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Time Course of Pharmacodynamic and Pharmacokinetic Effects of Physostigmine Assessed by Functional Brain Imaging in Humans

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Physostigmine Pharmacodynamics Pharmacokinetics rCBF PET Cognition Working memory Brain Humans

TO evaluate the effects of psychopharmacological compounds in humans, most studies have relied on clinical or cognitive measures or on measures in peripheral models of physiological effects (e.g., effects on enzymatic activities in platelets). The development of sophisticated brain imaging methods, including positron emission tomography (PET) and functional magnetic resonance imaging (fMRI), has made it possible to investigate the neurometabolic correlates of behavior and cognition in vivo in humans. By combining functional imaging studies with the pharmacological modulation of selective neurotransmitter systems, we can now determine in vivo how psychotropic agents alter brain function to result in changes in cognition.

Pharmacokinetic and pharmacodynamic responses to a pharmacologic agent can vary over time, and may not stabilize together. Pharmacokinetics refer to the time-course plasma concentration measures of a drug, and pharmacody-

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namics refer to the time-dependent pharmacologic action of a drug, which can include peripheral and central effects. Furthermore, the central effects of a drug are not necessarily correlated with stable plasma drug concentration or with peripheral measures of pharmacodynamics. An extreme example of this phenomenon is the delayed clinical response to tricyclic antidepressant medications, where plasma concentrations remain stable up to several weeks before clinical effects are evident (4,18). Before studying the effects of pharmacological manipulation on the brain response to cognitive or behavioral paradigms, particularly when multiple brain scans are obtained under experimentally varied cognitive conditions, it is important to establish the time point that plasma drug levels and variables that reflect the pharmacodynamic effects of the drug become stable. This information helps to ensure that any difference observed between sequentially obtained brain images is due to changes in the experimentally controlled conditions (e.g., different cognitive task conditions), and not due to underlying changes in the effect of drug.

Working memory (WM) refers to a process that maintains a temporary, active representation of information so that it is available for further processing or recall (2,3). Physostigmine, an acetylcholinesterase inhibitor that reduces the rate of breakdown of acetylcholine at the synaptic junction and thereby prolongs its action on postsynaptic cholinergic receptors, is known to improve performance on WM tasks in animals (6,16,24) and in patients in Alzheimer disease (17,20,22). In a previous study using PET in conjunction with physostigmine (9), we demonstrated that performance on a WM for faces task improved with physostigmine and that this improvement correlated with specific changes in regional cerebral blood flow (rCBF) in a task-related brain area in the right prefrontal cortex. Thus, we showed that the acute administration of physostigmine alters both performance and brain response to the WM task, but the time course of these responses has yet to be determined.

In this report, we sought to identify the time points at which plasma physostigmine concentration, plasma pharmacodynamics (as indicated by measures of butyrylcholinesterase inhibition), the central effects of physostigmine on the rCBF response in right prefrontal cortex, and the effects of physostigmine on task performance become stable. We also wanted to evaluate the relations among pharmacokinetic and pharmacodynamic measures by testing the correlations among the above variables once they became stable. To examine the time course of these drug effects, we have reanalyzed the data from our previous report (9) breaking down the behavioral, pharmacokinetic, and pharmacodynamic measures by time point, and found that all measures achieved a stable plateau after 40 min of infusion. The results demonstrate that our experimental approach can reliably evaluate the effects of pharmacological agents on the brain functional response to cognitive and behavioral probes, and highlight the importance of ensuring that pharmacological responses are stable prior to obtaining cognitive and neural measures.

METHOD

Subjects

Twenty-one healthy, nonsmoking right-handed subjects participated in the study. Thirteen subjects comprised the physostigmine group (mean age ± SD = 48 ± 20 years; five females, eight males) and eight the control group (37 ± 15 years; six females, two males). Groups did not differ significantly in mean age, years of education, or gender distribution ($p < 0.05$). All participants gave written informed consent after the purpose of the study and potential side effects of the drug were fully explained. Five additional subjects recruited for the study were excluded from the data analyses—one due to excessive anxiety during the PET scan and four because they did not complete the entire sequence of 10 PET scans. The study was approved by the National Institute on Aging Institutional Review Board (NIH protocol 94-AG-89) (9).

Subjects were screened for diseases that might alter brain function. They received physical, neurological, and psychiatric evaluations; blood tests, including routing blood counts and clotting studies, serum chemistry; liver, renal, and thyroid function tests; cholesterol, triglycerides; HIV and venereal disease research laboratory test (VDRL); antinuclear antibody and rheumatoid factor; vitamins B12 and folate, chest X-ray, EKG, EEG, brain MRI, and audiological and visual assessments. History of medical, neurological, or psychiatric illness, such as hypertension, diabetes mellitus, malignancy, renal, cardiac or hepatic disease, epilepsy, stroke, transient ischemic attack, head trauma with loss of consciousness, exposure to toxic substances, psychosis, substance abuse, radiological evidence of intracranial pathology, including lacunes, were exclusionary criteria (19).

Experimental PET Design

A series of 10 PET scans that alternated between rest and task conditions was obtained for each subject, resulting in five sequential sets of scan pairs. The first scan pair was collected during a control infusion of saline for subjects in both groups. Subsequent scans for the control group also were obtained during a saline infusion, whereas subsequent scans in the drug group were obtained during physostigmine infusion that began immediately after completion of scan two and continued to the end of the study. A schematic representation of the design is presented in Fig. 1.

Rest Condition

rCBF was measured while subjects laid on the PET scanner bed in a quiet and dimly lit room with their ears unplugged and eyes open, and were instructed to remain awake.

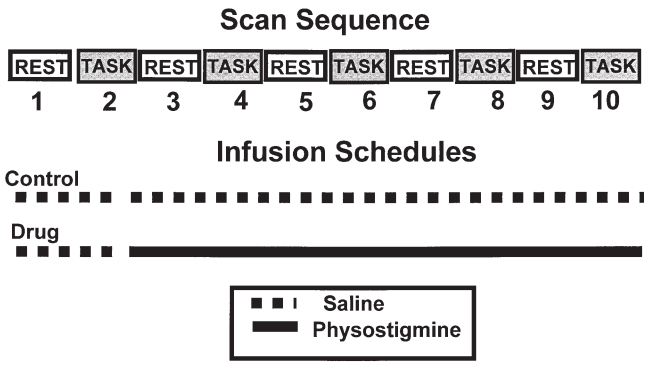

FIG. 1. The experimental design is summarized. All subjects received the 10 PET scan sequence shown, which alternated between rest and task conditions. The infusion schedules used for the two groups are summarized below the scan sequence. The subjects in the control group received a placebo infusion of saline (hatched line) throughout the 10-scan sequence; subjects in the drug group received a saline (hatched line) infusion during the first two scans, followed by a physostigmine (solid line) infusion that continued to completion of the study.

Working Memory for Faces Task

rCBF was measured while subjects performed a delayed match-to-sample test of face recognition, using a two alternative, forced-choice format (9,13). Stimuli were presented in three squares of equal size, one centered above two that were positioned side by side. Each item began with a 4-s presentation of a face to remember in the upper square, followed by a 6-s delay that consisted of a 1-s interstimulus interval, a 4-s presentation of the three square stimulus array each containing a gray square, and another 1-s interstimulus interval. After the delay, two test faces were presented for 4 s, one in each of the lower two squares, and the subjects indicated which test face matched the face presented at the beginning of the item by pressing a hand-held button with the right or left thumb. Subjects were instructed to attend carefully to the target face, so that they could recognize it after a delay. The correct choice face was randomly placed in the left or right square for the test item, so that the percent of correct responses was equally distributed between the right and left sides. Size and luminance levels of the visual displays were equivalent for all trials. Faces were of unknown individuals and were novel for each trial. Five such prepared task sets were assigned randomly to each of the five task scans for each subject. An example of a task memory item is presented in Fig. 2.

Prior to participation in the study, subjects were administered a working memory for faces pretest using the above described task design, to familiarize them with the task so as to obtain adequate performance during the PET study.

The task was controlled using a Macintosh IIx computer interfaced to an Apple Vision monitor that was positioned approximately 60 cm from the subjects eyes. SuperLab software (14) was used to present the stimuli and record subject responses including reaction time, made by pressing hand-held response buttons that were interfaced to the computer with a National Instruments card (NB-DIO-24).

Drug Administration

Physostigmine was administered intravenously at an average rate of 1 mg/h according to the following procedure: A loading dose rate of 1.93 mg/h for 10 min (0.322 mg) was used to rapidly achieve our target plasma level, then followed by a maintenance rate of 0.816 mg/h designed to maintain constant blood levels until the completion of the study. The rates of administration were based on pharmacokinetic data previously generated in our laboratory (1). Further, the total dose of 1 mg/h was selected based on indications that this dose has performance and rCBF effects in the absence of reported side effects (21,10,12).

To reduce the potential for peripheral side effects, such as changes in heart rate, nausea or emesis, 0.2 mg of the peripheral cholinergic antagonist glycopyrrolate was administered intravenously prior to physostigmine infusion. Heart rate, blood pressure, and blood oxygenation were monitored throughout the PET scan examination.

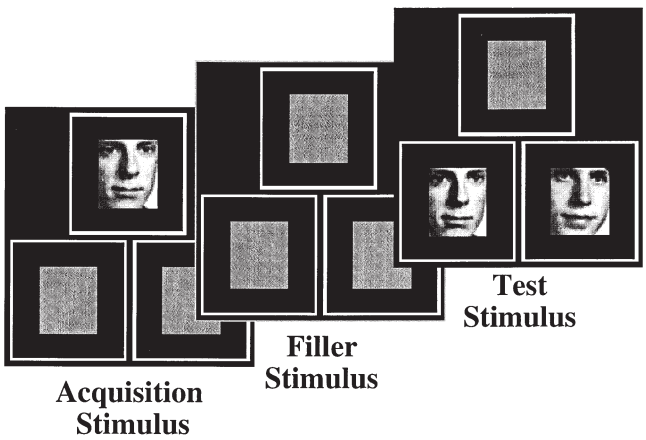

FIG. 2. Design of items for the working memory task. Each memory item began with a picture of an acquisition stimulus (face), followed by a 6-s delay period during which filler stimuli were presented, and by a test stimulus that included two faces, one of which matched the acquisition face. Subjects were instructed to indicate the matching face by pressing hand-held response buttons.

HPLC Assay of Plasma Physostigmine

In association with each PET scan, blood samples were collected in tubes spiked with pyridostigmine. Plasma (0.5 ml) was mixed with 250 μl phosphate buffer (0.1 M, pH 8.0) and *N*-methlyphysostigmine (Research Biochemicals International, Natick, MA), an internal standard. Analytes were extracted by a solid-phase extraction method using a cyanopropyl column (15), concentrated and reconstituted in HPLC mobile phase. After centrifugation (2000 × *g*), the supernatant was injected into HPLC. The components were resolved on a reversed-phase column (Basic, 2 × 250 mm, YMC Inc., Wilmington, NC) with a mobile phase consisting of 0.02 M acetic acid and acetonitrile (84:16;v:v) at 200 μl/min flow rate. Physostigmine and the internal standard were detected fluorometrically (1), and physostigmine concentrations were determined from the standard curve.

Plasma Butyrylcholinesterase Assay

A further fraction of each blood sample was placed in a heparinized tube, centrifuged, the plasma removed and frozen to −70°C for analysis of percent butyrylcholinesterase inhibition utilizing an assay described previously (1). These measures were obtained for 9 of the 13 subjects in the physostigmine group.

PET Procedure

All subjects fasted for at least 2 h and refrained from alcohol and caffeine for at least 24 h prior to PET scanning. Scans were obtained for each subject using a Scanditronix PC2048-15B tomograph (Uppsala, Sweden), with a reconstructed resolution of 6.5 mm in the transverse and axial planes (7). Fifteen cross-sectional planes parallel to the infero orbitomeatal line and separated by 6.5 mm, center to center, were obtained simultaneously, for a total axial field of view of 97.5 mm. Catheters were placed in a radial artery for drawing arterial blood samples and in antecubital veins of both arms for injection of the isotope and for drug and/or saline infusion. A thermoplastic mask was used to minimize head movements during the scans. A multislice transmission scan, obtained for each subject at the same level as the emission scans, was used to correct images for attenuation of radiation by skull and tissue (9).

A 10-ml bolus of 30 mCi $H_2{}^{15}O$ was injected intravenously for each PET scan. For task scans, subjects began the task 1 min prior to injection and continued to completion of the scan. Injections were separated by 10 min except for the injection between scans two and three, which were separated by 15

min to allow time for infusion of the physostigmine loading dose. Arterial blood radioactivity was determined continuously using an automatic blood counter. Scan data acquired during the 4-min scan period, together with the arterial time–activity curve, were used for image reconstruction using the rapid least squares algorithm (5) to give values for rCBF in ml/100g of brain tissue/min.

Image Processing

Scans were interpolated from 15 to 43 slices creating voxel dimensions of 2.0 × 2.0 × 4.0 mm. Scans were registered and then transformed into the Talairach stereotaxic atlas space (23) using Statistical Parametric Mapping (SPM: 8). After spatial normalization, scans were smoothed using a gaussian filter with a full width at half maximum of 20.0 × 20.0 × 12.0 mm. The smoothed images were ANCOVA adjusted and scaled to the mean global CBF of the first scan of all subjects within the group.

Statistical Procedures

ANOVA, repeated-measures ANOVA, and paired and unpaired *t*-tests were used where appropriate to assess within and between group differences in physostigmine plasma concentration, butyrylcholinesterase inhibition, reaction time, and mean values of rCBF.

Image Analyses

Voxel-by-voxel analyses were performed using SPM (8). Rest conditions were subtracted from task conditions to identify task activation patterns. Scan pairs 2 through 5 were analyzed to identify brain regions that were activated during task performance in the absence (control group) and in the presence (physostigmine group) of drug and to determine how these brain regions show a drug-dependent change in activation. The activation patterns of the two groups were compared directly, and within-group comparisons between scan pair one and scan pairs 2–5 also were performed to identify regions significantly altered by drug. Parametric maps of the *t*-statistic were transformed to *Z*-scores and thresholded at 2.33; statistical significance ($p < 0.05$) was determined based on an analysis of spatial extent (8).

RESULTS

In a repeated-measures ANOVA, mean plasma physostigmine concentration was found to differ among measures, $F(7, 84) = 9.89$, $p < 0.0001$. Individual comparisons using *t*-tests showed that measures obtained at 10 min during physostigmine infusions were significantly lower than all measures between 30 and 80 min of infusion; the mean plasma physostigmine level at 20 min was lower than those from 40 to 80 min; and the mean physostigmine level at 30 min was lower than those from 50 to 80 min (Fig. 3). No significant difference in physostigmine plasma concentration was observed among measures obtained from 40 to 80 min of drug infusion.

Mean plasma butyrylcholinesterase inhibition, when evaluated in a repeated-measures ANOVA, was found to differ among the sequentially obtained observations, $F(1, 8) = 11.18$, $p = 0.0001$. Individual comparisons using *t*-tests indicated that inhibition measures obtained at 10 min of physostigmine infusion were significantly lower than those obtained from 40 to 80 min; the measures obtained at 20 min were significantly lower than those obtained from 30 to 80 min; and those obtained at 30 min were lower than measures from 40 to 80 min (Fig. 4). No significant difference was observed among measures obtained from 40 to 80 min.

The median reaction time (RT) was determined in association with each task scan for each subject. The group mean RT

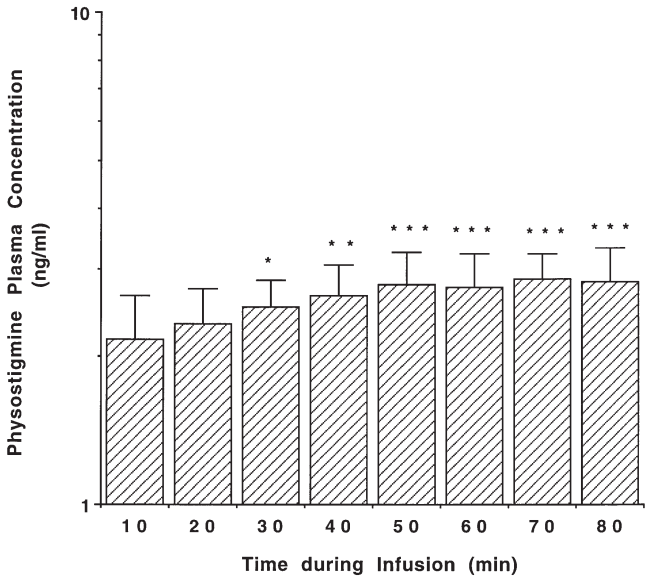

FIG. 3. Bars represent the group mean physostigmine plasma concentration (ng/ml) for each of the eight scans acquired during drug infusion. Error bars indicate standard deviation. *Significantly different from the measure at 10 min; **significantly different from the measures at 10 and 20 min; ***significantly different from the measures at 10, 20, and 30 min.

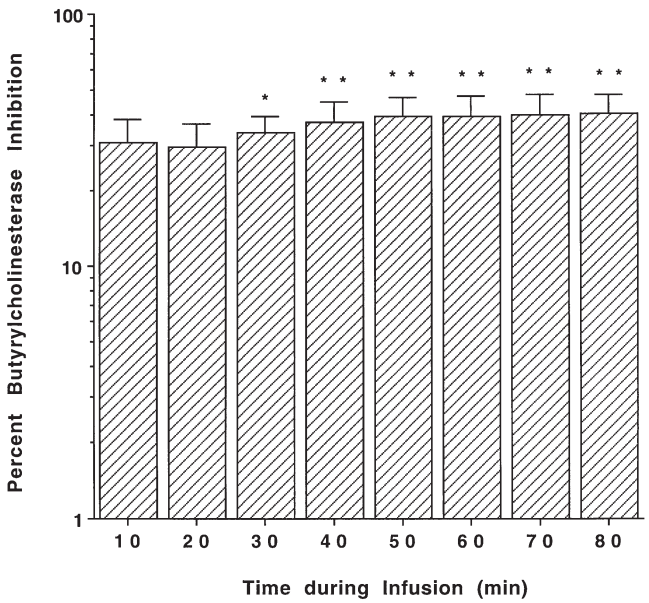

FIG. 4. Bars represent group mean percent butyrylcholinesterase inhibition for each of the eight scans acquired during infusion of physostigmine. Error bars reflect standard deviations. *Significantly different from measures at 20 min; **significantly different from measures at 10, 20, and 30 min.

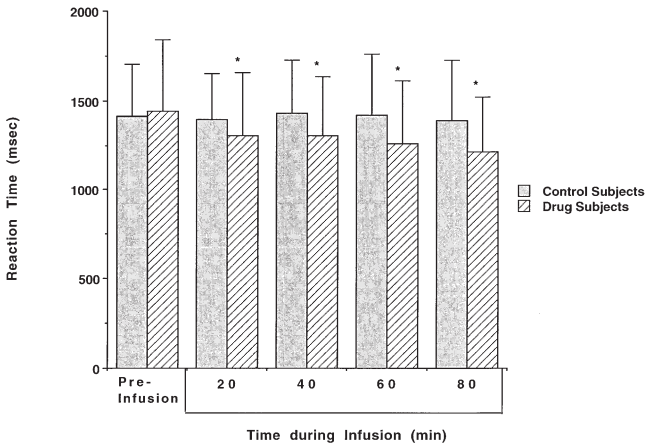

FIG. 5. Bars represent group mean reaction times (RT) for control subjects (filled bars) and for the physostigmine group (hatched bars). RT prior to drug is indicated by the pre-infusion bars and those obtained during drug, along with the comparable measures in the control group, are shown in association with time during infusion. Error bars represent standard deviation. *Significantly different from the pre-infusion measure (within group).

was calculated from individual median scores. Using repeated measure ANOVA, the group mean RT differed across scans in the physostigmine group, $F(4, 48) = 6.06$, $p = 0.0005$, but not in the control group, $F(4, 28) = 0.211$, NS. Within-group comparisons using paired t-tests indicated that all measures of mean RT obtained during physostigmine infusion were significantly lower than mean RT prior to drug (Fig. 5), and that none of these measures of mean RT during drug differed among themselves.

Figure 6 shows a map of voxels with Z-scores that exceeded a threshold of 2.33 ($p = 0.01$) from the within-group analysis between the saline scan pair and the scan pairs obtained during physostigmine. The result shows that the right prefrontal cortex had significantly larger rCBF increases during task performance in the absence of physostigmine as compared to during physostigmine. No region had significantly smaller rCBF responses during saline infusion in the drug group as compared to the physostigmine condition. No changes in task rCBF were observed over time in the control group.

Mean values of rCBF for each task scan were obtained from the right prefrontal region shown in Fig. 6. Subjects receiving physostigmine showed significant differences among sequentially obtained rCBF measures, $F(4, 48) = 6.70$, $p = 0.0002$, in right prefrontal rCBF during task (Fig. 7A), whereas saline control subjects did not show any difference, $F(4, 28) = 1.69$, NS. Paired t-test comparisons indicate that the first, pre-drug rCBF measure in the physostigmine group was significantly higher than rCBF measures obtained at 40 through 80 min during infusion; the measure at 20 min was significantly higher than the measure at 80 min; and the measure obtained at 80 min was significantly lower than the measure at 60 min but not at 40 min. Mean rCBF values from the same right prefrontal region obtained at rest (Fig. 7B) did not differ over time in the control group, $F(4, 28) = 1.70$, NS, or in the subjects receiving physostigmine, $F(4, 48) = 1.08$, NS.

The final measures obtained for the two peripheral pharmacologic variables of physostigmine plasma concentration and butyrylcholinesterase inhibition, values used to represent the stable measures of these variables, correlated positively ($r = 0.72$, $p = 0.027$). The magnitudes of the physostigmine effect on rCBF and RT were calculated by subtracting measures obtained in the saline control condition from the final measures obtained during physostigmine infusion. A significant positive correlation ($r = 0.57$, $p = 0.04$) was found between the magnitude of the effect of physostigmine on right

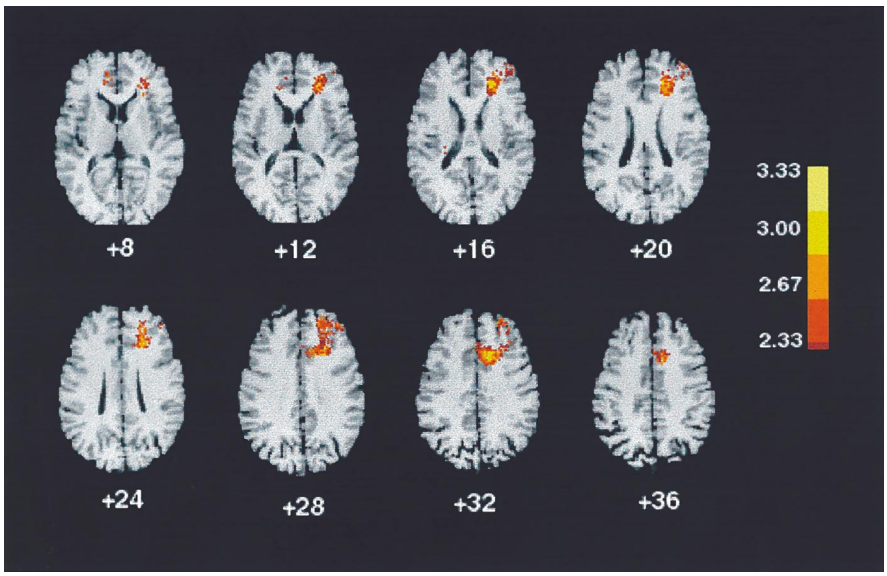

FIG. 6. Results of a within-group analysis showing brain regions with significantly higher rCBF during the WM task in the absence as compared to the presence of physostigmine. Pixels with Z-values that exceed 2.33 and that obtained significance after a blob analysis ($p < 0.05$) are superimposed on axial slices of a structural MRI template. The Z-coordinate from the Talairach and Tournoux Human Brain Atlas is indicated below each slice.

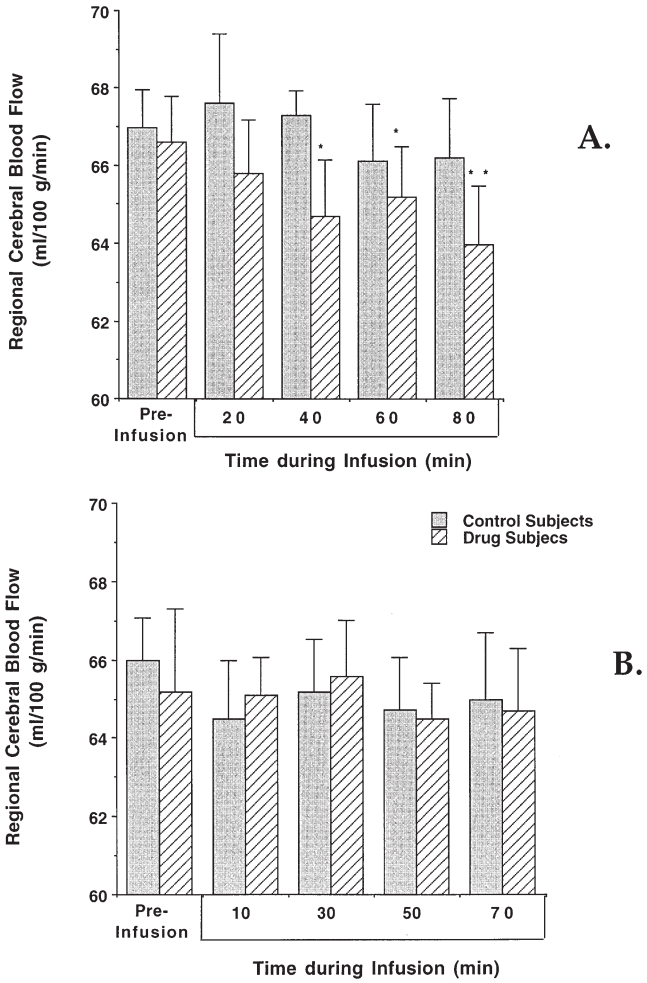

FIG. 7. Mean values of cerebral blood flow for the right prefrontal region that shows the task by drug effect during task performance (A) and at rest (B) for subjects receiving saline (filled bars) and subjects receiving physostigmine (hatched bars) infusions. Values obtained prior to infusion are indicated by the pre-infusion measures and subsequent measures are shown in association with time during infusion at which the scans were obtained. Error bars show standard deviation. *Significantly different from the pre-infusion measure; **significantly different from the pre-infusion measure and the measure at 60 min.

prefrontal rCBF (rCBF during the last task scan minus first task scan) and on task RT (task RT during the last scan minus first scan). While changes in task-related rCBF did not correlate significantly with either of the peripheral pharmacologic measures, the drug-induced change in RT correlated negatively with the final measure of percent butyrylcholinesterase inhibition ($r = -0.74$, $p = 0.02$), and did not correlate significantly with final plasma physostigmine concentration ($r = -0.34$, NS), but the strength of these correlations were not significantly different.

No subject had any serious side effect associated with the administration of physostigmine. At the end of the PET study and physostigmine infusion, 2 of the 13 subjects experienced mild nausea and emesis, which resolved within approximately 30 min. Several subjects reported dry mouth.

DISCUSSION

In this study, we measured rCBF and RT repeatedly during a memory task, as well as plasma drug levels and enzyme inhibition, prior to and during the intravenous administration of a preselected dose of physostigmine in healthy subjects, to determine the temporal characteristics of and the relation among peripheral and central indices of the pharmacological action of the drug. The evaluation of the time course of the peripheral and central responses to physostigmine indicated that a steady state was obtained for all variables of interest by 40 min of infusion. No differences were observed among group mean physostigmine concentrations or mean plasma butyrylcholinesterase inhibition levels from 40 min to completion of the study, indicating that physostigmine plasma concentration and the direct effect of physostigmine on enzyme inhibition became stable between 30 and 40 min of drug infusion. The effect of physostigmine on RT occurred rapidly, as shown by the fact that RT during infusion differed from baseline for the first time point (20 min), and did not show any further change for subsequent time points. The effect of physostigmine on right prefrontal rCBF was significant after 40 min of infusion and remained stable for the subsequent time points. In summary, the central effects of physostigmine, as indicated by altered RT and rCBF, were stable by 40 min of infusion, which is approximately the same time that steady-state plasma levels and enzyme inhibition were achieved.

Physostigmine has a brain/plasma concentration ratio of 1.2 in animals, indicating that plasma concentration closely reflects the concentration in the brain [see review, (11)]. As physostigmine nonselectively inhibits both acetyl- and butyrylcholinesterase equally, measurements of systemic butyrylcholinesterase inhibition closely reflect systemic acetylcholinesterase inhibition [see (11) for literature review]. Peripheral measures of plasma physostigmine concentration and butyrylcholinesterase inhibition, therefore, provide reasonable estimates of these parameters in the central nervous system.

Consistent with previous findings (1), the final steady-state index of the peripheral pharmacologic responses, including physostigmine plasma concentration and percent butyrylcholinesterase inhibition, correlate with each other and show that greater enzyme inhibition is observed with higher plasma drug concentrations. Additionally, the central effects of physostigmine are correlated, as shown by the relationship between improvement in task performance (reduction in RT) and rCBF decreases in right prefrontal cortex (9).

The relation between the peripheral pharmacologic measures and the central neural and cognitive responses is less clear. Only one correlation between peripheral pharmacologic measures and the central neural and cognitive responses was significant, suggesting there is a weak relation between these variables. Percent butyrylcholinesterase inhibition correlated with the magnitude of the RT response, but the strength of this correlation did not differ from the nonsignificant correlation between enzyme inhibition and the rCBF response or from the nonsignificant correlations between plasma concentration and the rCBF and RT responses.

Our results point to the need to design pharmacologic studies based on the time course of the central pharmacodynamic responses to drug. In our case, after a 10-min loading dose, the maintenance infusion schedule should be maintained for 20–30 min prior to the evaluation of physostigmine effects on rCBF using functional brain imaging. At 40 min, both rCBF and RT have stabilized, suggesting that the central effects of the drug remained constant.

The time at which the effect of physostigmine on RT and

rCBF became significant and stable differed by one time point. Although this difference may not be significant, given the measurement uncertainty associated with these behavioral and neurobiological measures, this result suggests that the time course of drug effects may not necessarily be the same for different measures. For example, the cognitive benefit of a drug may saturate at a different point than does the metabolic effect. Therefore, the time course of a drug effect must be determined specifically for each experimental variable of interest. Further, if neurobiologic or cognitive measures are to be obtained at multiple times during drug exposure, or if experimental conditions are to be systematically varied to assess the effects of drug under different conditions, it is important that the central drug effects of experimental interest be unchanging to ensure that differences in responses are due to experimental manipulation and not due to inconstant pharmacodynamics. Given that the rCBF response is likely to be small, not taking into account time course effects in drug responses can add variability to the rCBF data that could make it difficult to identify the true drug effects.

In the event that the time course of the pharmacodynamic responses cannot be established, it would be important to obtain measurements of experimental variables at the same time relative to drug administration across subjects so that the drug is having the same effect. Importantly, while counterbalancing of experimental conditions will control for order effects that may influence these measures, counterbalancing also may increase error variance because of inconstant drug effects.

In summary, we have shown that brain imaging methodologies combined with cognitive testing can be used to identify the time course of pharmacologically induced changes in brain function. These findings indicate that functional brain imaging in conjunction with pharmacologic probes that affect neurotransmitter systems offer an important methodological approach to study brain function. Properly designed functional imaging studies may expand our understanding about neural systems and their dysfunction in diseases, including neurodegenerative disorders, by determining the ways drugs that target specific neurotransmitter systems alter function in diseased brain; or by assessing how potential pharmaceutical treatments affect central neural systems.

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