Acetylcholine Increases Intracellular Calcium of Arterial Chemoreceptor Cells of Adult Cats

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Shirahata, Machiko, Robert S. Fitzgerald, and James S. K. Sham. Acetylcholine increases intracellular calcium of arterial chemoreceptor cells of adult cats. J. Neurophysiol. 78: 2388–2395, 1997. Several neurotransmitters have been reported to play important roles in the chemoreception of the carotid body. Among them acetylcholine (ACh) appears to be involved in excitatory processes in the cat carotid body. As one of the steps to elucidate possible roles of ACh in carotid body chemoreception in the cat, we examined the effect of ACh on intracellular calcium concentration ([Ca²⁺]) of cultured carotid body cells. The carotid body from adult cats was dissociated and cultured for up to 2 wk. [Ca²⁺], measured from clusters of cells with a microfluorometric technique using Indo-1 AM, experiments were performed at 37°C, and cells were continuously superfused with modified Krebs solutions equilibrated with 5% CO₂–16% O₂–79% N₂. ACh (100 μM) caused a marked increase in [Ca²⁺], in ~70% of clusters, and the responses to 1–300 μM of ACh were concentration dependent. The magnitude and kinetics of the ACh response were mimicked by the application of nicotine, whereas muscarinic agonists, pilocarpine, and muscarine failed to evoke a similar response. ACh-induced increase in [Ca²⁺] was dependent on extracellular Ca²⁺: it was greatly reduced or completely abolished by a transient removal of extracellular Ca²⁺. The response was consistently but only partially reduced by caffeine (5 mM) or nifedipine (10 μM). The effect of mecamylamine (100 μM) was inhibitory but small. Moreover, the increase in [Ca²⁺], in response to ACh was also observed in some clusters that did not respond to high K (100 mM) Krebs. These results suggest that ACh increases [Ca²⁺], of cultured carotid body cells by activating neuronal nicotinic ACh receptors, leading to Ca²⁺ influx via nicotinic channels. In addition, other pathways such as Ca²⁺ influx through L-type calcium channels, perhaps secondary to membrane depolarization, and Ca²⁺ release from intracellular stores may participate in increasing [Ca²⁺], in response to ACh. Muscarinic receptors appear to play only a small role, if any.

INTRODUCTION

The carotid body is a major arterial chemoreceptor organ that is stimulated by hypoxia, hypercapnia, and acidosis (natural stimuli). It is innervated by the carotid sinus nerve, a branch of the glossopharyngeal nerve. The stimulation of the carotid body reflexly affects respiratory, cardiovascular, renal, and endocrine systems (Daly 1986; Fitzgerald and Lahiri 1986; Fitzgerald and Shirahata 1997; Marshall 1994). Although the precise mechanisms are not known, substantial evidence strongly suggests that neurotransmitters play an important role in carotid body chemoreception (Fidone et al. 1990; Gonzalez et al. 1994). The contribution of each neurotransmitter for the carotid body function seems to vary among different species. In the cat, several studies suggest that acetylcholine (ACh) is involved in an excitatory process of the chemoreceptor afferent neurons. Exogenously applied ACh evokes a fast and consistent increase in carotid sinus nerve chemoreceptor discharge (for a review see Eyzaguirre et al. 1983). Administration of cholinergic blockers to the carotid body with various methods inhibits hypoxia-mediated elevation in chemoreceptor neural activity (Fitzgerald and Shirahata 1994; Landgren et al. 1952; Nishi and Eyzaguirre 1971), although the results of such studies remain controversial (Biscoe 1965; Sampson 1971).

Other data support a role for ACh functioning as a neurotransmitter in the carotid body: choline acetyltransferase, an ACh-synthesizing enzyme, has been localized in glomus cells (Wang et al. 1989); a high-affinity, Na-dependent cholinoceptor uptake mechanism, which is specific for cholinergic neurons, has been shown in cat glomus cells (Fidone et al. 1977). Further, cholinergic receptors have been demonstrated on the carotid sinus nerve (Fitzgerald and Shirahata 1990). Perhaps the simplest scenario in the cat would have natural stimuli causing glomus cells to release ACh, and the binding of ACh to cholinergic receptors generating action potentials in the afferent fibers. However, the processes involved in chemoreception appear to be more complex. Cholinergic receptors are also localized on glomus cells of the cat (Dinger et al. 1981, 1985; Hirano et al. 1992). Moreover, exogenous application of nicotine releases catecholamines from the carotid body (presumably from the glomus cells) (Dinger et al. 1985; Obeso et al. 1987). The effect is partially blocked by α-bungarotoxin, a nicotinic receptor antagonist, and almost abolished by removal of extracellular calcium (Dinger et al. 1985; Obeso et al. 1987).

Taken together, the data suggest that ACh has multiple actions in the carotid body, and one of them may be that presynaptic neuronal nicotinic ACh receptors on glomus cells modulate the release of neurotransmitters. As one of the steps to elucidate this possible role for ACh in carotid body chemoreception, we examined the effect of ACh on intracellular calcium concentration of cultured carotid body cells from adult cats. We hypothesized that ACh increases intracellular calcium ([Ca²⁺]) of glomus cells via nicotinic receptor activation. However, this hypothesis, although reasonable, is proposed guardedly because the testing of it is impeded by the problem of identifying precisely which cells...
in culture are glomus cells. This issue is addressed in the Discussion. Our data suggested that ACh increased \([Ca^{2+}]_i\) of carotid body cells via several mechanisms.

**METHODS**

**Cell culture**

Carotid body cells were cultured as previously described with a slight modification (Shirahata et al. 1994a). In short, carotid bodies were harvested from adult cats that were deeply anesthetized with pentobarbital sodium (30–40 mg/kg ip, then 50 mg/kg iv) and decapitated. Both carotid bodies were cleaned and dissociated with collagenase (0.1–0.2%, Sigma, type X1) and gentle trituration. The cells were then centrifuged (100–200 g, 5 min), the pellet was resuspended in a chemically defined culture medium, and the cell suspension was divided into 10–15 wells. Each well consisted of a glass coverslip (25 mm diam) at the center of which a plastic cloning cylinder (Specialty Media; 6 mm diam) was placed. The cells were then centrifuged (100 ± 200 g, 5 min), the pellet was resuspended in a chemically defined culture medium, and the cell suspension was divided into 10–15 wells. Each well consisted of a glass coverslip (25 mm diam) at the center of which a plastic cloning cylinder (Specialty Media; 6 mm diam) was placed. The coverslip was previously coated with poly-D-lysine (Sigma), and then diluted basement membrane complex (Matrigel, Collaborative Biomedical Products; 1/10 dilution) was applied. Three hours later the solution containing Matrigel was aspirated, and the coverslip was washed with culture medium before plating cells. The well was placed in a regular 35-mm culture dish, and the cells were cultured in a 5% CO₂/air incubator at 37°C. The chambers were placed on an inverted microscope (TMD, Nikon), and the cells were superfused with a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12 medium (DMEM/F12) for 45 min to wash out excess Indo-1. To measure \([Ca^{2+}]_i\), Indo-1 in the cells was repeatedly excited at a wavelength of 395 nm for 35 ms every 2 s. Two wavelengths of emission light (405 nm, \(F_{405,\text{min}}\); 495 nm, \(F_{495,\text{min}}\)) were recorded from a microscope field with a dual emission fluorometer (Biomedical Instrumentation Group, University of Pennsylvania), by which the fluorescent signals were converted to voltage changes. The microscopic field contained a small cluster of the carotid body cells. A PC-based computer and pCLAMP software (Axon Instruments) were used for the acquisition of the data (Sham et al. 1995). \([Ca^{2+}]_i\) was calculated with a standard method (Gryniewicz et al. 1985): \([Ca^{2+}]_i = K_d \times \beta \times (R - R_{\text{min}})/(R_{\text{max}} - R)\), where \(R = (F_{495,\text{min}} - F_{495,\text{bg}})/(F_{495,\text{max}} - F_{495,\text{bg}})\), \(\beta = F_{495,\text{min}}/F_{495,\text{max}}\), and \(K_d\) of Indo-1 is 288 nm. \(R_{\text{max}}\) and \(R_{\text{min}}\) were determined at the end of each experiment by permeabilizing the cells with 10 μM calcium ionophore (4-bromo-A23187; Calbiochem-Novabiochem) in Tyrode solutions containing 10 mM \([Ca^{2+}]_i\), or 10 mM ethylene glycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA). Background signals \((F_{495,\text{bg}} - F_{405,\text{bg}})\) were measured by quenching Indo-1 with MnCl₂. They were similar to the signals obtained from the area without any cells.

During the experiments the clusters were continuously superfused with Krebs solution with or without chemical agents. Twenty to 30 min intervened between the application of cholinergic agents to avoid the desensitization of nicotinic ACh receptors. Between experimental challenges together with the period for washing out the pre-existing chemical agents were obtained from Sigma Chemical unless otherwise stated. Subsequently, standard ABC techniques were applied using Vectastain Elite ABC kits (Vector). As chromogen, 3-aminobenzidine was used. Vector SG was used.

**Measurement of \([Ca^{2+}]_i\)**

**GENERAL.** \([Ca^{2+}]_i\) was measured with microfluorometric methods using acetylmethylester Indo-1 (Indo-1 AM, Molecular Probes). Indo-1 AM (50 μg) was dissolved in 20 μl of Pluronic F-127 (20% in dimethylsulfoxide; Molecular Probes) and subsequently diluted with culture medium. The dye was loaded by exposure of cells for 1 h to Indo-1 (4 μM) in the 5% CO₂/air incubator at 37°C. Subsequently, a coverslip with cells was pasted with silicon glue to the bottom of a recording chamber that was made from stainless steel. The chamber was placed in a heating chamber in which warm water circulated to maintain the temperature in the recording chamber at 37°C. The chambers were mounted on an inverted microscope (TMD, Nikon), and the cells were superfused with a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12 medium (DMEM/F12) for 45 min to wash out excess Indo-1. To measure \([Ca^{2+}]_i\), Indo-1 in the cells was repeatedly excited at a wavelength of 395 nm for 35 ms every 2 s. Two wavelengths of emission light (405 nm, \(F_{405,\text{min}}\); 495 nm, \(F_{495,\text{min}}\)) were recorded from a microscope field with a dual emission fluorometer (Biomedical Instrumentation Group, University of Pennsylvania), by which the fluorescent signals were converted to voltage changes. The microscopic field contained a small cluster of the carotid body cells. A PC-based computer and pCLAMP software (Axon Instruments) were used for the acquisition of the data (Sham et al. 1995). \([Ca^{2+}]_i\) was calculated with a standard method (Gryniewicz et al. 1985): \([Ca^{2+}]_i = K_d \times \beta \times (R - R_{\text{min}})/(R_{\text{max}} - R)\), where \(R = (F_{495,\text{min}} - F_{495,\text{bg}})/(F_{495,\text{max}} - F_{495,\text{bg}})\), \(\beta = F_{495,\text{min}}/F_{495,\text{max}}\), and \(K_d\) of Indo-1 is 288 nm. \(R_{\text{max}}\) and \(R_{\text{min}}\) were determined at the end of each experiment by permeabilizing the cells with 10 μM calcium ionophore (4-bromo-A23187; Calbiochem-Novabiochem) in Tyrode solutions containing 10 mM \([Ca^{2+}]_i\), or 10 mM ethylene glycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA). Background signals \((F_{495,\text{bg}} - F_{405,\text{bg}})\) were measured by quenching Indo-1 with MnCl₂. They were similar to the signals obtained from the area without any cells.

**EXPERIMENTAL PROTOCOL.** Preliminary studies showed that ~70% of the clusters of cells responded to ACh by increasing \([Ca^{2+}]_i\). When clusters were repeatedly exposed to higher concentrations of ACh (>100 μM) for 2 min, the responses became smaller after the second exposure. However, if the exposure to ACh did not exceed 30 s, the repeated administration of ACh generated reproducible responses. At the beginning of experiments, small clusters were exposed to ACh (100 μM) for 30 s, and clusters responding to ACh were considered appropriate clusters for testing. Most clusters were exposed to ACh more than once, and 20–30 min intervened between challenges to avoid any desensitization of nicotinic receptors. Five sets of experiments (1–5) were performed.

1) **The dose-response relationship.** In this set of experiments, several doses of ACh (1, 3, 10, 100, or 300 μM) were tested by...
superfusing ACh-containing Krebs for 2 min. The reason for allowing a longer perfusion period for ACh was to ensure the concentration of ACh in the recording well reached the desired level. When the cluster was exposed to two or three concentrations of ACh, the lowest concentration was used first to avoid prolonged exposure of nicotinic receptors to high concentrations of ACh. The difference in concentrations between the two successive ACh superfusions was >10 times.

2) Effects of nicotinic and muscarinic agonists. The responses to nicotine and to pilocarpine and muscarine (muscarinic agonists) were compared with the response to ACh. Control Krebs was switched to Krebs containing 100 μM ACh, nicotine, pilocarpine, or muscarine for 45 s. The concentration of ACh and the duration of perfusion were chosen for observing clear [Ca2+]i increase and concurrently avoiding desensitization of nicotinic receptors. The order of exposures was randomized.

3) Effect of mecamylamine. The ability of mecamylamine (a nicotinic antagonist) to reduce [Ca2+]i, response to ACh was tested. Control Krebs was switched to Krebs containing mecamylamine (100 μM to 1 mM) for 90 s, then to Krebs containing the same concentration of mecamylamine and 100 μM of ACh for 45 s. This exposure was followed by Krebs containing mecamylamine for 60 s.

4) Source of calcium. To investigate whether the increase in [Ca2+]i, was due to the influx of Ca2+ from extracellular space or due to the release of Ca2+ from intracellular storage sites, the effects of either Ca2+-free Krebs or Krebs containing caffeine were examined. These modified Krebs solutions were superfused before (90 s), during (45 s), and after (60 s) ACh administration. Composition of Ca2+-free Krebs was similar to normal Krebs except for the inclusion of 4 mM EGTA and exclusion of CaCl2. Caffeine was dissolved in Krebs at the concentration of 5 mM.

5) Involvement of L-type calcium channels. The possibility that L-type calcium channels are involved in a [Ca2+]i response to ACh was tested using nifedipine. The protocol was similar to 4). Nifedipine was dissolved in dimethyl sulfoxide and then added to Krebs to a final concentration of 10 μM. The effect of nifedipine on the [Ca2+]i response to high-K Krebs (100 mM) was also tested for comparison. For the high-K Krebs solution, the osmolality was maintained by reducing the concentration of NaCl.

DATA ANALYSIS. The control value of [Ca2+]i, was defined as the mean of values taken between 20 and 30 s from the start of experiments (14 data points). Maximal [Ca2+]i, was the mean value of 14 data points around the peak response. An increase in [Ca2+]i, was defined as the difference between maximal and control values. All values were reported as means ± SE. The Student's t-test and analysis of variance (ANOVA) with Duncan's new multiple-range test was used for statistical evaluation, and P < 0.05 was considered significant.

RESULTS

Cell identification

Immunocytochemical experiments have revealed that nearly all clusters contained tyrosine hydroxylase-positive cells (Fig. 1). Results are similar to our previous studies (Shirahata et al. 1994a,b). The presence of tyrosine hydroxylase is used as a marker for glomus cells. Other cells expressing tyrosine hydroxylase in the carotid body may be sympathetic ganglion cells, but they are extremely fewer than glomus cells (for a review see McDonald 1981). Microglia-like cells reacted with avidin-biotin complex and caused false-positive staining, but they were easily distinguished from other cells by their spiny appearance. Further, most of them were washed away before the measurement of [Ca2+]i. Glomus cells dominated in some clusters but not in all.

[Ca2+]i measurement

THE DOSE-RESPONSE RELATIONSHIP. The mean [Ca2+]i, measured from clusters of cultured carotid body cells at rest was 97 ± 9 nM (mean ± SE; n = 27). Figure 2A shows the effect of ACh perfusion (10 and 100 μM) on [Ca2+]i, of one cluster of cultured carotid body cells. Exposure to ACh increased [Ca2+]i, quickly. The maximum response was well sustained at low concentrations of ACh (up to 10 μM). However, at higher concentrations of ACh (e.g., 100 μM), [Ca2+]i, first increased but decayed very quickly, suggesting that desensitization occurred almost at the same time as the activation of the receptors. Removal of ACh from the superfusate produced a return of [Ca2+]i, to the control level. The magnitude of the response differed among the clusters most probably as a function of the number of responding cells in the cluster. Nevertheless, the [Ca2+]i, response to ACh appeared concentration dependent (Fig. 2B).

EFFECTS OF NICOTINIC AND MUSCARINIC AGONISTS. Five clusters were exposed to ACh, nicotine, and pilocarpine in random order. Nicotine provoked a response very similar to that of ACh at a concentration of 100 μM, but pilocarpine (100 μM) either had no effect (Fig. 3) or caused a small and slow increase. The increases in [Ca2+]i, in response to ACh, nicotine, and to pilocarpine challenges were 124 ± 30, 123 ± 28, and 20 ± 11 nM, respectively (n = 5). The effect of pilocarpine was significantly smaller than those of...
FIG. 2. A: perfusate was changed from normal Krebs to Krebs containing acetylcholine (ACh) as indicated by the horizontal bars. Dead space between the reservoir and the recording well was 2 ml, and the rate of perfusion was 2 ml/min. B: $\Delta[Ca^{2+}]$, was calculated as follows: $\Delta[Ca^{2+}] = \text{maximal}[Ca^{2+}] - \text{control}[Ca^{2+}]$. Control $[Ca^{2+}]$ was defined as the mean of value taken between 20 and 30 s from the start of experiments (14 data points). Maximal $[Ca^{2+}]$ was the mean value of 14 data points around the peak value. Each $\Delta[Ca^{2+}]$, value in response to ACh; $\bar{c}$, mean $\Delta[Ca^{2+}]$, values $\pm$SE. Mean values of $[Ca^{2+}]$, for 1, 10, and 100 $\mu$M ACh were statistically analyzed with analysis of variance (ANOVA), and they were significantly different from each other.

ACh and nicotine. In two clusters, pilocarpine did not have any effect, although ACh and nicotine clearly increased $[Ca^{2+}]$. The durations between the start of the perfusion of the cholinergic agents and the peak increases were 103 ± 14 s for ACh, 99 ± 9 s for nicotine, and 183 ± 31 s for pilocarpine, which was significantly longer than those of the other two agonists. In four other clusters the effects of ACh (100 $\mu$M) and muscarine (100 $\mu$M) were examined. The increase in $[Ca^{2+}]$ due to muscarine was significantly less, between 0 and 36% of ACh response (19 ± 9%; $n = 4$).

EFFECT OF MECAMYLAMINE. The effect of ACh (100 $\mu$M) was slightly attenuated by mecamylamine (100 $\mu$M), a nicotinic antagonist. In four experiments the increase in $[Ca^{2+}]$, in response to ACh without mecamylamine was 67 ± 20 nM. In response to ACh with mecamylamine $[Ca^{2+}]$, rose by 60 ± 18 nM over control. The effect of mecamylamine was consistently inhibitory, but statistically insignificant. Even 1 mM of mecamylamine did not completely block the effect of ACh.

SOURCE OF CALCIUM. Exposing the clusters to Ca$^{2+}$-free Krebs did not significantly change resting $[Ca^{2+}]$, until 2.5 min after the start of Ca$^{2+}$-free perfusion, but subsequently $[Ca^{2+}]$, rapidly decreased. The response to ACh was almost completely inhibited or significantly attenuated during Ca$^{2+}$-free superfusion (Fig. 4). Increases in $[Ca^{2+}]$, by ACh during control was 105 ± 38 nM ($n = 5$). No increase was observed in three clusters, and increases of 30 and 56 nM (68 and 29% of control, respectively) were observed in two clusters during Ca$^{2+}$-free Krebs perfusion. Prolonged superfusion of clusters with Ca$^{2+}$-free Krebs decreased basal levels of $[Ca^{2+}]$, and, at the end of 4 min perfusion of Ca$^{2+}$-free Krebs, $[Ca^{2+}]$, was 53 ± 16% of the preperfusion level. This decrease in $[Ca^{2+}]$, was restored by changing the perfusate from Ca$^{2+}$-free Krebs to normal Krebs (Fig. 4, beginning of the right panel).

Caffeine (5 mM) increased $[Ca^{2+}]$, in two of seven clusters. Continual administration of caffeine decreased the response of $[Ca^{2+}]$, to ACh (Fig. 5). In seven clusters, ACh did not significantly change resting $[Ca^{2+}]$, or Krebs containing ACh as indicated by the horizontal bars. Ca$^{2+}$-free Krebs did not significantly change resting $[Ca^{2+}]$, for the initial 90 s (2.5 min after the start of Ca$^{2+}$-free Krebs perfusion), but before administration of ACh $[Ca^{2+}]$, started decreasing. At this point the response to ACh was almost completely blocked (middle panel). When the perfusate was returned to normal Krebs, $[Ca^{2+}]$, returned to the control level, and ACh showed the same increase in $[Ca^{2+}]$, as seen at the 1st exposure to ACh. Responses were recorded from 1 cluster.

FIG. 3. Perfusate was changed from normal Krebs to Krebs containing ACh as indicated by the horizontal bars. Nicotine (100 $\mu$M) mimicked the effect of ACh (100 $\mu$M), but pilocarpine (100 $\mu$M) did not. Responses were recorded from 1 cluster.

FIG. 4. Perfusate was changed from normal Krebs to Ca$^{2+}$-free Krebs or Krebs containing ACh as indicated by the horizontal bars. Ca$^{2+}$-free Krebs did not significantly change resting $[Ca^{2+}]$, for the initial 90 s (2.5 min after the start of Ca$^{2+}$-free Krebs perfusion), but before administration of ACh $[Ca^{2+}]$, started decreasing. At this point the response to ACh was almost completely blocked (middle panel). When the perfusate was returned to normal Krebs, $[Ca^{2+}]$, returned to the control level, and ACh showed the same increase in $[Ca^{2+}]$, as seen at the 1st exposure to ACh. Responses were recorded from 1 cluster.

FIG. 5. Perfusate was changed from normal Krebs to Krebs containing caffeine or Krebs containing ACh as indicated by the horizontal bars. Caffeine partially blocked the increase in $[Ca^{2+}]$, in response to ACh (100 $\mu$M). Responses were recorded from 1 cluster.
Nifedipine reduced the ACh response to 67 ± 6% of control. The inhibitory effect of nifedipine on the ACh response was significantly less than that on the high K⁺ response. In some clusters high K⁺ Krebs failed to elicit [Ca²⁺] increase, suggesting a lack of functional voltage-gated calcium channels, while ACh continued to increase [Ca²⁺]. Figure 6C represents one of these clusters. The effect of nifedipine (10 μM) was tested in this cluster. The increases in [Ca²⁺] produced by ACh without or with nifedipine were 121 and 131 nM, respectively.

**DISCUSSION**

This study demonstrated that exogenously administered ACh increased [Ca²⁺], of cultured carotid body cells harvested from adult cats. The results indicate that these cultured cells possessed functional ACh receptors, and that the [Ca²⁺] response of these cells to ACh was likely mediated by the activation of neuronal nicotinic ACh receptors. First, in our preparation ACh over a range of 1–300 μM produced a concentration-dependent increase in [Ca²⁺], seeming to plateau at 300 μM. The EC₅₀ of ACh on neuronal nicotinic ACh receptors in the peripheral nervous system has been reported to be between 35 and 133 μM (McGhee and Role 1995; Role 1992). Second, desensitization of nicotinic receptors is a common feature (Montes et al. 1994; Role 1992), and in our study desensitization was clearly observed at the higher doses or prolonged exposures to ACh. Third, nicotine, but neither pilocarpine nor muscarine, mimicked the speed and magnitude of the ACh response, further suggesting that nicotinic receptors are critically involved in the [Ca²⁺] response to ACh in the cat carotid body cells.

We have studied clusters of cells rather than individual cells due to methodological reasons. First, the fluorescent signal from a single cell was too low to register any changes in [Ca²⁺], under our experimental conditions. Second, differentiating single glomus cells from single sheath cells based on simple morphology was extremely difficult. Third, the combining the microfluorometric methods of [Ca²⁺], with the immunocytochemical methods that would allow us to differentiate glomus cells from sheath cells was not practical, because the calibration procedure for [Ca²⁺], severely damaged the cells. In contrast, the fluorescent signal from clusters of cells was strong, and the complementary immunocytochemical study confirmed the presence of glomus cells in all clusters. Therefore glomus cells appear to be, at least partly, responsible for the [Ca²⁺], signal measured from clusters of cells. However, the important question remains as to whether ACh receptors are localized only on glomus cells. A previous electrophysiological study (Eyzaguirre and Monti-Bloch 1982) and receptor binding studies (Dinger et al. 1981, 1985; Hirano et al. 1992) have indicated the presence of nicotinic receptors on glomus cells in cats. We have previously shown with immunocytochemical techniques that α4 subunits of neuronal nicotinic ACh receptors are localized on cultured glomus cells (Ishizawa et al. 1997). Nevertheless, these studies do not exclude the possibility that sheath cells in culture express some types of ACh receptors. Recent studies have shown the presence of muscarinic receptors on glial cells (for a review see Brismar 1995). It is not clear whether nicotinic receptors are also localized on
mammalian glial cells. Because sheath cells are glialike cells, they may express some types of ACh receptors. Further, primary culture of the carotid body theoretically contains many other types of cells such as vascular smooth muscle cells and vascular endothelial cells, which may respond to ACh. However, under our culture conditions the majority of cells that remained in culture for more than 3 days are glomus cells, sheath cells, and microglia-like cells (Shirahata et al. 1994b). Microglia-like cells were easily distinguished from other cells by their spiny morphology, and most of them were washed away during initial superfusion. Taken together, [Ca\(^{2+}\)]

response to ACh very likely reflects the response of glomus cells. Contribution of sheath cells to the response needs further investigation and should not be dismissed at this point.

The expression of ACh receptors seems to vary among clusters. Some clusters responded only to ACh but not to high K\(^+\) (Fig. 6C); others responded only to high K\(^+\) (data not shown), and still others responded to both stimuli (Fig. 6, A and B), suggesting a nonuniform distribution of voltage-gated calcium channels and ACh receptors. It could be asked whether the heterogeneous distribution of these channels and receptors is inherent to the cat carotid body or due to the culture conditions. Several studies suggest that the former may be the case. Earlier morphological studies distinguished four types of glomus cells in cats, two types in rats, and three types in human (for reviews see Heath and Smith 1992; McDonald 1981). Some recent studies have addressed heterogeneous responses of rat or rabbit glomus cells to cyanide (Donnelly 1993) or to hypoxia (Bright et al. 1996; Pérez-García et al. 1992). Further, several types of neuronal nicotinic ACh receptors may coexist in the carotid body. To date, eight neuronal \(\alpha\) (\(\alpha2\)–9) and three neuronal \(\beta\) (\(\beta2\)–4) subunits have been encoded in rodent and chick. Colocalization of neuronal nicotinic receptors that have different subunit composition in a given neural structure is a common feature, and the calcium permeability of the nicotinic receptor depends on the composition of subunits (McGehee and Role 1995; Role 1992). In the cat carotid body, \(\alpha4\) subunits (Ishizawa et al. 1996) and possibly \(\alpha7\) subunit (\(\alpha\)-bungarotoxin binding sites) (Dinger et al. 1981, 1985; Hirano et al. 1992) have been identified, but the distribution of these subunits is not yet clear. Additionally, even if one assumes that only glomus cells respond to ACh, the difference in the number of glomus cells in the different clusters would create variability of [Ca\(^{2+}\)], changes among clusters.

Inward currents through nicotinic receptor channels, which were easily desensitized, have been recorded in rat glomus cells (Peers et al. 1994; Wyatt and Peers 1993). These currents were completely inhibited by mecamylamine. On the other hand, the effect of mecamylamine on the [Ca\(^{2+}\)], response to ACh was consistently in the inhibitory direction, but the value was not significantly different from control in our preparation. This result is puzzling. Based on the effects of nicotine, pilocarpine, and muscarine, it is hard to believe that a major component of the ACh effect is mediated by the muscarinic receptors. The effect of muscarinic agonists on [Ca\(^{2+}\)], response was slow and small or negligible. Muscarinic receptors may play a minor role, if any. A previous study supporting this view is that \(\alpha\)-bungarotoxin binding sites (one type of neuronal nicotinic ACh receptor) outnumber quinuclidinyl benzilate binding sites (muscarinic receptors) in the cat carotid body (Hirano et al. 1992). An alternative explanation for the poor effect of mecamylamine might be that nicotinic ACh receptors that are less sensitive to mecamylamine play a role in the [Ca\(^{2+}\)], response in the cat. In contrast, glomus cells of the rat might have more mecamylamine-sensitive nicotinic receptors. The composition of nicotinic receptor subunits determines the sensitivity to agonists and antagonists. Alkondon and Albuquerque (1993) have shown at least four types of ACh-activated currents in cultured rat hippocampal neurons, and structurally distinct nicotinic ACh receptors appeared to carry each current (Albuquerque et al. 1995). ACh receptors that seemed to bear an \(\alpha4\) or \(\alpha7\) subunit were less sensitive to mecamylamine in their preparation. Currently the \(\alpha\)-bungarotoxin binding site, possibly the \(\alpha7\) subunit, and the \(\alpha4\) subunit have been demonstrated in the cat carotid body. Perhaps nicotinic receptors with these subunits in the carotid body may be also less sensitive to mecamylamine.

A major fraction of calcium ions involved in the increase in [Ca\(^{2+}\)], of carotid body cells was extracellular in origin. The [Ca\(^{2+}\)], level was sustained during the superfusion of the Ca\(^{2+}\)-free Krebs for 90 s, then it started declining. At this point the [Ca\(^{2+}\)], response induced by ACh was greatly diminished or almost abolished. However, release of Ca\(^{2+}\) from intracellular storage sites cannot be totally discounted as a cause of the increase in [Ca\(^{2+}\)], by ACh, because a prolonged exposure to Ca\(^{2+}\)-free Krebs decreased [Ca\(^{2+}\)], possibly due to the depletion of Ca\(^{2+}\) from intracellular storage sites. The fact that caffeine reduced the increase in [Ca\(^{2+}\)], in response to ACh suggests some contribution from intracellular calcium. Previous studies suggested that caffeine was an agonist for the ryanodine receptor, but more recent studies have indicated that it acts on two classes of intracellular Ca\(^{2+}\)-release channels (for a review see Ehrlich et al. 1994). Further studies are necessary to elucidate the role of intracellular calcium channels in the ACh-evoked increase in [Ca\(^{2+}\)].

Another possible mechanism of the ACh-induced increase in [Ca\(^{2+}\)], is that ACh may depolarize glomus cells and activate voltage-gated calcium channels followed by an influx of calcium ions. Several investigators have shown that glomus cells of the cat, mouse, and rat were depolarized by ACh (Eyzaguirre and Monti-Bloch 1982; Peers et al. 1994; Wyatt and Peers 1993). This possibility was tested by using nifedipine, an L-type calcium channel blocker. The presence of the L-type of calcium channels has been shown in glomus cells of rabbits and rats. In our preparation ~50% of clusters responded to high-K\(^+\) Krebs by increasing [Ca\(^{2+}\)], suggesting the presence of voltage-gated calcium channels in these cells. The effect of high-K\(^+\) was greatly attenuated by 10 \(\mu\)M nifedipine, indicating the presence and contribution of L-type channels (Fig. 6A). The residual effect of high-K\(^+\) Krebs may be due to the activation of other types of voltage-gated calcium channels. The partial inhibition of the ACh response by nifedipine suggests the contribution of L-type calcium channels to the increase in [Ca\(^{2+}\)], by ACh. However, ACh can increase [Ca\(^{2+}\)], without the involvement of voltage-gated calcium channels. This was indicated by the fact that, whereas some clusters did not respond to high-K\(^+\), suggesting the absence or scantiness of functional volt-
age-gated calcium channels, the same clusters still responded to ACh (Fig. 6C). The fact that in one of these clusters nifedipine did not affect the ACh response further supports this possibility. It has been well recognized that neuronal nicotinic ACh receptors are highly permeable to Ca$^{2+}$. The permeability ratio of Ca$^{2+}$ to Na$^+$ ranges from 1 to 20, depending on the types of subunits composing the neuronal nicotinic ACh receptor. Among the ligand-gated ion channels, a7 subunit-containing ACh receptors (α-bungarotoxin binding sites) are the most Ca$^{2+}$ permeable (Albuquerque et al. 1995; McGehee and Role 1995; Patrick et al. 1991; Role 1992). And cat carotid body cells are reported having these receptors (Dinger et al. 1981, 1985; Hirano et al. 1992).

These nicotinic ACh receptors may play a role in the release of neurotransmitters from glomus cells. Recent findings support the physiological significance of presynaptic nicotinic ACh receptors in modifying the release of catecholamines, γ-aminobutyric acid, serotonin, glutamate, and ACh in other neural tissues (Albuquerque et al. 1995; McGehee and Role 1995; Albuquerque et al. 1995). Several investigators have shown that administration of nicotinic agonists increases the release of catecholamine from the cat and rabbit carotid body (Dinger et al. 1985; Gomez-Niño 1993; Obeso et al. 1987). The ACh-nicotinic receptor interaction on the glomus cells may influence the further release of ACh as well as other neurotransmitters present in the carotid body. Further, electrophysiological and immunocytochemical studies are necessary to identify and localize the subtypes of nicotinic ACh receptors on glomus cells and/or sheath cells before the role of these cholinergic receptors can be completely and accurately appreciated.

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REFERENCES


