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Carbonic anhydrase in the carotid body and the carotid sinus nerve

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Summary. It is well known that carbonic anhydrase plays an important role in the physiological responses of carotid-body chemoreceptors to hypercapnia. Nevertheless the precise location of the enzyme within the carotid body has been a matter of controversy for many years. Using the Hansson method we found histochemical evidence that this enzyme is localized in type I cells. Type II cells and nerve terminals did not show enzymatic activity. These results allow us to define the carotid body as a secondary receptor in the context of the "acidic hypothesis" of transduction in the carotid body.

Introduction

The presence of carbonic anhydrase (CAH) in the carotid body has been demonstrated biochemically (Lee 1968) and histochemically (Becker et al. 1967; Lee 1968; Laurent et al. 1969), but its precise location is a matter of dispute. Becker et al. (1967) have suggested that the enzyme is located in type II cells, while Lee (1968) has argued for its presence in type I cells. Laurent et al. (1969) have stated that, with available histochemical methods, it is impossible to ascribe the enzyme to a given glomic structure.

It has been shown that CAH plays an important role in the response of carotid-body chemoreceptors to hypercapnic stimulus. This response, which is recorded in the carotid sinus nerve (rapid increase to a maximum followed by adaptation to a steady lower level and undershoot on removal stimulus), is modified by acetazolamide which makes the response slower and leads to the disappearance of the transients (McCloskey 1968; Black et al. 1971).

On the other hand, CAH is a key component in the acidic hypothesis of chemosensory transduction (Torrance 1974, 1977; Hanson et al. 1981). As non-penetrating CAH inhibitors do not modify the response to hypercapnic stimulus, Hanson et al. (1981) have proposed that the enzyme is intracellular and must be located in either sensory nerve endings or type I cells. The structure containing the enzyme could be considered to be the transducer element (for a review, see Belmonte and Gonzalez 1983).

The present study was undertaken in order to identify the location of CAH in the carotid body and, in the context of the acidic hypothesis to define this organ as a primary or secondary receptor (Grundfest 1971).

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Materials and methods

Eight male cats weighing about 3 kg were used. The carotid bodies of two of them were denervated by sectioning the carotid sinus nerve close to the chemoreceptor organ. These two animals were killed 3 and 30 days after denervation. In two other cats, the axonal transport in the carotid sinus nerve was stopped, by unilaterally placing a thin ligature (6-0 silk) about 4 mm above the carotid body in order to determine CAH activity in the nerves proximal to the ligature; these two cats were killed 72 h later. The rest of the animals were untreated.

All of the cats were anaesthetized with pentobarbitone (30 mg/kg i.p.; Sigma London, UK), heparinized (16 mg/kg, i.v.; Leo S.A. Madrid); and transcardially perfused (1) with phosphate-buffered saline (PBS; 0.1 M NaCl, 0.01 M phosphate, pH = 7,2) containing $NaNO_2$ (1 g/l) for 30 s; and (2) with 3 L 2% glutaraldehyde (EM grade; Merck, Darmstat, FRG) in 0.1 M phosphate buffer, pH = 7.4. After perfusion, the carotid bifurcation was surgically exposed, removed and placed in a dissection chamber where the carotid body and carotid sinus nerve were freed from the surrounding tissue and post-fixed in the same fixative for 1.5 h at 4° C.

After a brief rinse in PBS, the carotid bodies were dehydrated in 50%, 70% and 90% water mixtures of component A of JB-4 resin with catalyst (10 min for each step; Polysciences, Northampton, UK) and transferred to component A with catalyst for 1 h with four changes of 15 min. Polymerization with component B was performed in vacuo for 3 h at room temperature. Tissue blocks were kept at −20° C until sectioning [the blocks can be stored for months under these conditions (Ridderstrale 1980)]. For inmediate sectioning, they were transferred to a container with P_2O_5 at room temperature.

Sections (about 1 μm thick) were cut using a Sorvall JB-4 microtome (Du Pont, Conn. USA) mounted on single-hole grids and then processed for CAH demonstration according to the method of Ridderstrale (1976). The incubation time was adjusted to 5 min.

Grids were examined under a Leitz-Orthoplan microscope (Leitz, Wetzlar, FRG) and photographed before and after counterstaining (about 30 s) with toluidine blue. Other sections (0.25 μm thick) were cut using a Jeol ultramicrotome (Jeol, Tokyo) and studied under a Zeiss-1 C electron microscope (Zeiss, Oberkochen, FRG) operating at 100 KV. Some sections were also post-stained with uranyl acetate and lead citrate (Hayat 1972) in order to identify the structures reacting positively for CAH. The specificity of the histochemical reaction was double-checked by adding acetazolamide (10^{-5} M; Sigma London, UK) or by eliminating the substrate (sodium bicarbonate) of the incubation medium.

Results

The CAH-activity reaction which has previously been described in the kidney by Ridderstrale (1976) gave excellent results in the carotid body when the incubation time was

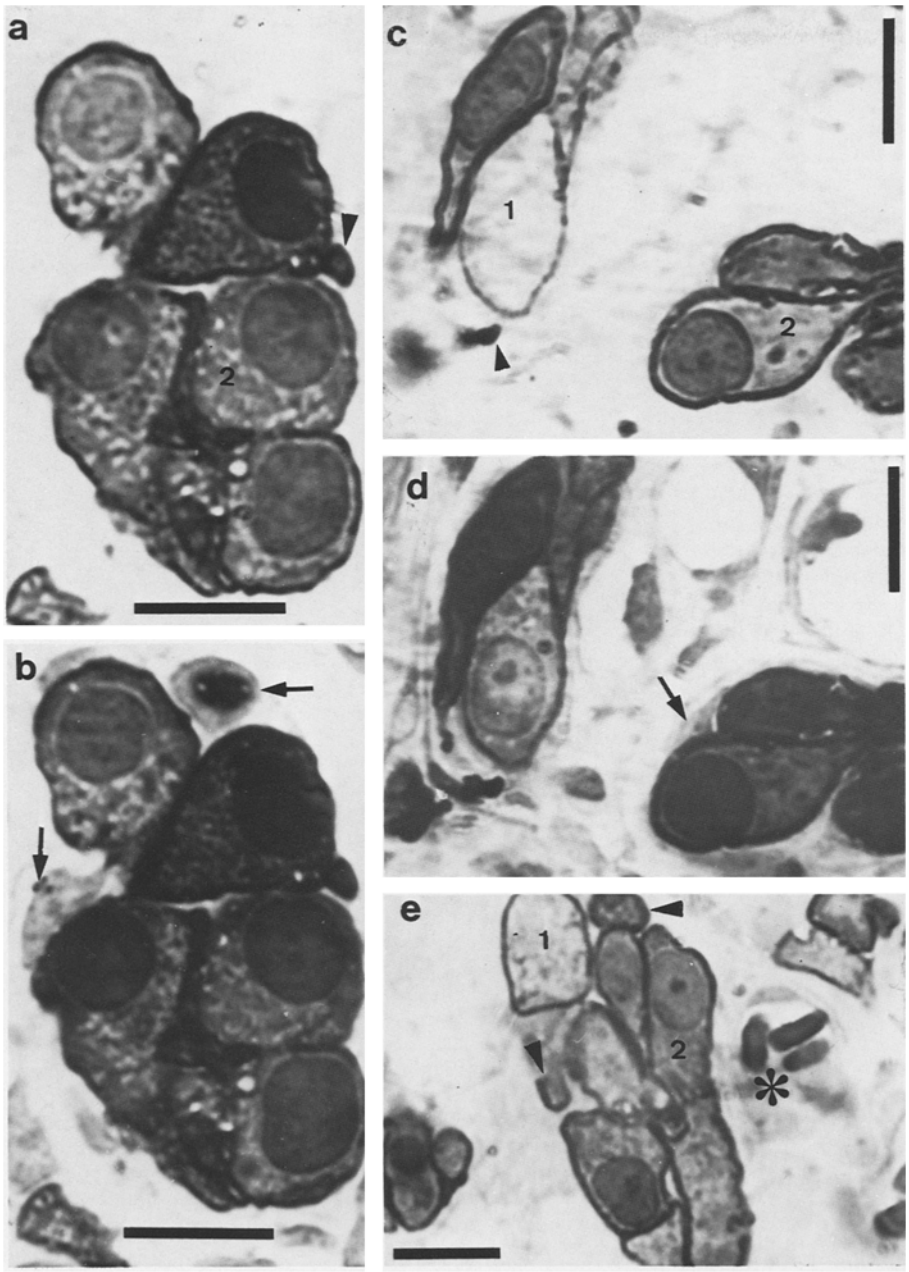

Fig. 1. a, c, e, Reaction pattern seen in different glomoids.
b, d counterstained sections. *Arrows*, type-II cells; *arrowheads*, unidentified structures; *asterisk*, erythrocytes. Note cells where the reaction is restricted to the cytoplasmic membrane (1), and cells with diffuse reaction (2). *Bars*, 10 μm

adjusted to avoid unspecific precipitations. The reaction was completely absent in the presence of acetazolamide and also when the substrate was eliminated.

Figure 1 shows some glomoids in which it was possible to distinguish cells in which the reaction was confined to the cytoplasmic membrane and cells in which the reaction product was also present over the nucleus and cytoplasm (Fig. 1a, c, e). The nuclei of some of the cells which exhibited membrane cytoplasmic reaction could only be distinguished after counterstaining (compare Fig. 1c and d). The appearance of all these reactive cells corresponded well with that of type I cells (McDonald 1981). As expected, erythrocytes were also intensively stained by the enzyme (Fig. 1e). Type II cells were identified as non-reactive structures in the counterstained sections (Fig. 1b, d). These results allow us to conclude that type I cells react positively with CAH, while type II cells do not. Intermingled with the reactive cells, we observed some irregular structures that were difficult to identify at the light-microscopic level; these exhibited reaction in both normal and denervated carotid bodies (Fig. 1a, c, e) and may have been either type-I cell processes or sensory nerve endings.

Sections were also examined under the electron microscope at the maximum accelerating voltage in order to obtain the best possible resolution. The reactive product appeared as a fine dust-like deposit densely covering the cell membrane and sometimes diffusing inwards to the cytoplasm. Darkened nuclei were also seen in several cells (Fig. 2a). When sections were counterstained, dense vesicles could be seen in the reactive cells (Fig. 2a, b); their size and density corresponded well with the dense core vesicles which are the most important identifying characteristic of type I cells at the electron microscopic level (McDonald 1981). In some instances, we were able to identify the al-

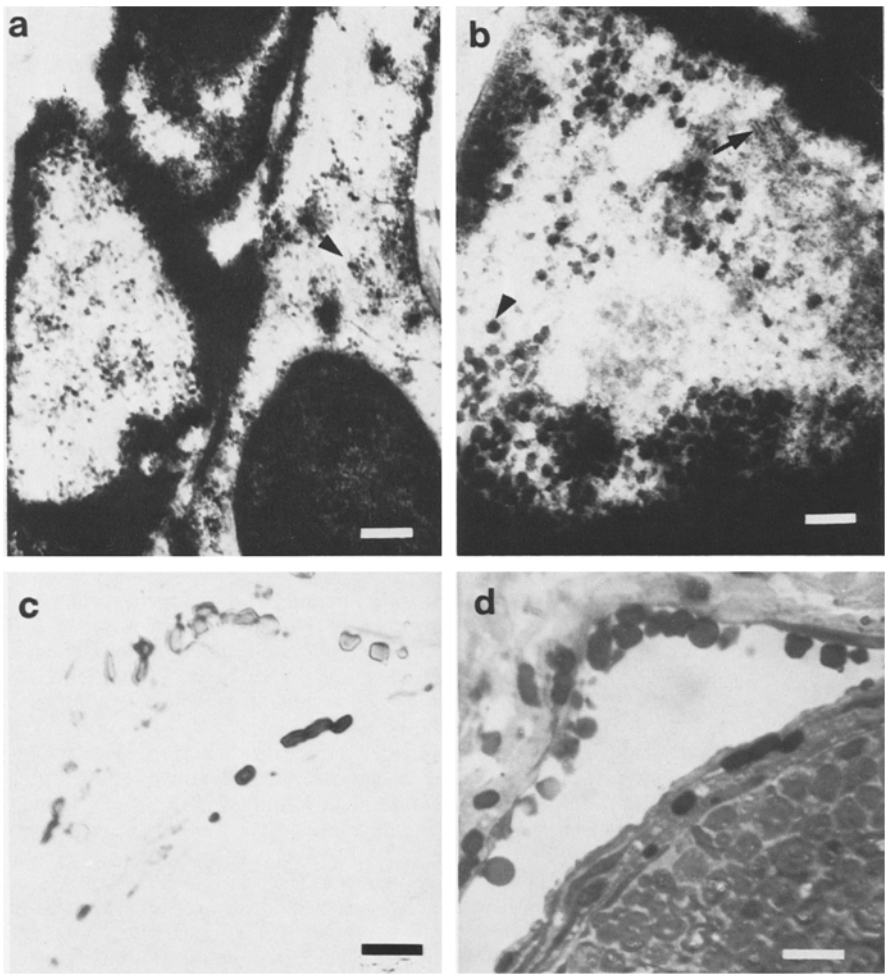

Fig. 2. a, b, Electronmicrographs showing CAH reaction after weak counterstaining. *Arrow*, endoplasmic reticulum; *arrowheads*, dense vesicles. **c** CAH reaction in the carotid sinus nerve proximal to the ligature. **d** Same section counterstained. *Bars*: **a** 1 μm; **b** 0.5 μm; **c**, **d** 10 μm

ready mentioned irregular structures as type I cell processes, because on counterstaining, they showed a great density of dense core vesicles and also cisternae of endoplasmic reticulum (Fig. 2b). However, when endoplasmic reticulum was not present, it was impossible to decide whether these structures were type I cell processes or nerve endings. Therefore, we decided to stop the axonal transport in the carotid sinus nerve and check for CAH activity proximal to the ligature. Erithrocytes exhibited a strong reaction which was never observed on the axoplasms (Fig. 2c, d).

Discussion

The specificity of the Hansson reaction (Hansson 1967) has been a subject of controversy for many years. However, recent studies have suggested that the procedure detects real CAH activity (Troyer 1980; Dobyan and Bulger 1982).

Biochemical studies have shown that the enzymatic activity is reduced by the glutaraldehyde fixation required for electron microscopy; nevertheless, the histochemical reaction is more intense in fixed than in unfixed sections. Thus, glutaraldehyde fixation provides a better immobilization of the enzyme, wich is advantageous when the aim is to localize the presence of CAH (for more details, see Muther 1972; Lönnerholm 1974; Dobyan and Bulger 1982).

The irregular structures reacting positively with CAH remain to be identified. Taking into consideration the fact that the nerve endings in the carotid body represent about 0,5% of the total organ volume (Pallot 1983), it is impossible, on the basis of denervation, to exclude the possibility that some of these irregular structures were nerve endings. If this is the case, CAH must reach the endings by axonal transport. Therefore we decided to stop the axonal flow for 72 h, as this time allows optimal accumulation of radioactivity in the carotid body when the petrosal ganglion is injected with labelled proline (Fidone et al. 1977). As we never detected CAH activity in the axons proximal to the ligature, we assumed that all of the non-identified structures were probably type-I cell processes.

The difference in the intensity of the reaction observed among type I cells can be attributed to the reasons suggested by Ridderstrale (1980), and also to the existence of different subpopulations among these cells (McDonald 1981). It seems well established that the enzyme is intracellularly located, because benzolamide (a non-penetrating CAH inhibitor) does not modify the response of the carotid body to the hypercapnic stimulus. The subcellular localization of the enzyme cannot be reliably ascertained using Hansson's method (Ridderstrale 1980). Nevertheless, the exclusion of type II cells and nerve endings permit us to draw some functional conclusions. Firstly, as type I cells are the structures containing CAH, they must be responsible for the transients in the response to high pCO_2 as re-

corded in the carotid sinus nerve. Secondly, in the context of the acidic hypothesis (Hanson et al. 1981), the carotid body should be considered to be a secondary receptor. In accordance with this view, it is worth mentioning that acidic stimulation of the carotid body is accompanied by the release of dopamine from type I cells (Rigual et al. 1984), and that high pCO_2 levels at normal pH also induce the release of dopamine (unpublished results) at a rate proportional to the activity recorded in the carotid sinus nerve.

Note added in proof. While this work was under consideration for publication a paper was published (Y. Ridderstrale and M.A. Hanson, Ann NY Acad Sci 429:398–400) which essentially agrees with our results.

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