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Classical protein kinase C and its hypoxic stimulus-induced translocation in the cat and rat carotid body

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ABSTRACT: The presence, subcellular distribution, species specificity and possible hypoxic stimulus-induced translocation of classical protein kinase C (cPKC) isozymes were examined in the carotid body.

Carotid bodies were dissected from cats exposed *in vivo* to normoxic or acute hypoxic conditions and from normoxic rats. For immunohistochemistry isoform-specific monoclonal antisera to PKCα, PKCβI, PKCβII and PKCγ were used. The immunoreactivity was visualized by fluorescein isothiocyanate (FITC) labelling. FITC/Texas red double-labelled specimens for the cPKC isozymes/tyrosine hydroxylase were used to demonstrate the chemoreceptor cell localization of cPKC isozymes. The immunofluorescence was detected using laser scanning confocal image technology.

The results showed expression of the PKCα and PKCγ but not PKCβ isoforms in the cytoplasm of carotid body chemoreceptor cells. The double labelling provided evidence for the chemoreceptor cell localization of the cPKC isoforms detected. The immunostaining was most intense in the periphery of the perikarya, the nuclear envelope and, occasionally, the nucleoplasm. No major differences were found in the immunolocalization of PKCα and PKCγ under normoxic and hypoxic conditions or between species. However, the immunoreactivity tended to accumulate more in the peripheral cytoplasm and away from the nucleus in the hypoxic chemoreceptor cell.

This study demonstrates the presence of classical protein kinase C enzymes in chemoreceptor cells. The intensity of the immunoreactivity may suggest a role for the classical protein kinase C signalling pathway in shaping the hypoxic response at the carotid body. However, this study failed to provide firm evidence of this.

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The carotid body is a peripheral sensory organ of neural crest origin [1]. It contains respiratory chemoreceptors that respond to reductions in arterial oxygen tension (P_{a,O_2}) and generate preventive respiratory reflexes at the brain level. These reflexes maintain oxygenation of arterial blood through increased pulmonary ventilation. The mechanism of detection of the hypoxic stimulus (by the chemoreceptor cell), albeit unsettled, is involved in a variety of subcellular mechanisms such as closure of membrane K^+ channels, membrane depolarization, increased Ca^{2+} levels, release of putative neurotransmitters and breakdown of membrane phospholipids [2]. All of them may involve the enzyme protein kinase C (PKC), whose function has not yet been fully explored in the carotid body.

PKC is a family of isozymes that are present in mammalian neuronal tissues and are elaborated in response to a stimulus. There are three major PKC categories: classical, novel, and atypical, each having different activators and consisting of several isoforms. The classical PKC (cPKC) isoforms: α, βI, βII, and γ are activated by diacylglycerol (DAG) and Ca^{2+}, the activation being enhanced by free fatty acids such as arachidonic acid (AA) [3]. These activators are formed and released during the phospholipase C (PLC)-mediated cleavage of membrane phospholipids [4]. Thus they are operational in the phosphoinositide signalling pathway in which cPKC isoforms play the role of effector molecules. Of the cPKC isoforms, PKCα seems ubiquitously distributed in all tissues so far examined, whereas the other isoforms usually display a high degree of tissue, cell and substrate specificity [3].

Previous studies from the authors′ laboratory have shown that levels of both PLC acting against phosphatidylinositol 4,5-bisphosphate and AA metabolites are increased in hypoxic cat carotid body tissue [5, 6]. Furthermore, presence of PKCα in carotid body chemoreceptor cells was demonstrated [7]. However, the wide distribution of PKCα rather discounts a specific involvement in shaping the chemoreceptor cell response to hypoxia. This involvement would be more conceivable if PKCα were the only isoform present in the chemoreceptor cell and translocated intracellularly after hypoxic stimulation, as is the case in other cell lines [8].

This issue was addressed in the present study by immunocytochemically examining the presence, subcellular distribution, species specificity and differences in distribution between quiescent (normoxic) and reactive (hypoxic), carotid body tissue of all the cPKC isoforms.

Materials and methods

Preparation

Twelve cat and four rat carotid bodies were used for this study. The organs were dissected from cats anaesthetized

with α-chloralose and urethane (35 and 800 mg·kg^{-1}, respectively), and from rats anaesthetized with ether. The cats were tracheostomized, ventilated, and subjected (two each), to acute normoxia (P_{a,O_2} 12.0 kPa (90 mmHg)) or hypoxia (P_{a,O_2} 2.7 kPa (20 mmHg)) for 15 min under otherwise constant blood gas and acid/base conditions. The rats were normoxic. The animals were sacrificed with an overdose of anaesthetic. Carotid bodies were rapidly excised and fixed by immersion in 4% paraformaldehyde for 2 h at 4°C, and then cryoprotected in 30% sucrose until further use.

Immunohistochemistry

Cryostat (Leica SM1900; Leica, Heidelberg, Germany) sections of 20 μm were cut and attached to glass slides coated with poly-L-lysine.The sections were treated with 0.3% Triton X-100 in 0.1 M phosphate-buffered saline (PBS), pH 7.4 for 30 min, rinsed with PBS and incubated in 0.3% hydrogen peroxide/methanol. After sequential rinsing with PBS, sections were incubated with 5% normal goat serum for 30 min to inhibit nonspecific binding. They were washed again with PBS and incubated with mouse monoclonal antibodies directed against rat PKCα, PKCβI, PKCβII or PKCγ (Transduction Laboratories, Lexington, Kentucky, USA) at a final concentration of 1 μg·mL^{-1} overnight at room temperature (21°C).

For immunofluorescence staining, sections were incubated with fluorescein isothiocyanate (FITC)-labelled antimouse immunoglobulin G (IgG; dilution 1:200; Jackson Immunoresearch West Grove, PA, USA) for 1 h. To confirm the chemoreceptor cell localization of the PKC antigens, other sections were double-labelled by simultaneous incubation with a rabbit polyclonal antibody directed against rat tyrosine hydroxylase (TH; dilution 1:500; Chemicon International, Inc., Temecula, CA, USA) and visualization with Texas red-labelled antirabbit IgG (dilution 1:200; Jackson Immunoresearch). The immunofluorescence was detected using a confocal laser scanning microscope and workstation (TCS NT; Leica, Heidelberg, Germany). Sections from the normoxic and hypoxic carotid body specimens were processed together on the same glass slides. Antisera were omitted from the immunocontrols. No immunostaining was recognizable under these conditions.

Results

A confocal microscopic examination of FITC-labelled normoxic cat carotid body specimens (figs. 1A and 2A) revealed clusters of cells showing intense PKCγ immunoreactivity. The round or oval-shaped cells, with a large nucleus of 10–15 μm in diameter, had all the characteristics of type I, *i.e.* chemoreceptor cells. Immunoreactivity was present in the nuclear envelope and throughout the cytoplasm, forming a dense granular layer encircling the nucleus and spreading towards the periphery of the perikarya and the plasma membrane. The cytoplasmic distribution of the immunoreactivity encompassed that of the dense-core secretory vesicles. However, the vesicles could not be visualized using light microscopy. Less frequently, immunoreactivity was also seen in the nucleoplasm (fig. 2A). No attempt was made to make an exact count of cells with stained nuclei but it was appraised, from a survey of 20 sections from four carotid bodies, that approximately a quarter of the chemoreceptor cells contained immunostained nuclei. PKCγ was also found to be similarly distributed in rat chemoreceptor cells (not shown). Immunoreactivities varied in intensity and appearance between cells and clusters.

The double FITC/Texas red labelling in figure 1 shows FITC-labelled cells, (expressing PKCγ) Texas red-labelled cells (expressing TH, a marker of chemoreceptor cells) and an overlapping image of the two fluorescent probes; the yellow spots indicate colocalization of the two enzymes. A near-perfect overlap provides evidence for the presence of PKCγ in the chemoreceptor cell. The same pattern of expression was also found for PKCα, whose presence was confirmed not only in the cat but also in the rat carotid body (fig. 3A and B).

No appreciable differences were noted in the distribution and intensity of PKCγ and PKCα immunostaining with the technique of visualization employed. Nor could any striking differences between the normoxic and hypoxic specimens be seen. However, a tendency was observed for an increasing accumulation of immunoreactivity towards the cytoplasmic periphery in the hypoxic cell, which made the cell interior appear somewhat devoid of immunoreactivity. Also, the nucleoplasmic localization of immunoreactivity seen in the normoxic cell was absent in the hypoxic cell (fig. 2B). No other consistent differences between the two gas conditions studied were observed.

The PKCβ isoforms were not expressed in the specimens studied. Therefore, their presence in the chemoreceptor cell was positively excluded. No other elements of the carotid body parenchyma, such as sustentacular cells or nerve fibres, were found to express PKC immunoreactivity.

Discussion

In this study, *ex vivo* cat and rat carotid body tissues were screened for the presence of cPKC. Normoxic and hypoxic carotid bodies were also compared. The latter were dissected from cats subjected to acute hypoxic conditions, similar to those often used for both carotid body and ventilatory excitation in experimental hypoxia tests. The study demonstrates the presence of PKCγ but not PKCβ isoforms and confirms that of PKCα in the carotid body. Additionally, the study provides evidence for the chemoreceptor cell localization of cPKC.

The cPKC isoforms are Ca^{2+}-dependent and DAG-activated. They display substantial activity at the basal, endogenous, level of Ca^{2+} [3], which may underlie their expression in the normoxic carotid body. The greatest activity of both isoforms has been reported in brain cells, but their distribution differs. PKCα is found not only in neural cells but also in other cell types, whereas PKCγ has so far been ascribed exclusively to brain cells [3]. The existence of PKCγ in the carotid body, an organ outside the brain, is now demonstrated. In brain, PKCγ is localized predominantly in the cytoplasm and, to a lesser extent, in the nucleoplasm and PKCα in the periphery of the perikarya [9]. The distribution of both isoforms was found, in general, to conform to that present in brain. In the nuclear membranes of brain cells, PKC exists as a

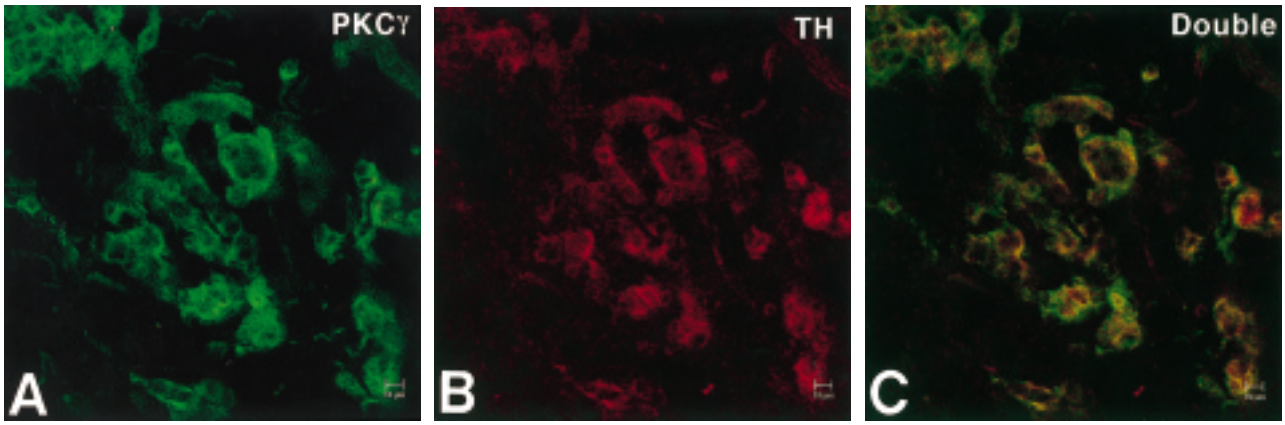

Fig. 1. – Confocal fluorescence microscopy of double immunolabelling of a section of a normoxic cat carotid body tissue stained with mouse anti-protein kinase C (PKC)γ and rabbit anti-tyrosine hydroxylase (TH). The localization of PKCγ is visualized in green with fluorescein isothiocyanate labelled antimouse immunoglobulin G (IgG) (A), whereas that of TH in red with Texas red-labelled antirabbit IgG (B). An overlapping image is shown in C; the yellow spots indicate colocalization of the two fluorescent probes. The near-perfect colocalization identifies the PKCγ as being in the chemoreceptor cell. (Internal scale bars=10 μm.)

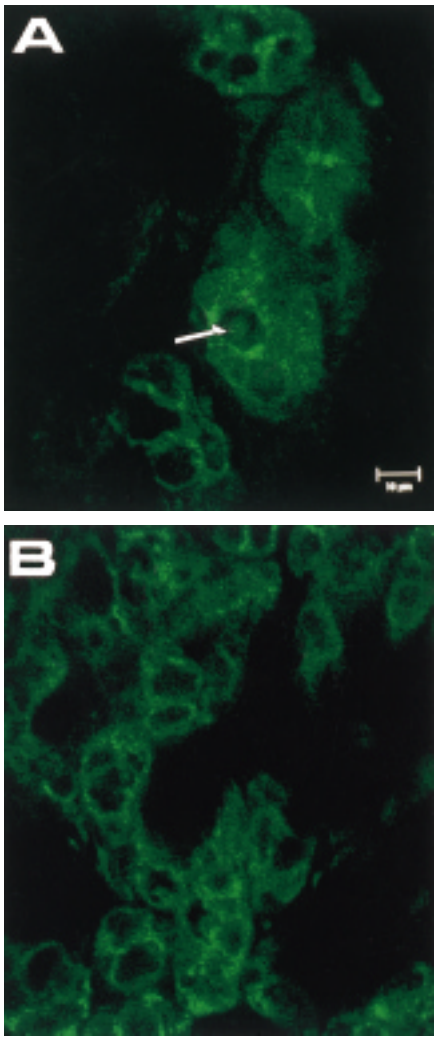

Fig. 2. – Confocal microscopic image of protein kinase Cγ in: A) normoxic; and B) hypoxic cat carotid body specimens. Clusters of chemoreceptor cells displaying immunoreactivity can be seen in the cytoplasm. The nucleoplasmic localization in the normoxic condition, as indicated by an arrow in A, was absent in the hypoxic one. No other striking differences between the two conditions were noted. (Internal scale bar=10 μm.)

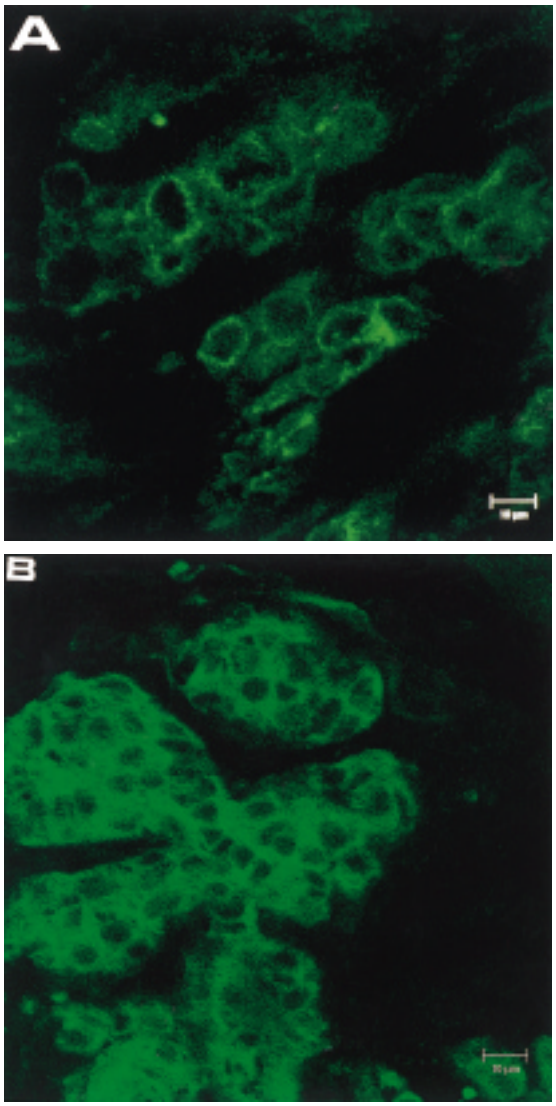

Fig. 3. – Confocal microscopic images of protein kinase Cα in normoxic: A) cat; and B) rat carotid body specimens. Immunoreactivity is present in the cytoplasm of chemoreceptor cells. (Internal scale bars=10 μm.)

constitutively active form that increases nuclear phosphoinositide turnover and Ca^{2+} release [10, 11]. Both increased cytosolic Ca^{2+} levels and a viable PKC pathway are essential to the induction by hypoxia of TH gene expression in oxygen-sensitive cells, such as the cell line PC12 and carotid body chemoreceptor cells [12, 13].

Involvement of cPKC in carotid body responses would probably require some sort of intracellular translocation. A lack of nucleoplasmic immunolocalization and a tendency for a shift of immunostaining towards the plasma membrane in the hypoxic chemoreceptor cell were found. These differences, albeit subtle, suggest that intracellular cPKC content is affected by the stimulus. The interpretation of PKC changes is confounded by an apparent lack of correlation between the degree of translocation and that of the physiological effects observed. In lymphocytes, as small a shift of PKC from the cytosolic to membranous pool as 5 or 9% suffices to activate the enzyme and to cause the cell to respond [14, 15]. Therefore, although the differences noted in the present study fall short of proving that translocation occurs, the authors believe that they may still fit in with several potential schemes of cPKC participation in shaping the chemosensory response.

Of major interest with respect to carotid body function, aside from TH gene induction, is the role of PKC in modulation of synaptic transmission, membrane channels, receptors and intracellular Ca^{2+} levels. These elements are tuned to the intensity of the chemical stimulus [2]. PKC may affect synaptic transmission, acting either on the presynaptic or postsynaptic side. The presynaptic one has often to do with the activation of nonchannel-linked G protein-dependent receptors by an agonist, followed by depolarization-induced activation of PLC and, in turn, breakdown of membrane phospholipids. The process entails activation of PKC downstream of the transduction pathway. The carotid body is well suited to such an action. Plasma membrane receptors, such as the dopaminergic D_2 receptor, are present on the chemoreceptor cell [16]. A G protein-dependent polyphosphatidylinositol-specific PLC activity, and consequently intracellular PKC activators, such as DAG and Ca^{2+}, is increased in hypoxic cat carotid body tissue [5, 17].

In contrast, RIGUAL *et al.* [18] found no appreciable effect on phosphoinositide hydrolysis, and thus on PLC activity, of short-term hypoxia in the rabbit carotid body. This discrepancy may be due to methodological or animal reasons. The study by RIGUAL *et al.* [18] was carried out on *in vitro* rabbit carotid body tissue, whose bathing solution was supplemented with ^{3}H-*myo*-inositol and equilibrated with a mixture of low oxygen tension. Such an experimental paradigm can hardly be compared with that of *ex vivo* hypoxic carotid body tissue, where the organ is dissected and the enzyme activity studied after *in vivo* exposure to a hypoxic test, used in the cat study [5]. The rabbit also seems different from the cat and rat in some other aspects of carotid body function. Dopamine is excitatory to chemoreceptor afferent activity in the former but inhibitory in the latter species [19]. Detection of the role of the phosphoinositide cascade in carotid body function requires studies across various species and experimental designs. Such extensive studies have not yet been carried out, making the results of the existing studies, to some extent, preliminary in nature.

The contentious issue of PLC activation in the carotid body does not quite preclude a role of PKC in the chemosensory response. PKC may be stimulated by DAG, also produced by the action of phospholipase D from the substrates in the phosphatidylcholine cycle other than those for PLC [20]. Finally, PKC, even if insufficient in itself to excite the carotid body mechanism, may interact with other neuroactive substances or putative carotid body neurotransmitters to do so. Endothelins, which are present in the carotid body, stimulate the hypoxia-evoked chemosensory discharge in the carotid sinus nerve in the rabbit, the process being linked to increases in inositol phosphate and intracellular Ca^{2+} levels [21]. In brain tissue, the stimulus-evoked release of noradrenalin and dopamine is modulated by the PKC pathway [22].

Modulation of postsynaptic currents through channel-linked receptors, present on the carotid body sensory nerve terminals, such as nicotinic binding sites [23], may be another target for PKC action. PKCγ, localized chiefly postsynaptically in brain [24], may be of importance in this respect. PKC could modulate the ionic channel conductivity by phosphorylation of channel protein. PKC activation inhibits Ca^{2+}-dependent K^+ channels but not Ca^{2+} channels in the rat carotid body [25]. This inhibition seems not to interfere with that caused by hypoxia, which would speak against the involvement of PKC in this mode of hypoxic action. That is not to say that PKC has no part in chemical signal transduction, as inhibition of K^+ currents as an underlying mechanism of hypoxic carotid body excitation is disputed [26].

In conclusion, the present study demonstrates the existence of protein kinase Cγ and protein kinase Cα in cat and rat carotid chemoreceptor cells and infers a regulatory role of the protein kinase C signalling pathway in carotid body function. Rigorous evidence to this end may be difficult to obtain in this area riddled with numerous intertwined subcellular mechanisms, involving neurotransmitters, receptors, ionic channels and messenger molecules. Alternatively, protein kinase C may be a constitutive enzyme subserving functions other than those linked to chemical signal transduction.

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