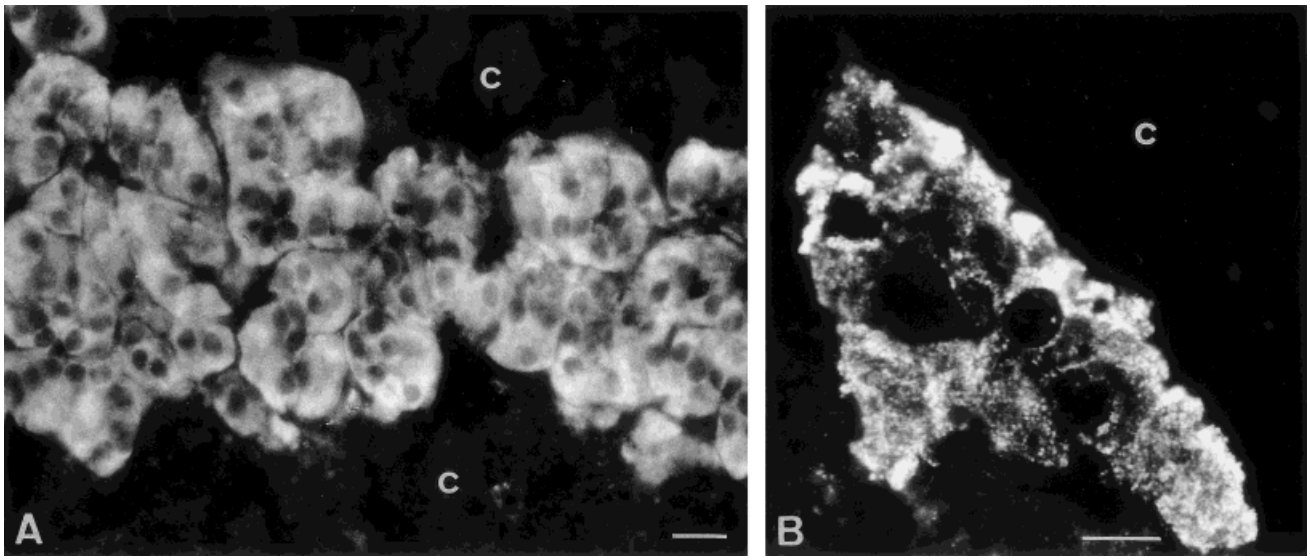

Fig. 1. Immunoreactivity to p22phox in guinea-pig adrenal gland. **a:** In this 14-μm-thick section labelled with antiserum coded R5553, all medullary cells exhibit p22phox-immunoreactivity, while cortical cells (C) are unlabelled.**b:** Higher magnification of a 6-μm-thick section labelled with antiserum coded p22/16b; adrenal medullary cells exhibit an intracellular granular labelling pattern. Bars = 20 μm (a), 10 μm (b).

Immunolabelling was absent when the R5553-antiserum had been preabsorbed with its corresponding synthetic peptide prior to use. Sustentacular cells of the medulla, endothelial capillary cells, and adrenal cortical cells were devoid of p22phox-immunolabelling.

Western blotting of protein extracts of guinea-pig adrenal medulla showed two bands immunoreactive to the p22phox-antiserum coded p22/16b (Fig. 5). One band migrated with an apparent molecular weight of 22 kD, and an additional more diffuse band, considered to represent dimers (see Quinn et al., 1992), at approximately 44 kD.

DISCUSSION

This study demonstrates the presence of the small subunit of cytochrome b558, p22phox, in guinea-pig adrenal medullary A-cells, thereby extending previous results obtained at paraganglionic cells of the carotid body (Kummer and Acker, 1995; Youngson et al., 1997) and sympathetic ganglia (Kummer and Acker, 1997) and establishing it as a general constituent of paraganglionic cells in this species. The absence of classical NA-cells, identifiable by their vesicular morphology, from our material is most probably not due to the omission of glutaraldehyde fixation as well as osmication, but reflects the near absence of this cell type in the guinea-pig adrenal gland as reported earlier (Unsicker et al., 1978).

The proposal that cytochrome b558 serves as oxygen sensor in paraganglionic cells originates from spectrophotometric recordings of the rat carotid body demonstrating a hypoxia-dependent reduction of a non-mitochondrial cytochrome with peak absorption at 558 nm (Acker et al., 1989, 1992; Cross et al., 1990). Since patch-clamp recordings have identified hypoxia-sensitive K^+-channels in excised patches of the plasma membrane of paraganglionic cells (rabbit carotid body, Ganfornina and López-Barneo, 1992), an oxygen sensor molecule shall be located within the plasma membrane. This observation was also in favour of cytochrome b558 serving as this sensor, since a plasmalemmal distribution has been reported previously in two other cell types: In human neutrophil granulocytes and in spheroid cultures of the oxygen-sensing human hepatoma cell line, HepG2, cytochrome b558-immunoreactivity has been detected in a clustered distribution in the plasma membrane as well as in the membrane of specific granules (Nakamura et al., 1988; Ehleben et al., 1997; Wientjes et al., 1997). High-resolution of its subcellular localization in paraganglionic cells had not yet been achieved. Youngson and coworkers (1997) performed conventional immunofluorescence on cultured rat carotid body cells and localized immunolabelling "to the plasma membrane and/or cytoplasm" but finally proposed a plasma membrane localization of cytochrome b558 due to the fact that immunolabelling was also obtained when a permeabilizing agent was omitted from the incubation protocol (Youngson et al., 1997). However, in the present study of guinea-pig adrenal medullary cells, specific immunolabelling was absent from the plasma membrane but was concentrated on the dense core vesicles as evidenced by electron microscopy. According to the present findings, the cytochrome b558 is very unlikely to represent a plasmalemmal oxygen receptor of paraganglionic cells. This interpretation is supported by the recent finding, that hypoxic reduction of this cytochrome in the rat carotid body occurs *after* the first hypoxic responses (electric activity in the carotid sinus nerve) can be recorded (Lahiri and Acker, 1999).

Although the absence of cytochrome b558-immunoreactivity from the plasma membrane of adrenal medul-

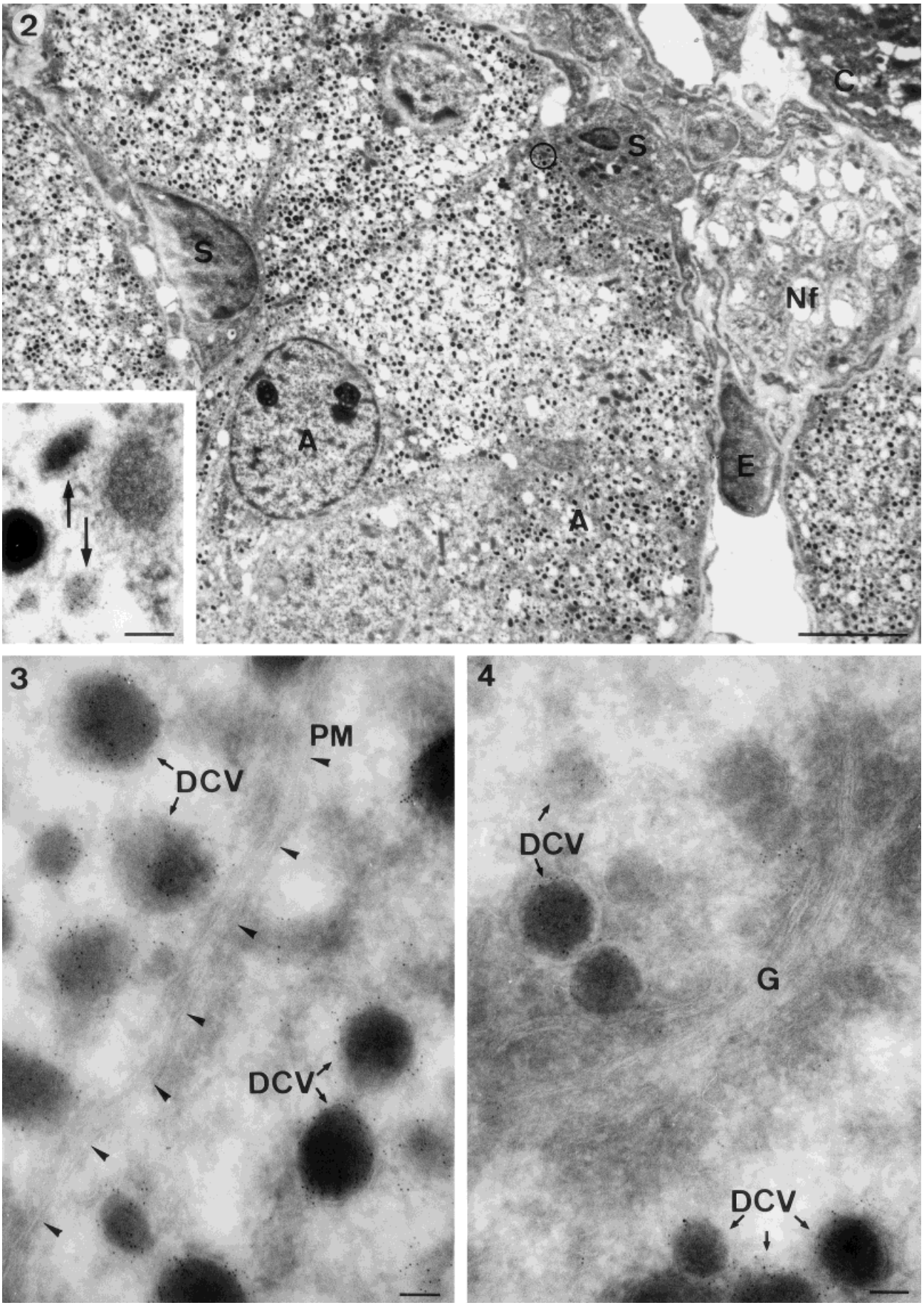

Fig. 2–4.

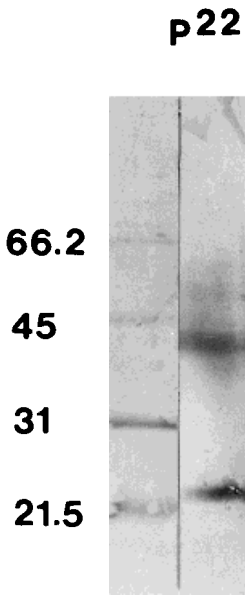

Fig. 5. Western blotting revealed p22phox-immunoreactive bands at 22 kD (monomers) and 44 kD (dimers) in protein extracts of guinea-pig adrenal medulla; antiserum p22/16b; **left lane:** molecular weight markers.

lary cells does not meet the expectation based on a model of plasmalemmal oxygen sensors, the presence of intracellular vesicular cytochrome b558 is not unique to these cells: In neutrophil granulocytes, most of the NADPH oxidase activity is recovered from the specific granules while only a minor fraction is retained in the plasma membrane (Johansson et al., 1995). The localization of p22phox in the dense core vesicles of adrenal medullary A-cells raises the question whether it is functionally associated with other proteins of these specialized organelles. In view of the fact that the NADPH oxidase in neutrophil granulocytes greatly facilitates transmembrane H^+-conductance (Nanda et al., 1994), an attractive candidate for interaction with p22phox in the dense core vesicle membrane is its vacuolar type of H^+-pumping ATPase (Cidon and Nelson, 1983; Percy et al., 1985; Schmidt et al., 1982). However, this issue needs direct functional investigation.

Fig. 2. Guinea-pig adrenal gland embedded in LR-white resin, immunogold-labelling (5 nm colloidal gold) for p22phox (antiserum R5553). In the medulla, endocrine A-cells (A), sustentacular cells (S), capillary endothelial cells (E), and a nerve fiber bundle (Nf) can be discriminated. C = cortical cell. **Inset:** Encircled region at higher magnification, demonstrating immunolabelling at two secretory vesicles with electron-lucent core while it is undetectable at an adjacent individual darker vesicle. Bars = 5 µm, 0.5 µm (inset).

Fig. 3. Cryosection showing two opposing parts of A-cells, immunolabelled (5 nm colloidal gold) for p22phox (antiserum R5553). Immunoreactivity is confined to the large dense-cored secretory vesicles (DCV) of A-cells while the plasma membrane (PM, arrowheads) is devoid of labelling. Bar = 100 nm.

Fig. 4. Cryosection, immunolabelled (5 nm colloidal gold) for p22phox (antiserum R5553). Immunolabelling is present on vesicles in the vicinity, but not on the cisternae of the Golgi apparatus (G). DCV = dense core vesicles. Bar = 100 nm.

In conclusion, the present study documents the presence of the small subunit of cytochrome b558 in guinea-pig adrenal medullary A-cells, but its vesicular localization does not support the initial interpretation of cytochrome b558 serving as a plasmalemmal oxygen sensor in paraganglionic cells.

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