Cutaneous blood flow and sweat rate responses to exogenous administration of acetylcholine and methacholine

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METHODS

Twelve healthy subjects (6 men and 6 women) participated in protocol 1; their age, height, and weight (mean ± SE) were as follows: 36 ± 2 yr, 176 ± 4 cm, and 79 ± 6 kg. Seven healthy subjects (5 men and 2 women) participated in protocol 2; their age,

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height, and weight were as follows: 34 ± 2 yr, 171 ± 4 cm, and 61 ± 3 kg. Each subject was informed of the purpose and risks of the study before providing their written consent. The Institutional Review Boards of the University of Texas Southwestern Medical Center at Dallas and Presbyterian Hospital of Dallas approved all protocols and informed consents.

**Instrumentation and Measurements**

On entering the laboratory, each subject rested in the supine position during placement of four intradermal microdialysis probes in the dermal space of the dorsal forearm. Each probe was separated by 2.5 cm. The membrane window for each probe was 10 mm. For placement of the probes, a 25-gauge needle pierced the dermal space without local anesthetic and exited 20–25 mm from the point of entry. The microdialysis probe was threaded through the lumen of the needle. The needle was then removed, leaving the probe in place. The depth of probe placement was not identified, although a depth of 0.3–1.0 mm was reported in a prior study using similar procedures (17). After membrane placement, the probes were perfused with lactated Ringer solution at 2 μl/min via an infusion pump (Pump 11, Harvard Apparatus, Natick, MA). Chambers with a small (10 × 5 mm, i.e., 0.5-cm² surface area) window were positioned over each membrane to measure SR via capacitance hygrometry (Viasala, Woburn, WA) using compressed nitrogen gas at a flow of 300 ml/min. Absolute humidity over each probe was converted to SR from gas parameters (17). After membrane placement, the probes were perfused with lactated Ringer solution at 2 μl/min via an infusion pump (Pump 11, Harvard Apparatus, Natick, MA). Chambers with a small (10 × 5 mm, i.e., 0.5-cm² surface area) window were positioned over each membrane to measure SR via capacitance hygrometry (Viasala, Woburn, WA) using compressed nitrogen gas at a flow of 300 ml/min. Absolute humidity over each probe was converted to SR from gas parameters (17).

**Protocol 1**

The primary purpose of protocol 1 was to compare local SR and CVC responses to exogenous ACh and MCh administration. A secondary goal was to examine intersite variations in SR and CVC for each drug. After microdialysis membrane placement and ≥90 min for the hyperemic response associated with membrane placement to subside, baseline SR and CVC data were collected for 5 min. Subsequently, two membranes were perfused with increasing doses of ACh (1 × 10⁻⁷–1 M; 8 doses at 10-fold increments) dissolved in Ringer solution, while the other two membranes were perfused with the same concentrations of MCh, with each dose being administered for 7 min at a perfusion rate of 2 μl/min. ACh and MCh were administered to randomly assigned sites. After the last dose of the drug, 50 mM sodium nitroprusside (SNP) was administered for 10 min at all sites. This duration of SNP administration is adequate to maximally dilate the skin when sites previously received high doses of vasodilating drugs such as ACh or MCh. CVC was normalized relative to each site’s maximal CVC.

**Protocol 2**

Protocol 2 was performed on a subset of subjects to investigate the effects of AChE on local SR and CVC responsiveness to ACh and MCh administration. Four microdialysis membranes were placed in forearm skin as described for protocol 1. One site was perfused with increasing concentrations of ACh (1 × 10⁻⁷–1 M; 8 doses at 10-fold increments), a second site was perfused with the same concentrations of MCh, a third site was perfused with the same concentrations of ACh + 10 μM neostigmine (an AChE inhibitor), and a fourth site was perfused with the same concentrations of MCh + 10 μM neostigmine. The dose of neostigmine was based on prior studies in which 10 μM was demonstrated to effectively inhibit AChE (34–36). Similar to protocol 1, each dose of drug was administered for 7 min at a perfusion rate of 2 μl/min. After the last dose, 50 mM SNP was administered for 10 min to maximally vasodilate the assessed areas. Sites were randomly selected to receive ACh, MCh, ACh + neostigmine, and MCh + neostigmine.

**Data Collection and Statistical Analysis**

Data were continuously obtained and digitized at a sampling rate of 50 Hz (Biopac System, Santa Barbara, CA). For each dose, SR and skin blood flow were averaged at 10-s intervals, and the 60-s average around the maximum 10-s value from each stage was selected for analysis. This 60-s period typically occurred during the last 1–2 min of the perfused dose. Dose-response curves for CVC and SR at each of the four sites in both protocols were constructed using a nonlinear fitting technique, with a Hill slope of 1, from which the effective concentration causing 50% of the maximal response (EC₅₀) was identified (Prism, Graphpad Software). For the intersite analysis for protocol 1 (i.e., evaluation of responses between ACh sites and between MCh sites), SR and CVC between the proximal and distal sites receiving ACh, as well as between the proximal and distal sites receiving MCh, were statistically analyzed across doses of drug via a repeated-measures two-way ANOVA. In addition, the EC₅₀ values of the proximal and distal sites for each drug were compared using paired t-tests. For the between-drug analysis (i.e., ACh responses compared with MCh responses), SR and CVC for the sites receiving the same drug were first averaged for each dose. These averaged responses were then modeled via the nonlinear fitting technique, and differences in responses between drugs across the dose range were compared via a repeated-measures two-way ANOVA. Moreover, differences in the EC₅₀ between ACh and MCh dose-response curves were compared using paired t-tests. Because of a technical error, SR was not measured in two subjects for protocol 1. Thus, for protocol 1, SR is reported from 10 subjects and CVC is reported from 12 subjects. For protocol 2, the EC₅₀ values for SR and CVC to ACh and MCh at neostigmine-treated and untreated sites were compared using a repeated-measures one-way ANOVA. Differences in responses between ACh and MCh at the neostigmine-treated and untreated sites across the dose range were compared via a repeated-measures two-way ANOVA. Values are means ± SE. The level of statistical significance was set at P < 0.05.

**RESULTS**

**Protocol 1**

**Within-drug comparisons.** The EC₅₀ for SR and CVC dose-response curves showed no significant difference between proximal and distal sites for ACh or MCh (P > 0.05; Figs. 1 and 2). A linear relation for SR existed between the two ACh sites (slope = 1.29 ± 0.14, R = 0.96 ± 0.01) and the two MCh sites (slope = 0.68 ± 0.09, R = 0.96 ± 0.01). Consistent with this observation, a linear relation for CVC existed between the two ACh sites (slope = 0.96 ± 0.05, R = 0.92 ± 0.02) and the two MCh sites (slope = 0.84 ± 0.05, R = 0.94 ± 0.01). There was no significant difference in CVC between sites receiving ACh or MCh (i.e., main effect of site P > 0.05; Fig. 2). However, for SR, there was a significant interaction between site and dose for ACh and MCh trials (P < 0.05; Fig. 1).

**Between-drug comparisons.** SR was significantly greater at the MCh than at the ACh sites during administration of moderate...
doses of these drugs (1 × 10^{-4}–1 × 10^{-2} M; Fig. 3). The EC_{50} for SR was significantly higher at the ACh sites (-1.50 ± 0.16 log-molar concentration of ACh) than at the MCh sites (-2.46 ± 0.21 log-molar concentration of MCh, P = 0.007). Moreover, the concentration of the drug at the onset of sweating was significantly lower for MCh (-4.86 ± 0.29 log-molar concentration of MCh) than for ACh (-2.38 ± 0.25 log-molar concentration of ACh, P < 0.001; Fig. 3). CVC was significantly greater at the MCh sites at the ACh sites during administration of moderate doses of these drugs (1 × 10^{-5}–1 × 10^{-3} M; Fig. 4). The EC_{50} was significantly higher for the ACh sites (-3.68 ± 0.17 log-molar concentration of ACh, P = 0.002) than for the MCh sites (-4.56 ± 0.12 log-molar concentrations of MCh).

Protocol 2

Administration of ACh and MCh increased SR at neostigmine-treated and untreated sites. Neostigmine at the ACh site induced a leftward shift of the SR dose-response curve compared with the non-neostigmine-treated site, as evidenced by a significant difference in the EC_{50} between sites (-2.18 ± 0.39 and -0.87 ± 0.22 log-molar ACh concentration for neostigmine-treated and untreated sites, respectively, P = 0.002). The EC_{50} for SR to various doses of MCh was unaffected by neostigmine in the perfusion solution (-2.48 ± 0.26 and -2.36 ± 0.16 log-molar concentration of MCh for neostigmine-treated and untreated sites, respectively, P > 0.05). There was no significant difference in the EC_{50} between the MCh (-2.36 ± 0.16 log-molar concentration) and ACh sites when neostigmine was included at the ACh site (-2.18 ± 0.39 log-molar concentration, P > 0.05). At the neostigmine-treated sites, ACh and MCh induced sweating at the lowest concentrations of drug (1 × 10^{-7} M), while sweating did not occur at the non-neostigmine-treated sites until a higher concentration of ACh was administered.

Fig. 1. Sweat rate (SR) dose-response curves to exogenous administration of 10^{-7}–1 M ACh (A) and methacholine (MCh, B) at proximal and distal sites. EC_{50} values were not significantly different between proximal and distal sites treated with the same drug. *Significant difference between sites at indicated dose (P < 0.05).

Fig. 2. Cutaneous vascular conductance (CVC) dose-response curves to exogenous administration of 10^{-7}–1 M ACh (A) and methacholine (MCh, B) at proximal and distal sites. EC_{50} values were not significantly different between proximal and distal sites treated with the same drug.
DISCUSSION

The primary aim of this study was to compare local SR and CVC responses to exogenous ACh and MCh administration when these agents were administered via intradermal microdialysis. The main finding of the study is that SR and CVC responses to ACh were significantly less sensitive, as indexed by the EC50 of the dose-response curve, than SR and CVC responses to MCh. Furthermore, the onset of sweating occurred at a lower dose of MCh than ACh. On the basis of these findings, protocol 2 was included to evaluate whether the observed differences between ACh and MCh responses could be attributed to different degrees of sensitivity of ACh and MCh to AChE. Coadministration of an AChE inhibitor caused a significant leftward shift of the ACh dose-response curve for CVC and SR, demonstrating that the lower EC50 for SR and CVC to MCh than to ACh is likely due to the susceptibility of ACh to hydrolysis by AChE. Finally, the intersite differences (i.e., within-drug comparison) for CVC were similar when the EC50 to various doses of ACh, as well as MCh, was evaluated. However, SR between sites receiving the same drug were significantly different at higher doses of ACh and MCh.

Sweating Responses

As internal and skin temperatures increase, sweating occurs on the release of ACh from cholinergic nerves and subsequent binding to muscarinic receptors on the eccrine sweat gland (28, 29, 37). ACh is rapidly hydrolyzed to choline and acetate by AChE. Previous studies investigating the effects of AChE inhibition on SR have shown that AChE inhibition increases the number of activated sweat glands and that AChE tends to increase SR (23, 24). Consistent with previous findings, our laboratory has shown that inhibition of AChE with coadministration of neostigmine caused a leftward shift of the dose-response curve to exogenous ACh (34). Together, these findings suggest that SR responsiveness to ACh can be affected by the activity of AChE. A key objective of protocol 1 was to evaluate differences in responses between ACh and an analog of ACh, MCh, which is not as rapidly degraded by AChE (9, 22). The EC50 of the sweating response was significantly lower with administration of MCh than with administration of ACh (Fig. 3). In addition, the onset of sweating occurred at a lower concentration of MCh than ACh. At higher concentrations of these drugs (i.e., 1 × 10⁻¹⁻¹ M), sweating responses between ACh and MCh became similar. This observation suggests that the effect of hydrolysis of ACh on sweating responses can be overcome as the exogenous dose of ACh approaches 1 × 10⁻¹⁻¹ M. When AChE activity was inhibited with coadministration of neostigmine, sweating responsiveness to ACh (EC50 and threshold for the onset of sweating) was similar to that observed during MCh administration (Fig. 5). At higher concentrations of ACh (i.e., approaching 1 M), sweating responses were unaffected by AChE inhibition, consistent with the aforementioned observation of similar sweating responses at higher doses of ACh and MCh in the absence of neostigmine. Coadministration of neostigmine with MCh did not significantly alter the dose-response curve relative to administration of MCh alone. Together, these observations strongly suggest that differences in sweating responses between exogenous ACh and MCh are due to the effect of AChE on hydrolysis of ACh.
of these drugs. A number of variables could result in different responses to the same drug at different sites, including heterogeneity of skin blood flow, sweat gland density, and the potential for differences in characteristics of each microdialysis membrane (6–8, 15). Thus a secondary goal of this study was to examine intersite variability of ACh- and MCh-induced increases in SR and CVC. This objective was accomplished by comparing dose responses to identical drug administration at proximal and distal sites. Although CVC was not significantly different between sites receiving the same drug, SR was significantly different between sites at higher doses for ACh and MCh (Fig. 1). These data were further analyzed by evaluation of the linear regression between the two sites receiving the same drug. Although the R values for these analyses were very high (i.e., averaging >0.92), the slope of the relation between the proximal and distal sites for SR averaged 0.68 ± 0.09 when MCh was administered and 1.29 ± 0.14 when ACh was administered, whereas the slope of the relation between sites for CVC averaged 0.84 ± 0.06 when MCh was administered and 0.96 ± 0.05 when ACh was administered. These observations raise a concern regarding the intersite variability when cutaneous vascular and sweating responses to exogenous ACh and MCh administration are evaluated. Unfortunately, we do not have a clear explanation for these varying responses, despite what may appear to be otherwise reproducible findings. Nevertheless, these observations raise concern about the appropriateness of comparing CVC and SR responses between sites, unless the expected difference is greater than the observed variability between sites.

In conclusion, the present data demonstrate attenuation of sudomotor and cutaneous vasodilator responses to exogenous ACh compared with MCh administration. These different responses are likely due to the responsiveness of AChE in hydrolysis of ACh, whereas MCh is more resistant to AChE hydrolysis. Perhaps because of its accessibility, a number of investigators are using the skin to evaluate the effects of various pathologies on vascular and sudomotor responsiveness (1–4, 11, 19–21, 30). Commonly, endothelium-dependent vasodilation and sudomotor responses are assessed through

**Cutaneous Vasodilator Responses**

Increasing concentrations of exogenous ACh and MCh resulted in progressive increases in CVC. The mechanism by which ACh induces cutaneous vasodilation has recently been investigated. It is generally agreed that ACh mediates increases in CVC through a combination of nitric oxide, prostaglandins, and possibly endothelium-derived hyperpolarizing factor mechanisms (5, 12, 14, 16–18, 26, 27, 31, 33). In the present study, it is assumed that one or all of these mechanisms were responsible for the elevation in CVC in response to ACh and MCh. At moderate doses of MCh (1 × 10⁻⁵–1 × 10⁻⁷ M), the increase in CVC was significantly greater than that observed in response to ACh administration (Fig. 4), resulting in a significant reduction in the EC₅₀ of MCh relative to ACh. As outlined above, these differences are proposed to be due to differences in AChE susceptibility between these agents. In support of this hypothesis, when neostigmine was added to the ACh solution, the EC₅₀ values of CVC dose-response curves to ACh were not different from those to MCh, whereas coadministration of neostigmine with MCh did not significantly alter the EC₅₀ of the MCh site (Fig. 6). Given these observations, differences in CVC between ACh and MCh in the absence of neostigmine were not due to differences in the ability of these drugs to evoke nitric oxide-, prostaglandin-, and endothelium-derived hyperpolarizing factor-stimulated vasodilation. Rather, different CVC responses of these drugs were likely due to greater sensitivity of ACh to AChE. Similar to its effect on sweating, CVC responses were unaffected by AChE inhibition at higher concentrations of ACh, suggesting that the dose of ACh can overwhelm the capacity of AChE to hydrolyze ACh.

**Intersite Variability**

Despite the widespread use of exogenous ACh and MCh administration in evaluating cutaneous vascular and sudomotor responses, little is known regarding the intersite variability of this procedure, particularly during microdialysis administration

**Fig. 5.** Effect of inhibition of acetylcholinesterase (AChE) on SR dose-response curves to exogenous administration of ACh and MCh. Inhibition of AChE shifted ACh dose-response curve to the left compared with control site, such that EC₅₀ was significantly greater for ACh than for ACh + neostigmine (Neo), MCh, or MCh + neostigmine. †Significant difference between EC₅₀ values (P < 0.05).

**Fig. 6.** Effect of inhibition of AChE on CVC dose-response curves to exogenous administration of ACh and MCh. Inhibition of AChE shifted ACh dose-response curve to the left compared with control site, such that EC₅₀ was significantly greater for ACh than for ACh + neostigmine, MCh, or MCh + neostigmine. †Significant difference between EC₅₀ values (P < 0.05).
exogenous administration of ACh via intradermal injection, iontophoresis, and intradermal microdialysis. However, given the effects of AChE in inactivation of ACh, findings from these studies may be clouded by the possibility that different responses could be attributed to the effect of that pathology in alteration of AChE-mediated hydrolysis of ACh, as opposed to a direct effect of that pathology on endothelium-dependent vasodilation and sudomotor responsiveness. To overcome this potential limitation, data from the present investigation suggest that MCh may be preferred over ACh for evaluation of sudomotor and endothelium-dependent cutaneous vasodilator responses.

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GRANTS

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