Cholinergic dopamine release from the in vitro rabbit carotid body

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Bairam, Aida, Habib Néji, and François Marchal. Cholinergic dopamine release from the in vitro rabbit carotid body. J Appl Physiol 88: 1737–1742, 2000.—The aim of this study was to test whether cholinergic mechanisms regulate dopamine (DA) release from the carotid body (CB) and interact with DA D2 autoreceptors. One hundred forty-two CBs from adult rabbits were infused in vitro in a surviving medium bubbled with O2 (Bairam A, Marchal F, Cottet-Emard J M, Basson H, Pequignot J M, Hascoet J M, and Lahiri S. J Appl Physiol 80: 20–24, 1996). CB DA content and release were measured after 1 h of exposure to various treatments: control, cholinergic agonist (0.1–50 µM carbachol), full muscarinic antagonist (1 and 10 µM atropine), antagonists of M1 and M2 muscarinic receptors (1 and 10 µM pirenzepine and 10 µM AFDX-116, respectively), and the DA D2 receptor antagonist domperidone (1 µM), alone and with carbachol (1 µM). Compared with control, the release of DA was significantly increased by carbachol (1–50 µM), AFDX-116, and domperidone and decreased by atropine (10 µM) and pirenzepine (10 µM). The effects of domperidone and carbachol were not significantly different but were clearly additive. It is concluded that, in the rabbit CB, M1 and M2 muscarinic receptor subtypes may be involved in the control of DA release, in addition to the DA D2 autoreceptors.

dopamine D2 autoreceptors; muscarinic receptor agonists and antagonists; domperidone

DOPAMINE (DA) is present in the carotid body (CB) of many animal species and is released in response to hypoxia (see Ref. 16 for review). We recently described a fairly simple approach to study the in vitro release of DA by the rabbit CB. The CB is isolated and placed in a surviving medium in which a known gas concentration is constantly bubbled. This preparation of the “infused” CB was used to demonstrate significant release of DA by 1 h of hypoxia (2). The mechanisms that regulate CB DA release under basal or stimulated conditions are only partially unveiled. A negative-feedback control has been suggested, dependent on DA D2 receptors presynaptic to afferent carotid sinus nerve (CSN) endings (16). These receptors have been described as autoreceptors, since they are located on chemosensory cells that themselves contain numerous DA vesicles. Specific DA D2 receptor antagonists are known to increase CB DA release (16).

Other substances may be involved in the regulation of DA release, since carotid chemosensory cells contain a number of neurotransmitters. The physiological significance of ACh is confirmed by observations of its effects on the CSN chemosensory discharge (15, 16). However, marked differences between species have been reported. For instance, the CSN chemosensory discharge is stimulated by ACh or carbachol in the cat, whereas it is inhibited in the rabbit (23). On the other hand, the cholinergic agonist nicotine stimulates CSN discharge in the cat and rabbit (11, 12, 23). Cholinergic receptors of the nicotinic and the muscarinic type have been located on the type I cell plasma membrane (14–16). The nicotinic-to-muscarinic receptor ratio has been shown to approximate 2:1 in the cat and 1:12 in the rabbit (20), and this may account for the observed chemosensory excitation in the former and inhibition in the latter species in response to ACh (11, 12, 23). In vitro catecholamine (CA) release has been shown to be stimulated by nicotine in isolated CBs of the cat (12, 24) and rabbit (11). Data on the effect of carbachol on CA release by the rabbit CB are scanty. The information is important because of the marked species difference in distribution of types of cholinergic receptor on the type I cell.

In a recent neurophysiological study, Fitzgerald et al. (19) suggested that the adult cat CB contains M1 and M2 muscarinic receptors. Experiments on rat striatum showed antagonist effects of these receptor subtypes on DA release that were enhanced by M2 and inhibited by M2 receptor stimulation (8, 10, 30). In the rabbit, CA release evoked by nicotine is reduced by stimulation with the muscarinic agonists oxotremorine and bethanechol (11). However, to our knowledge, information on specific muscarinic receptor presence and effects on DA release is not available in the rabbit CB.

This study was undertaken to characterize the cholinergic mechanisms involved in basal release of DA by carotid chemoreceptors in the infused rabbit CB preparation. The specific aims were 1) to titrate the effects of carbachol on DA release, 2) to test the hypothesis that M1 and M2 receptor subtypes are present in the rabbit CB and to evaluate their contribution to DA release, and 3) to assess the interactions between dopaminergic autoreceptor and muscarinic receptor mechanisms in the regulation of DA release.

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The study shows that carbachol stimulates DA release. M₁ receptor blockade appears to inhibit, whereas M₂ receptor blockade appears to enhance, this release. Carbachol and DA D₂ receptor blockade by domperidone appear to have additive effects.

**MATERIALS AND METHODS**

Experiments were carried out on 142 CBs sampled from 72 adult New Zealand White rabbits. On the basis of previous observations of the variability of DA concentration in this preparation, we aimed at obtaining a minimum of 10 CBs in each of the treatment groups or subgroups defined below.

Preparation. Animals were anesthetized with a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg) injected intramuscularly. The procedure has been reported previously (2, 4). Briefly, a tracheostomy was performed, and artificial ventilation was initiated with 100% O₂ to minimize CB stimulation during surgery. Both CBs were identified, carefully dissected, freed from connective tissues, and exposed.

Protocol. The CBs were quickly removed and immediately placed each in a vial containing surviving medium. The vials were enclosed in a closed circuit where pure O₂ was circulated and also bubbled in the surviving medium (2, 4). The specimens were incubated at 37°C for 1 h.

The medium consisted of 400 µl of the following solution (in mmol/l): 140 NaCl, 5 KCl, 1.5 CaCl₂, 1 MgCl₂, 6 glucose, 10 HEPES, and 0.029 EDTA (7); pH was adjusted to 7.40 with 0.1 N NaOH after the relevant drug was added to the medium. Each drug concentration was prepared daily by dissolution in bidistilled water, except for domperidone, which was dissolved in ascorbic acid (1% solution).

Carbachol (carbamylcholine chloride) was tested at 0.1, 1, 10, and 50 µM. The cholinergic antagonist atropine (atropine sulfate) and the selective muscarinic M₁ receptor antagonist pirenzepine (pirenzepine dihydrochloride) were used at 1 and 10 µM. AFDX-116, a selective M₂ receptor antagonist, was tested at 10 µM. Finally, domperidone (1 µM), a specific DA D₂ receptor antagonist, was also tested alone or in the presence of 1 µM carbachol. All drugs were purchased from Research Biochemicals International (Natick, MA), except AFDX-116, which was a generous gift from Boehringer-Ingelheim.

At the end of incubation, the CB was separated from the surviving medium and placed in 440 µl of a preservative solution composed of 0.1 M perchloric acid and 1 M sodium bisulfate (400 µl). Forty microlitres of this solution were added to the medium. CB and medium were immediately frozen on dry ice with alcohol and stored at −80°C for later CA assay.

CA assay. DA, its major metabolite 3,4-dihydroxyphenylacetic acid (DOPAC), and norepinephrine (NE) were measured from the homogenized CB and the surviving medium by HPLC and electron detection, as described (25) and used previously (2, 4). Data analysis. The amount of CA was calculated as the total of DA, DOPAC, and NE in the CB (i.e., CA content of CB (CA_CB)) and in the surviving medium (i.e., CA released (CA_r)). The results for DA were expressed by pooling DA and DOPAC in the CB (DA_CB) and in the surviving medium. The release of DA (DA_r) was expressed as percentage of the total CA (CA_CB + CA_r).

The effects of the various treatments were assessed using a factorial ANOVA. When the variance ratio (F) was significant at P < 0.05, Fisher’s test was used to compare two individual groups. Values are means ± SE.

**RESULTS**

Analysis of control CA_CB showed that DA was by far the predominant amine and accounted for 78 ± 2.5% of baseline CAs. An HPLC trace is shown in Fig 1.

Carbachol concentration-response curves. Carbachol (1 µM and up to 50 µM) resulted in significantly lower CA_CB than control (P < 0.01; Fig 2A). This was associated with a significantly lower DA_CB than in control (P < 0.01; Fig 3A). Parallel to the effect on CA_CB, CA_r was significantly larger than control at 1, 10, and 50 µM (P < 0.03; Fig. 2B). DA_r was significantly increased with ≥1 µM carbachol compared with control (P < 0.04; Fig. 2B).

Cholinergic antagonists. Data for cholinergic antagonists are presented in Figs. 4 and 5. Atropine (1 or 10 µM) was associated with no significant difference from control in CA_CB or DA_CB (Figs. 4A and 5A). On the other hand, CA_r and DA_r were significantly lower than control after 10 µM atropine (P < 0.04 and P < 0.01, respectively; Figs. 4B and 5B).

With both concentrations (1 and 10 µM) of the selective M₁ antagonist pirenzepine, neither CA_CB nor CA_r showed a significant difference from control (Fig. 4). After 1 µM pirenzepine, DA_CB was significantly increased compared with control (P < 0.01), and there was no change in DA_r (Fig. 5). After 10 µM pirenzepine, there was no significant difference in DA_CB, and DA_r was significantly decreased (P < 0.01; Fig. 5).

With the M₂ antagonist AFDX-116 (10 µM), CA_CB was significantly increased compared with control (P < 0.01; Fig 4A) in association with a slight and nonsignificant increase in CA_r (Fig. 4B). On the other hand, DA_CB was significantly decreased (P < 0.01; Fig 5A), and DA_r was significantly increased (P < 0.01; Fig. 5B).

Comparison of the effects of equivalent concentrations (10 µM) of antagonists showed that CA_CB, DA_CB, CA_r, and DA_r were comparable after atropine and pirenzepine. After AFDX-116, CA_CB and DA_CB were significantly lower than after atropine (P < 0.004 and P < 0.0005, respectively) or pirenzepine (P < 0.0001 and P < 0.002, respectively). Accordingly,CA_r and DA_r were significantly higher after AFDX-116 than after atropine (P < 0.0014 and P < 0.0001, respectively) or pirenzepine (P < 0.0007 and P < 0.00001, respectively).

Domperidone and carbachol + domperidone stimulation. Domperidone induced a significant decrease in CA_CB (P < 0.01; Fig. 4A) and increase in CA_r from control (P < 0.04; Fig. 4B), which were not significantly different from those observed with carbachol. Similarly, after domperidone, there was a significant decrease in DA_CB (P < 0.01; Fig. 5A), which appeared larger than with carbachol (P < 0.04; Fig. 5A), and an increase in DA_r (P < 0.01; Fig. 5B), which was not different from that after carbachol.

Domperidone + carbachol stimulation basically produced about the same effect as either drug alone on CA_CB or DA_CB, since there was no difference compared with domperidone or carbachol (Figs. 4A and 5A). However, the increase in CA_r and DA_r was significantly larger than for either drug alone (P < 0.001; Figs. 4B and 5B).
DISCUSSION

The main findings in this study are that carbachol stimulates CA and DA release from the isolated, infused rabbit CB exposed to high ambient O₂ concentration. DA, is inhibited by atropine and 10 µM pirenzepine and enhanced by 10 µM AFDX-116. The stimulant effect of domperidone on DAr, appears to be additive to that of carbachol.

DA is known as the major amine in the CB of a number of animal species and represents about two-thirds of total CAs in the rabbit CB (22). The DA proportion obtained in this study was comparable to those previously reported in normoxia (22) or during incubation in 100% O₂ for 1 h (2). DAr, was expressed as a fraction of the total CA in the preparation to minimize the effects of confounding factors, such as intersubject variation of CB size and CA metabolism (4, 16, 21). The intersubject variability of DA content of the whole CB preparation is not negligible. This may, for instance, explain why the effect of pirenzepine on CA CB was not picked up with both concentrations, whereas the effect on DAr (as percentage of total CA) was dose dependent.

A significant number of presynaptic muscarinic receptors have been suggested to be present on sympathetic

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![Fig. 1. Chromatograms of standard and carotid body samples. Each standard contains 10 pmol of norepinephrine (NE), 3,4-dihydroxyphenylacetic acid (DOPAC), and dopamine (DA). Volume injected for standard or carotid body was 25 µl.](image)

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![Fig. 2. Effects of carbachol on catecholamine content (A, CA CB) and release (B, CA r) of carotid bodies infused in vitro and bubbled with 100% O₂. Carbachol added to surviving medium decreased CA CB and increased CA r in a concentration-dependent manner. Significant effects occurred at ≥1 µM carbachol. Numbers within bars represent number of carotid bodies. C, control. Significantly different from control: *P < 0.01; **P < 0.03.](image)
endings innervating the CB vasculature (11). Their stimulation/inhibition may modify the release of NE (27) but is less likely to affect DA.

Cholinergic mechanisms. The concentration-effect curves of carbachol showed significant CA at 1 µM, without further increase when the concentration was increased up to 50 µM. Under the experimental conditions, 1 µM carbachol may thus be considered enough to produce full cholinergic CA. The aspect of the concentration-effect relationship for DA was similar, with a plateau occurring at 1 µM. In keeping with the significant release of CA induced by 1 µM carbachol, there was a significant decrease in CA and DA in our preparation. We are not aware of previous reports on the effect of carbachol on basal CA or DA secretion by the rabbit CB with which our study could be compared (11). Qualitatively, however, our results are similar to those of Shaw et al. (26), who used rat CB superfused with 100% O2-equilibrated solution. Subsequent addition of 10 µM carbachol for 10 min significantly increased DA concentration in the effluent. Thus carbachol appears to affect in vitro CB DA release similarly in rabbits and rats during exposure to 100% O2. This is likely to express the stimulation of the nicotinic receptors, since carbachol is a nonspecific cholinergic agonist and nicotine has been shown to stimulate CB DA release in a number of species, including rabbits (11). Furthermore, the muscarinic agonists oxotremorine and bethanechol were found to negatively modulate nicotine-evoked DA release in the rabbit CB (11). It is interesting that the dominant effect of carbachol in the rabbit CB consists in a stimulation of basal DA release, despite the fact that muscarinic receptors have been shown to be far more numerous than nicotinic receptors (20).

At 1 µM the muscarinic antagonist atropine did not affect CB CA or DA content or release, but at 10 µM it
significantly decreased CAr and DAr. Similarly, in the in vitro rat CB, superfusion with a solution containing 100 µM atropine has been shown to suppress radiolabeled CA and Ca2+ released in response to carbachol stimulation (26). A first interpretation was to relate the effects to the overall blockade of the muscarinic receptors. On the other hand, voltage-clamp studies of neonatal rat type I cells showed that atropine at 10 or 100 µM, but not 1 µM, inhibited the inward current evoked by nicotine stimulation, an indication of the inhibition of nicotinic receptors by high concentrations of atropine (29). How much the bioavailability and effect of this antagonist or the nicotinic-to-muscarinic receptor density ratio compares between neonatal rat isolated type I cell and adult rabbit whole organ preparations is unknown. We are also unaware of data on effects of atropine on DAr evoked by 1 µM carbachol in the isolated CB preparation.

Opposite effects of selective M1 and M2 muscarinic antagonists were nonetheless clearly observed in our study. The M1 muscarinic antagonist pirenzepine was found to reproduce the effects of atropine on DAr. In contrast, AFDX-116, the M2 antagonist, resulted in a significant decrease in DACB and a significant increase in DAr. As previously discussed by Fitzgerald et al. (19), the opposite effects of the M1 and M2 blockers would be difficult to explain on the sole basis of nonspecific action on the CB. ACh is known to be contained in the type I cell (13, 17, 18), and muscarinic receptors have been located in its plasma membrane (11). The present data suggest that M1 and M2 receptor subtypes are present in the rabbit CB. Alteration in the type I cell cytosolic free Ca2+ concentration ([Ca2+]i) is reported to be a major result of stimulating or inhibiting cholinergic receptors. Nicotine (9, 29) and muscarine (9) stimulation appear to increase [Ca2+]i in the rat type I cell. The increase in [Ca2+]i, in turn, is known to promote CA release (16). Little information is available on the respective interaction of receptor subtypes M1 and M2 with CB DA release. The inhibitory effect of pirenzepine shown in this study suggests that DA is stimulated by M1 muscarinic receptors. On the other hand, M2 receptors located on autonomic postganglionic neurons are autoreceptors (3, 6; see Ref. 8 for review) and, as such, regulate ACh release. Taken in this context, it is conceivable that the M2 receptors on type I cells inhibit ACh release, which would in turn lessen DA release. Furthermore, the effects of the M1 antagonist pirenzepine and the M2 antagonist AFDX-116 at baseline may be taken as indirect evidence that, in this preparation, there exists a basal release of ACh negatively regulated by M2 muscarinic receptors. The system of the M1-M2 receptors could act as a mechanism for fine tuning of DA release, controlling initiation and termination of DA secretion. Overall, our data on effects of selective M1 and M2 muscarinic receptor antagonists concur with studies in which DA release was measured in vivo by microdialysis of brain tissues (8, 10).

Interaction between cholinergic and dopaminergic mechanisms. The specific DA D2 receptor antagonist domperidone significantly reduced CACB and DACB and increased CAr and DAr. The changes are in agreement with previous data obtained by using spiperone in adult cats (16) or haloperidol in rabbits (4) and are consistent with a mechanism of control of endogenous DA release by presynaptic DA D2 receptors located on type I cells. The Ca2+-dependent DA release has recently been shown to inhibit Ca2+ currents in the CB, resulting in a negative-feedback control of the secretion of this amine (5).

An interesting finding in our study is that effects of domperidone and carbachol appeared to be additive, since the increase in CA or DA with domperidone +...
carbachol was much larger than with either drug alone. The findings are in keeping with data on neurons in the central nervous system, where activation of presynaptic DA D2 autoreceptors regulates DA release by cholinergic stimulation (8; see Ref. 28 for review) and limits ACh release (28). Similarly, DA may be involved in the control of ACh release in the CB. Carotid nerve responses to ACh application on the petrosal ganglion were shown to be modulated by DA in the cat (1). The clearly additive effects of carbachol and domperidone in promoting CB DA release reported in our study could thus express the combination of the enhanced Ca2+-dependent DA release by exogenous cholinergic stimulation and the suppression of inhibition of ACh secretion by D2 receptor blockade.

Together, the present data suggest that DA and ACh regulate endogenous release of DA from the infused rabbit CB. M1 and/or nicotinic receptor stimulation appears to contribute to DA release. Activation of M2 receptors by ACh and of D2 autoreceptors by DA appears to limit further release. The data are also consistent with a control of ACh release by DA D2 receptors. The relevance of these findings on control of basal DA release and DA-ACh interaction in understanding the chemosensory function of the type I cell requires further investigations.

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