Effects of almitrine on the release of catecholamines from the rabbit carotid body *in vitro*

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1. Almitrine increases ventilation by stimulating the carotid body (CB) arterial chemoreceptors but neither its intraglomerular target nor its mechanism of action have been elucidated.
2. We have tested the hypothesis that chemoreceptor cells are targets for almitrine by studying its effects on the release of ³H-catecholamines in an *in vitro* rabbit CB preparation.
3. It was found that almitrine (0.3 and 1.5×10^{-6} M; i.e. 0.2 and 1 mg l^{-1}) increases the resting release of ³H-catecholamines from CBs (previously loaded with [³H]-tyrosine) incubated in a balanced 95% O₂/5% CO₂-equilibrated solution.
4. Almitrine at a concentration of 3×10^{-6} M (2 mg l^{-1}) also augmented the release of ³H-catecholamines elicited by incubating the CBs in a hypoxic solution (equilibrated with 7% O₂/5% CO₂ in N₂), by high external K⁺ (35 mM) and by veratridine (2×10^{-5} M), but did not modify release induced by dinitrophenol (7.5×10^{-5} M).
5. At the same concentration (3×10^{-6} M), almitrine increased the rate of dopamine synthesis and was ineffective in modifying the cyclic AMP levels in either normoxic or hypoxic CBs.
6. It is concluded that chemoreceptor cells are the intraglomerular targets for almitrine. The mechanisms of action of almitrine on chemoreceptor cells are discussed.

Keywords: Carotid body; arterial chemoreceptors; catecholamine; almitrine

Introduction

Almitrine bismesylate intravenously administered produces a potent and long-lasting stimulation of carotid body (CB) chemoreceptors leading to an equally long-lasting hyper-ventilatory response in different animal species including man (Laubie & Schmitt, 1980; Bisgard, 1981; O'Regan *et al.*, 1983; McQueen *et al.*, 1989; Lahiri *et al.*, 1989). In spite of the abundant literature on almitrine, not only its mechanism of action but even its intraglomerular target(s) (chemoreceptor cells, sensory nerve endings or blood vessels) remain undefined.

On the other hand, it is well documented that all chemostimulants tested, including the natural stimuli low P_{O_2} and low pH/high P_{CO_2} and many pharmacological agents produce, in parallel, an increase in action potential frequency in the carotid sinus nerve (CSN) and in the release of catecholamines (CA) from chemoreceptor cells (see Fidone *et al.*, 1990, for a review). In the present study we have tested the hypothesis that almitrine also activates the release of CA from chemoreceptor cells. Our results show that almitrine produces a long-lasting increase in the release of ³H-CA from chemoreceptor cells in resting normoxic conditions, augmenting also the release response evoked by low P_{O_2} and high external K⁺. Almitrine was ineffective in modifying the release evoked by dinitrophenol (DNP) and did not alter adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels in the CB in either normoxic or hypoxic conditions.

Methods

Carotid bodies were isolated from adult New Zealand rabbits (1.5–2.5 kg) under pentobarbitone anaesthesia as previously described (Fidone & Gonzalez, 1982). After CB removal the animals were killed with an overdose of pentobarbitone. The release of CA from the *in vitro* CBs was studied following the

protocol and analytical procedures recently described by Rocher *et al.* (1991). In brief, the CA stores were labelled by incubating the organs for 2 h in a metabolic shaker at 37°C in 0.5 ml of 100% O₂-equilibrated Tyrode solution, containing 2×10^{-5} M of [³H]-tyrosine (3,5-[³H]-tyrosine, 30 Ci mmol⁻¹), 10^{-4} M DL-6-methyl-5,6,7,8-tetrahydropterine and 10^{-3} M ascorbic acid. Under these conditions the CBs synthesized an average of 20 pmol mg⁻¹ tissue 2 h⁻¹, equivalent to 3.5×10^5 c.p.m. The ³H-CA synthesized were [³H]-dopamine and [³H]-noradrenaline ([³H]-NA) in a proportion near 10:1 (Fidone & Gonzalez, 1982; Rocher *et al.*, 1991). Individual CBs were then incubated for the rest of the experiment in 4 ml of [³H]-tyrosine-free and CO₂/HCO₃⁻-buffered Tyrode (pH = 7.42, 95% O₂:5% CO₂). During the first 2 h, the incubating solutions were renewed every 30 min and discarded; thereafter the solutions were renewed every 10 min and collected for subsequent analysis of their ³H-catechol content. Test solutions were applied in this latter period of the experiments as indicated in the Results section. ³H-catechols present in the collected samples were adsorbed onto alumina at a pH of 8.6, batch eluted with 1 N HCl (Weil-Malherbe, 1971) and counted in a scintillation spectrometer. In typical experiments from each series, the alumina eluates were subjected to thin layer chromatography and it was found that about 90% of all the ³H-catechols present corresponded to [³H]-dopamine plus its catabolite [³H]-dihydroxyphenylacetic acid, the rest being [³H]-NA and its catabolites (Rocher *et al.*, 1991). This indicates that the released material comes mostly from chemoreceptor cells (Fidone & Gonzalez, 1982).

The synthesis of ³H-CA was studied by incubating the CBs with [³H]-tyrosine as in the loading period of the release experiments, but the specific activity of the precursor was lower (4 Ci mmol⁻¹) and the incubation lasted 1 h. At the end of the incubation period, and after 3 min of washing out in ice cold saline, the CBs were homogenized in 0.4 N perchloric acid and centrifuged. ³H-CA and free [³H]-tyrosine present in the supernatants were isolated by high performance liquid chromatography (h.p.l.c.) and quantified by

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liquid scintillation spectrometry. [^3H]-tyrosine incorporated into proteins was measured in the pellet.

The protocols for the experiments in which cyclic AMP was determined have also been recently described (Perez-Garcia *et al.*, 1990). The nucleotide was assayed with a radioimmunoassay kit in pairs of CBs incubated in control (95% O_2 :5% CO_2 equilibrated) and in hypoxic (7% O_2 :5% CO_2 :88% N_2 equilibrated) solutions, both, in the presence and in the absence of almitrine.

In all the experiments (except in the labelling periods for the release experiments; Rocher *et al.*, 1991) the incubating solution was a modified CO_2 -bicarbonate Tyrode with the following composition (mM): NaCl 120, KCl 4.7, CaCl 2.2, MgCl 1.1, NaHCO_3 24, HEPES 5 and glucose 5.5. The solutions were equilibrated with a 5% CO_2 -containing gas mixture and adjusted to pH = 7.42 at 37°C. The control solution was equilibrated with 95% O_2 :5% CO_2 to achieve true resting conditions for the CB chemoreceptors (Fidone *et al.*, 1982).

Statistical significance of the differences observed were assessed with Student's *t* test for paired and unpaired data according to the experimental design, except for those release experiments in which the time course of almitrine action was studied. In these later experiments the significance of the differences was determined by a one tail test of homogeneity of slopes model (General Lineal Models, 'GLM-SAS').

Drugs

Almitrine bismesylate was provided by Servier International. The rest of the chemicals used, including sodium pentobarbitone, were obtained from Sigma. The radioimmunoassay kit for cyclic AMP measurement and [^3H]-tyrosine were purchased from Amersham.

Results

Effects of almitrine on the basal release of ^3H -catecholamines

In previous studies (Rigual *et al.*, 1991; Rocher *et al.*, 1991) it has been established that weak acids, when applied alone, do not modify the release of ^3H -CA at concentrations below 1 mM. In spite of that, malic acid (used as solvent for almitrine) was added to the control CBs incubation media at the same concentrations used in almitrine-treated organs. Figure 1a shows that malic acid applied for 20 min (3 and 15×10^{-6} M) did not modify the typical slowly declining basal release of ^3H -CA (Almaraz *et al.*, 1986). In contrast, in the experimental group (contralateral CBs), the basal release of ^3H -CA showed a tendency to increase after the treatment with the same concentrations of malic acid plus 0.3 and 1.5×10^{-6} M of almitrine. This tendency to increase is long-lasting for both concentrations of almitrine, but is greatest with the higher concentration. The effect is more clearly shown in Figure 1b where the data have been normalized. In another group of experiments dimethyl sulphoxide (DMSO) was used as the solvent for almitrine (3×10^{-6} M) and we found that DMSO itself did not modify the release of ^3H -CA at concentrations below 0.2%; almitrine was also effective in augmenting basal release with this solvent (not shown).

Effects of almitrine on stimulus-induced release of ^3H -catecholamine

The effect of almitrine on the release of ^3H -CA induced by low PO_2 , DNP and high external K^+ was studied following the experimental protocol shown in Figure 2. Figure 2a and b show the release response obtained in a pair of CBs in

Figure 1 Effects of almitrine (Almitr, open bar) and malic acid (Malic ac, solid bar) on the basal release of ^3H -catecholamines (^3H -CA). (a) The data are expressed as c.p.m. present in each collected sample. (b) The data were normalized; the ^3H -CA present in the sample collected immediately prior to drug application was taken as 100%. (●) Malic acid-treated carotid bodies (CBs); (△) almitrine plus malic acid-treated CBs. Concentrations as shown in the figure. Data are means with s.e.mean (vertical bars) of $n = 5$ (left panels) and of $n = 9$ (right panels). * $P < 0.01$ (one tail test of homogeneity of slopes).

Figure 2 Effects of almitrine on low PO_2 -induced release of ^3H -catecholamines (^3H -CA). Single experiment with two carotid bodies (CBs) to illustrate the general protocol followed to study the effects of almitrine on stimulus-induced release. (a) One CB (control) was subjected to three consecutive hypoxic stimulations (10 min each; hatched columns). Malic acid at a concentration of $30 \times 10^{-6}\text{ M}$ was present in the incubation solution during the 10 min period prior to and for the second presentation of the stimulus. (b) The contralateral CB was similarly stimulated, but during the second presentation of the stimulus $30 \times 10^{-6}\text{ M}$ malic acid and $3 \times 10^{-6}\text{ M}$ almitrine were present in the bathing solution. In both graphs each column represents the c.p.m. present in the incubation solutions that were collected every 10 min. Open columns: solution equilibrated with 95% O₂:5% CO₂; hatched columns: solutions equilibrated with 7% O₂:5% CO₂ in N₂. The horizontal lines through the columns in (a) and (b) separate the stimulus-induced release (above) from the basal release (below). (c) Shows the evoked release (c.p.m.) calculated as described for the three presentations of the stimulus (S₁, S₂ and S₃) in the control and in the experimental CB; (d) shows the ratios of the evoked release in the second (S₂) and third (S₃) presentation of the stimulus to that obtained in the first (S₁) for the control (C) and for the experimental (E) CB. Note that only at the second presentation of the hypoxic stimulus to the experimental CB was almitrine present in the incubation solution.

response to three consecutive applications of a hypoxic stimulus (incubation with a 7% O₂:5% CO₂:88% N₂-equilibrated solution for 10 min; dashed columns). In the control CB (a) the 10 min period prior to and during the presentation of the second stimulus, the incubating solution contained $30 \times 10^{-6}\text{ M}$ malic acid. At this concentration malic acid did not modify basal release. In the experimental CB (b), and also in relation to the second presentation of the stimulus, the incubation solution contained $3 \times 10^{-6}\text{ M}$ almitrine in addition to malic acid. It is evident that almitrine augmented the evoked release response, which corresponds to the c.p.m. above the horizontal lines in (a) and (b) and is represented in part (c) of the Figure. The evoked release in the first presentation of the stimulus, for both control and experimental CB, amounted to about 3000 c.p.m., which corresponds to approximately 2.5% of the total ^3H -CA stored in the organs (mean CB weight is 0.4 mg). As normally found, in the control CB the evoked release decreased on the second and third presentation of the stimulus (S₂ and S₃, respectively), while in the experimental CB in the second presentation of the stimulus carried out in the presence of almitrine, the evoked release was similar to that seen in the first stimulus. Figure 2d shows the ratios of the evoked release for control and experimental CBs in the second and third presentation of the stimulus to that obtained in the first one (S₂/S₁ and S₃/S₁, respectively). This form of presenting the data corrects for individual variations in the absolute amount of ^3H -CA released and allows a better dissection of the effects due to the treatment.

Average results obtained in 14 pairs of CBs for low PO_2 stimulation, 5 pairs for DNP and 9 for high external K⁺ are shown in Figure 3. Left panels show mean evoked release responses in the successive presentations of the stimuli for control and experimental CBs and for the three different stimuli. The intensity of the different stimuli was chosen to produce similar absolute responses. Right panels show mean ratios of the evoked responses. Almitrine produced a 56% increase in the response evoked by low PO_2 ($P < 0.02$), a 34% increase in release evoked by high K⁺ ($P < 0.05$) and a non significant (22%) tendency to augment DNP-induced release of ^3H -CA.

Figure 4 shows the effects of almitrine on the release of ^3H -CA induced by veratridine ($2 \times 10^{-5}\text{ M}$). Due to the slow wash out of veratridine and to the fact that on successive applications the release responses decrease very markedly (Rocher *et al.*, 1988), the protocol followed to study the effects of almitrine on veratridine-induced release was different: it was studied in single presentations of the stimulus in 5 pairs of CBs as shown in Figure 4. Almitrine produced over 100% increase in the release response evoked by veratridine ($P < 0.02$).

Effects of almitrine on ^3H -catecholamine synthesis

Taking into consideration the mechanisms of the short-term control of tyrosine hydroxylase activity (Weiner *et al.*, 1972; Zigmond *et al.*, 1989), it would be expected from our release experiments that almitrine increases the rate of ^3H -CA synthesis. Figure 5 shows that this was indeed the case. In seven CBs incubated for 1 h with [^3H]-tyrosine ($2 \times 10^{-5}\text{ M}$) in the presence of $30 \times 10^{-6}\text{ M}$ malic acid plus almitrine ($3 \times 10^{-6}\text{ M}$) there was a small (15%) but significant ($P < 0.05$) increase in the synthesis of [^3H]-dopamine in comparison to that obtained in the contralateral organs incubated in the absence of almitrine. No change was observed in the rate of

Figure 3 Effects of almitrine on low PO_2 , dinitrophenol (DNP) and high external K⁺ induced release of ^3H -catecholamines (^3H -CA). Protocols as in previous figure. Left panels show average evoked release for the three presentations of the stimuli (S₁, S₂ and S₃) and for the three stimuli; in S₂, $30 \times 10^{-6}\text{ M}$ malic acid (open columns) or malic acid plus $3 \times 10^{-6}\text{ M}$ almitrine (solid columns) were present 10 min before and during the stimulation period. Right panels show mean S₂/S₁ and S₃/S₁ ratios for the three stimuli. The intensity of the stimuli is indicated in the figure. Data are means with s.e.mean (vertical bars) of 14 values for low PO_2 , 5 values for DNP and 9 values for K⁺.

* $P < 0.05$; ** $P < 0.02$; (two tailed Student's *t* test for unpaired observations).

Figure 4 Effects of almitrine on veratridine-induced release of ^3H -catecholamines (^3H -CA). (a) The data are expressed as c.p.m. in the collected incubation solutions (means with s.e.mean shown by vertical bars). Veratridine (2×10^{-6} M) was applied during 10 min (hatched columns); malic acid (30×10^{-6} M) and malic acid plus almitrine (3×10^{-6} M) were applied as indicated in the figure. (b) Mean evoked release in control and experimental CBs ($n = 5$). $*P < 0.02$; (two tailed Student's t test for unpaired observations).

Figure 5 Effects of almitrine on catecholamine synthesis in the carotid body (CB). The organs were incubated for 1 h in a solution equilibrated with 95% O_2 :5% CO_2 at a pH of 7.4 in the presence of 30×10^{-6} M malic acid (controls; open columns) or malic acid plus 3×10^{-6} M almitrine (experimentals; solid columns). $[^3\text{H}]\text{-noradrenaline}$ synthesis, not shown in the figure, was not different in control and experimental CBs and amounted to 0.25 pmol/CB. $*P < 0.05$. (Student's t test for paired observations; $n = 7$).

$[^3\text{H}]\text{-NA}$ synthesis (see Figure legend). There was neither a significant change in the accumulation of free $[^3\text{H}]\text{-tyrosine}$ nor in the incorporation of $[^3\text{H}]\text{-tyrosine}$ to proteins.

Effects of almitrine on cyclic AMP levels of the carotid body

Since low PO_2 increases cyclic AMP content in the CB (Perez-Garcia *et al.*, 1990) and, on the other hand, different

manoeuvres that increase cyclic AMP levels in the CB also increase the release of ^3H -CA induced by low PO_2 (Perez-Garcia *et al.*, 1991; Wang *et al.*, 1991), we tested the possibility that almitrine increased the levels of this nucleotide as a ready explanation for its effect on the release of ^3H -CA. Two groups of 14 CBs were used. In the first one, 7 organs were incubated in control solution (95% O_2 :5% CO_2 -equilibrated) containing 0.5×10^{-3} M isobutylmethylxanthine (a phosphodiesterase inhibitor) and 30×10^{-6} M malic acid, and their contralaterals in the same solution that contained, in addition, 3×10^{-6} M almitrine. In the second group 7 CBs were incubated for 10 min in a 7% O_2 :5% CO_2 -equilibrated solution that contained isobutylmethylxanthine and malic acid and their contralaterals were similarly incubated, but the solution also contained almitrine. As shown in Figure 6, almitrine did not modify cyclic AMP levels in either condition.

Discussion

The present results show that almitrine *in vitro*, at concentrations similar to those found in blood after administration of effective doses *in vivo* (Gordon *et al.*, 1987), augments the release of CA, mostly dopamine, from chemoreceptor cells in a preparation of the intact rabbit CB. Regardless of what the physiological role of the released CA is (see Fidone *et al.*, 1990 vs. Ponte & Sadler, 1989, for a discussion on this topic), the present results are consistent with previous studies in showing that almitrine, as any other chemostimulant agent tested, increases the release of CA from chemoreceptor cells (Fidone *et al.*, 1990). It should be pointed out also that the long-lasting effect of almitrine on the release of ^3H -CA, seen in the present study, accords with the well known long-lasting increases in ventilation (Laubie & Schmitt, 1980) and carotid sinus nerve (CSN) chemoreceptor potential frequency (Bisgard, 1981; O'Regan *et al.*, 1983; McQueen *et al.*, 1989; Lahiri *et al.*, 1989) observed when the drug is administered *in vivo*.

It has been reported (Pallot & Al Neamy, 1983) that almitrine causes a decrease in the content of CA in the CB, and the suggestion was made that almitrine increased the

Figure 6 Effects of almitrine on cyclic AMP content in control and hypoxic carotid bodies (CBs). Control CBs were incubated in a solution equilibrated with 95% O_2 :5% CO_2 and hypoxic ones in another equilibrated with 7% O_2 :5% CO_2 in N_2 . In both cases the incubation solutions contained 0.5×10^{-3} M isobutylmethylxanthine and 3×10^{-5} M malic acid; almitrine 3×10^{-6} M was present only in the experimental group (hatched columns). In all the cases the data are means with s.e.mean shown by vertical bars; $n = 7$. Means are not statistically different.

release of these biogenic amines. Pequignot *et al.* (1987) also found that almitrine (5 mg kg⁻¹) produced a decrease in the content of CA in the CB that was accompanied by a decrease in the turnover rate of these biogenic amines, the effect being greater in the case of dopamine than in the case of NA. No change in CA content was observed with lower doses of almitrine (1 and 3 mg kg⁻¹). In this later study, carried out for periods as long as 15 days, the possibility cannot be excluded that in the initial moments there was an increase in CA release because the turnover rates could not be assessed at intervals shorter than 2 h. In the present study the *in vitro* preparation allows an almost minute by minute analysis of the action of any experimental manoeuvre, and the results are clear in demonstrating that almitrine increases the release and synthesis of CA. In addition, in relation to previous studies and despite the observed modifications in dopamine metabolism, it was not possible in these studies to establish whether the action of almitrine was directly on chemoreceptor cells or, if it was reflexly mediated. It was also not possible to exclude the possibility that the observed effects on dopamine metabolism in the CB was produced by an efferent pathway present in the ganglioglomerular nerves which is known to be activated by almitrine (McQueen *et al.*, 1989). This uncertainty is eliminated in the present *in vitro* study where the CB is not subjected to reflex inputs. The possibility that almitrine is promoting the release of CA indirectly via retrograde influences on the nerve endings or via endothelial-derived factors is considered remote, for the following reasons. Almaraz & Fidone (1986) have shown that even supramaximal electrical stimulation of the carotid sinus nerve elicits a release of dopamine which is much lower than that observed here with almitrine treatment. The possibility that almitrine is acting on capillary endothelial cells promoting the genesis of a diffusible factor (e.g. NO), which in turn activates the release of dopamine from chemoreceptor cells, cannot be excluded. However, we have found (Obeso, unpublished) that inhibition of NO synthesis affects neither basal nor high external K⁺-induced release of dopamine. We therefore conclude that almitrine augments the release of CA by a direct action on chemoreceptor cells.

The precise mechanism of action of almitrine remains elusive. We have found an increase in the release of ³H-CA in all conditions tested except for DNP, a protonophore that at the concentration used, mimics acidic stimulus (Rocher *et al.*, 1991). Contrary to our observation on the lack of effect of almitrine on acidic (DNP)-induced release, it is well established that almitrine *in vivo* activates chemoreceptor activity in the CSN at any PO₂ and PCO₂. It should be pointed out, however, that while the chemoreceptor discharge evoked by almitrine appears indistinguishable from that evoked by hypoxia, these do not resemble the response to hypercapnic stimulus (Nye *et al.*, 1990). Since the actions of almitrine on the CSN responses or on the hyperventilation elicited by protonophores have not been studied, the possibility remains that these agents interfere with the action of almitrine.

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- Our findings can be interpreted in the light of observations made by Peers & O'Donnell (1990) that almitrine produces a long lasting inhibition of K⁺ currents in CB chemoreceptor cells. Thus, almitrine would be expected to cause a resting (normoxic) depolarization and would tend to increase low PO₂, high external K⁺ and veratridine-induced depolarization (Gonzalez *et al.*, 1990). As a consequence, an increase in the release of ³H-CA would be anticipated in all these situations. Our interpretation of the mechanism of action of almitrine rests on this inhibition of the K⁺ currents in chemoreceptor cells and it has the merit of unifying conceptually the mechanisms of action of almitrine as a chemostimulant and as potentiator of pulmonary hypoxic vasoconstriction (e.g. Naeije *et al.*, 1989). It has been found that chemoreceptor cells possess an oxygen-sensitive K⁺ channel (Lopez-Barneo *et al.*, 1988; Lopez-Lopez *et al.*, 1989; Ganfornina & Lopez-Barneo, 1991) and the proposal has been made that the inhibition of this channel by low PO₂ leads to depolarization of chemoreceptor cells, Ca²⁺ entry and release of neurotransmitters (Gonzalez *et al.*, 1990). Quite recently it has been suggested (Rodman & Voelkel, 1991) that the same mechanism could be operating in the smooth muscle cells of the pulmonary vessels, in such a way that in response to pulmonary hypoxia the muscular cells would be depolarized and vasoconstriction would follow. Almitrine, by blocking K⁺ channels in both cell types, would potentiate their natural responses.
- Our results also suggest that contrary to the situation with the natural hypoxic stimulus (Perez-Garcia *et al.*, 1990), the adenylate cyclase system does not appear to participate in the acute responses elicited by almitrine in chemoreceptor cells. This finding could explain why some of the well known effects produced by long-term hypoxic stimulation (e.g. increase in the turnover rate of CA in the CB) are not seen during long term treatment with almitrine (Pequignot *et al.*, 1987).
- In conclusion, we have studied the effects of almitrine on the synthesis and release of catecholamines in an *in vitro* preparation of rabbit CB and have found that almitrine, like any other chemostimulant agent tested, increases both parameters. This suggests that chemoreceptor cells are direct targets for the drug. In contrast to a natural stimulus (hypoxia), almitrine does not activate adenylate cyclase in the CB. It is suggested that the two pharmacologically important effects of almitrine, i.e. potentiation of the pulmonary hypoxic vasoconstriction and CB chemoreceptor activation, could be mediated via inhibition of O₂-sensitive K⁺ currents present in pulmonary vessels smooth muscle and CB chemoreceptor cells.
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