**ACUTE EFFECTS OF ANTICHOLINESTERASE AGENTS ON PUPILLARY FUNCTION**

**AUTHOR(s)**
Ezio Giacobini

**PERFORMING ORGANIZATION NAME AND ADDRESS**
University of Connecticut
Dept. Biobehavioral Sciences
Storrs, CT 06268

**CONTROLLING OFFICE NAME AND ADDRESS**
Air Force Office of Scientific Research/ATI.
Bolling AFB, DC 20332

**ABSTRACT**
The effect of anticholinesterase agents on pupillary function and parameters of cholinergic activity were investigated both in vitro and in vivo following topical administration. The study describes changes in three different aspects of cholinergic function: 1) uptake of choline, 2) release of acetylcholine and 3) AChE activity and pupil size. Our results are consistent with the concept of existence of a presynaptic muscarinic autoreceptor which is affected (DFP directly or through acetylcholine). DFP exerts multiple effects on various cholinergic parameters.
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UNIVERSITY OF CONNECTICUT
STORRS, CT 06268

Dr. Ezio Giacobini

Controlling Office: USAF Office of Scientific Research/NL
Bolling Air Force Base, DC 20332

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The strategy of this investigation is to correlate closely any impairment of pupillary function to parameters of cholinergic function in vitro and in vivo, following topical or systemic administration of antiChEs. The study focuses upon three aspects of cholinergic function: the synthesis and turnover of ACh, the cholinergic receptor and the release of ACh (Fig. 1a). A schematic diagram of the various steps involved in the analysis of multiple parameters of acetylcholine metabolism in a single isolated iris is reported in Fig. 1b.

The characteristics of the high and low affinity Ch uptake system which have been previously described by us for the developing and aging avian iris (Marchi et al., Dev. Neurosci. 3, 185, 1980 and Brain Res. 195, 423, 1980) have now been determined for the adult rat iris as well (Fig. 2).

**Uptake of Choline**

The uptake of choline by rat iris is linear over at least six concentrations of choline ranging from 0.1 μM to 10 μM (linear regression coefficient = 0.99). The rat iris exhibits two distinct Ch uptake systems. One component (Fig. 2), a Na+ dependent, temperature sensitive, high affinity system which is blocked by ouabain and hemicholinium, is most likely confined to cholinergic nerve terminals. A second component, probably localized in the iris muscle cells, is Na+ independent and shows low affinity. The substitution of 80% Na with lithium decreased uptake by 55%.

The kinetic curves show a low affinity and high affinity uptake system with a \( K_{m1} = 100 \mu M \) and \( K_{m2} = 6.67 \mu M \), respectively (Fig. 2).

The effect of various drugs on choline uptake is reported in Figure 3 as a % control. Choline uptake, as expected, could be inhibited by ouabain which is consistent with Na+ dependent uptake, and by hemicholinium. However, the iris preparation seems to be less sensitive to hemicholinium than brain preparations at a 100 μM concentration was necessary in order to produce a 35% inhibition of uptake. Of particular interest is the effect of DFP which at 1 mM concentration inhibits choline uptake by approximately 30% (Fig. 3). Scopolamine (1 mM) showed a 25% inhibition effect. This effect at higher concentrations may relate to its effect on release as seen in the following experiments.

**Release of Acetylcholine**

Acetylcholine was released from iris preloaded by incubation with 1 μM 3H-Ch by 20 mA, 5 ms, 100 Hz bipolar nearly square waves (Fig. 4). The release is reported in Figure 5 as a percent of the total tissue radioactivity due to ACh at each individual time. Four to six subsequential stimulations were performed starting after spontaneous release had levelled off. Released tritiated ACh and Ch were separated and counted at each point. The evoked release ratio (ERR) was calculated from the areas under the curve (Fig. 5) representing released radioactivity and compared with corresponding non-stimulated controls. Electrical stimulation evokes 1- to 2-fold increase in the...
release of $^3$H-ACh over the spontaneous release during prestimulation baseline. The stimulated release is frequency dependent, tetrodotoxin sensitive and Ca$^{2+}$ dependent.

Scopolamine 10 nM increased evoked ACh release by at least 2-fold while 1 µM scopolamine increased spontaneous release only. These effects support the hypothesis of the presence in the iris terminals of a presynaptic muscarinic autoreceptor which controls release (Fig. 5).

DFP (1 µM) reduced both the spontaneous and the evoked release by 30% (Fig. 5). The effect was stronger following the first stimulations. This concentration of DFP inhibits AChE by approximately 80% and corresponds to the concentration used in the following in vivo experiments. This increase was blocked by scopolamine.

Acute Effects of DFP on the Iris

In this series of experiments the acute effects (up to 120 min) of DFP (5 mM) on the pupil were studied (Fig. 6).

5 µl of a .1% solution of DFP (corresponding to 5 µg of a 5 mM concentration) was instilled in the conjunctival sac of rats and its effect on the pupil area or diameter was recorded.

1. AChE activity was inhibited by 65% at 1 min and 95% at the following times (up to 1 hr). At 2 hrs the AChE activity was still inhibited by 75%.

2. Pupil size was unchanged at 1 min but decreased by more than 50% at successive times up to 2 hrs.

3. ACh levels were unchanged at 1 min, were increased by 40% at 5 min and by 50 min and were still increased by 30% at 120 min.

4. Choline levels were decreased at 5 min, showing a tendency toward a slight increase after 5 min with an increase of 25% at 120 min. In part, this reflects the low rate of hydrolysis of ACh in the presence of DFP. It is interesting to note that the pupil was still constricted at a time (120 min) when ACh had almost returned to baseline values. This may be due to a prolonged effect of ACh maintaining constantly high levels at the postsynaptic site.

In conclusion, it can be said that our results are consistent with the concept of existence of presynaptic muscarinic autoreceptors that control release of ACh from the cholinergic nerve terminals in the rat iris. With regard to the mechanism of action of DFP on the iris, we propose that the drug may exert multiple effects (see Fig. 7), such as:

a. inhibition of AChE with following increase in ACh levels both intra- and extrasynaptically,

b. inhibition of Ch uptake, and

c. reduction of both spontaneous and stimulated release.

Points b and c need further examination.
PUBLICATIONS OR COMMUNICATIONS SUPPORTED BY THE PRESENT GRANT


EFFECT OF DFP ON ACETYLCHOLINE METABOLISM IN THE RAT IRIS.
Mattio, T.G., Giacobini, E. and J.S. Richardson. Dept. Pharm.,
Southern Ill. Univ. School of Medicine, Springfield, IL 62708

The iris contains cholinergic nerve endings whose cell bodies
are located in the ciliary ganglion. This makes this structure
a good model of nerve terminal function free from contamination
by cell body and glia effects. Following the characterization
of the uptake system for choline (Ch) and the release of acetyl-
choline (ACh) in the isolated rat iris we have studied, the
effect of the increase in ACh concentration following local
administration of the irreversible cholinesterase inhibitor
diisopropyl fluorophosphate (DFP). At various times after the
topical administration of 0.1% DFP in sesame oil onto the
corneal surface, the rats were sacrificed and the irises were
removed. Pupil diameter was measured, ACh as well as Ch levels
were determined and acetylcholinesterase (AChE) activity
measured in segments of the same iris. One minute after DFP, no
changes were found in pupil diameter and ACh levels, but AChE
activity was decreased by 65%. At 5 minutes, pupil diameter was
reduced by 60% (and remained at this level for the duration of
the experiment), Ch by 30%, AChE by 92%, and ACh was increased
by 38%. At 15 minutes ACh was increased by 28%, and Ch was
still reduced (10%) but continued to recover reaching control
levels at 60 minutes. Acetylcholine levels were still increased
at 60 and 120 minutes. AChE activity was still inhibited 86%
and 74% at 60 and 120 minutes, respectively. Our results show
that in peripheral cholinergic terminals, in spite of the con-
tinual inhibition of AChE activity and the functional pupillary
paralysis following a single exposure to antiChE agents, ACh and
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American Society for Neurochemistry

Monday, March 21, 1983

111 EFFECTS OF SOMAN ON HIGH AFFINITY CHOLINE UPTAKE BY RAT BRAIN SYNAPTOSOMES
Whalley, C.E.\(^8\) and Shih, T.-M. (SPON: B.E. Hackley, Jr.)
We have previously reported (Psychopharmacology, 24, 170, (1971) the regional variation in the effects of soman, a potent organophosphorus cholinesterase inhibitor, on brain levels of acetylcholine and choline. Available evidence (Kuchar et al., J. Neurochem., 30, 15, (1978) suggests that alterations of activity of cholinergic neurons in vivo parallel changes in sodium-dependent high affinity choline uptake. In the present study, we examined the changes of SDNACU in synaptosomes isolated from rat striatum (S), hippocampus (H), cortex (C), midbrain (M), brainstem (B), cerebellum (K) and whole brain, following soman treatment. Incubation of synaptosomes with various concentrations of soman in vivo (SDNACU) did not affect synaptosomal SDNACU in the whole brain or in any of the brain regions. On the other hand, SDNACU following in vivo soman treatment showed in S an increase at 4 and 24 hrs (60 and 71%, respectively); in C a decrease at 1 and 4 hrs (54% and 65%, respectively); and in H a decrease only at 1 hr (44%). In B or M no significant change occurred in synaptosomal SDNACU at any time studied, while in N, SDNACU was not detectable in either control or soman treated animals. These data indicate that brain synaptosomal SDNACU does not appear to be affected directly by soman at concentrations studied in vitro and it is postulated that the regional differences observed in and or control or soman treated animals reflect changes in activity of cholinergic neurons during acute intoxication.

112 EFFECT OF DFP ON ACETYLCHOLINE METABOLISM IN THE RAT IRIS
Matto, T. G.*; E. Glacobini, and J. S. Richardson
The iris contains cholinergic nerve endings whose cell bodies are located in the ciliary ganglion. This makes this structure a good model of nerve terminal function free from contamination by cell body and glia effects. Following the characterization of the uptake system for choline (Ch) and the release of acetylcholine (ACH) in the isolated rat iris we have studied, the effect of the increase in ACh concentration following local administration of the irreversible cholinesterase inhibitor disopropylfluorophosphate (DFP). At various times after topical application of 0.1% DFP in sesame oil onto the corneal surface, the rats were sacrificed and the irises were removed. Pupil diameter, required to move ACh as well as Ch, was determined and acetylcholinesterase (ACHE) activity measured in segment of the same iris. One minute after DFP, no changes were found in pupil diameter and ACh levels, but ACh activity was decreased by 65%. At 5 minutes, pupil diameter remained at 60% (and at this level at the duration of the experiment), Ch by 30%, ACh by 92%, and ACh was increased by 38%. At 15 minutes ACh was increased by 283%, and Ch was still reduced (103%) but continued to return reaching control levels at 60 minutes. Acetylcholine levels were still increased at 60 and 120 minutes. ACh activity was still inhibited 86% and 74% at 60 and 120 minutes respectively. Our results show that in peripheral cholinergic terminals, increase of functional curarization followed by a single exposure to anticholinesterase agents, ACh and Ch tend to return toward normal levels. (Supported by Grant AFOSR-B1-0229 to E. G.)
MONOCLONAL ANTIBODIES TO CHOLINE ACETYLTRANSFERASE FROM RAT BRAIN
Crawford, G., Salvaterra, P.M.
City of Hope Research Institute, Duarte, CA 91010
We have recently described the production and partial characterization of five monoclonal antibodies reacting with rat brain choline acetyltransferase. Antibodies purified from ascites fluids were found to recognize a cluster of determinants restricted to a small portion of the enzyme surface. (Crawford et al., PNAS, 1983.) One of the antibodies recognizes a determinant not destroyed by glutaraldehyde fixation and is presently being employed in a number of chemical investigations of choline acetyltransferase distribution in rat brain. Titration curves of the antibodies have been obtained by Scatchard's method. Each antibody displays a single affinity for the enzyme present in other rodent brains, such as mouse and guinea pig, primate brain from baboon and human as well as enzyme from human placenta. One of the antibodies also cross reacts with enzyme from chicken brain, frog spinal cord, and Aplysia ganglion. The determinants thus appear to be not only clustered in a small region of the enzyme surface but also highly conserved. (Supported by NS 8858.)

115 PLASTICITY OF NICOTINIC SYNAPTIC TRANSMISSION
Briggs, C.A.; T.H. Brown; D.A. McAfee
City of Hope Research Institute, Duarte, CA 91010
Previous studies have demonstrated long term potentiation (LTP) of synaptic transmission in the superior cervical ganglion of the rat (Brown and McAfee, 1985, Science 221:1411-1413). We have obtained these results by stimulating the ganglion in curare to reduce end-plate potentials, and then repetitively stimulating the preganglionic nerve at 20 Hz for only 20 seconds. While measuring the compound action potential in response to the repetitive stimulus once every minute, we observed a 2-fold increase in the response amplitude which decayed as a double exponential with time constants 1-3 minutes (PTP) and 30-230 minutes (LTP). These findings, based on extracellular measurements, have now been confirmed using intracellular techniques.

In 21 of 41 cells, stimulation of the preganglionic nerve at 20 Hz for 20 sec induced an increase in the nicotinic excitative postsynaptic potential (EPSP) or an increase in the amplitude of synaptic transmission to an end-plate potential. These effects were frequently obscured by synaptically driven action potentials which appeared after the tetanic stimulation. These effects lasted for 30 minutes to several hours, as long as the recording could be maintained. The potentiation was not accompanied by measurable changes in resting membrane potential or input resistance. Direct nonnastic stimulation of the postsynaptic neuron (20 Hz for 20 sec) failed to increase any synaptic transmission in cells. Thus, LTP in the ganglion appears to be due to an increase in the efficacy of nicotinic synaptic transmission. We have hypothesized that LTP is accompanied by an increase in acetylcholine release but this awaits direct measurement. Supported NSF BNS-82142.

116 L-TYROSINE ENHANCEMENT OF STRIATAL ACETYLCHOLINE RELEASE IN VIVO
Gewe, J., M., and Graber, B.
Dept. of Surgery, Henry Ford Hospital, Detroit, MI 48202
The effect of L-tyrosine, a specific dopamine-2,3-phenylalanine catabolic enzyme, is to effectively elevate dopamine activity (SPASM, 1979). In the release of acetylcholine (ACH) from rat striatal tissue slices which were incubated and were then incubated in Krebs-phosphate buffer containing glucose (4 mM) and choline (50 mM), and were immediately aliquoted to seven replicates. Each replicate was divided into two subgroups, containing no drug (control) or L-tyrosine (100, 100). After a further 30 min, either at 36°C or in Krebs-phosphate-glucose (200), the ACH release was measured immediately by a radioreceptor method (HUBER et al., Life Sci., 13:47-53, 1983). The liberation of the ACH released (20-2,000) was measured by a radioreceptor method. Incubation

condition control 10-5 M 10-4 M 10-3 M

L-Tyrosine

100

6.0 15.8

100

15.8

100

20 20

Each value is the mean ± SEM. All ACh released as protein 10 min/SEM of the specific activity in parentheses, each based on triplicate assays. (*) and (**) indicate a *p < 0.05, respectively, vs. appropriate control. (1) includes a *p < 0.05 difference between 100 and 50% control conditions. These data show a significant enhancement of ACh release under depolarized (1000 K) conditions and a lack of effect of L-tyrosine on resting release. Release of rat striatum. The results suggest that D-2 (100) and ACh are important in radiolabeled DA control of striatal release, and suggest that the competitive ACh release must be present for L-tyrosine's effect to be apparent. Supported by the Fund for Brain Research.
IRIS PREPARATION

a) Schematic diagram showing various steps involved in the regulation of acetylcholine synthesis in a cholinergic terminal.
b) Scheme of analysis of multiple parameters of acetylcholine metabolism in three segments (A, B, and C) of a single isolated iris.
Figure 2

KINETIC CURVES FOR CH UPTAKE IN THE RAT IRIS

\[ \frac{\text{pmole / half iris / 5 minutes}}{\text{Choline} [\mu M]^{-1}} \]

- \( K_m = 100 \)
- \( V_{max} = 100 \)

- \( K_m = 6.67 \mu M \)
- \( V_{max} = 16.7 \)
Figure 3 - EFFECT OF VARIOUS DRUGS ON CHOLINE UPTAKE

Choline Uptake (% Control)

[Drug] (μM)

- Scopolamine
- Physostigmine
- DFP
- Ouabain
- Hemicholinium
Figure 5

EFFECT OF VARIOUS DRUGS ON THE RELEASE OF
$^3$H-ACh FOLLOWING PRELOADING WITH 1 µM $^3$H-LH (20 mA, 5 ms, 100 Hz)

Graph showing the impact of different drugs on the release of $^3$H-ACh.

- Buffer
- 10 nM Scopolamine
- 1 µM Scopolamine
- 1 µM DFP

Release: Percent Lost per Minute
Superfusion Fractions 5, 10, 15, 20
Figure 6
CHANGES IN AChE ACTIVITY, PUPIL DIAMETER, ACh AND Ch LEVELS IN RATS INSTILLED WITH DFP (5 mM) IN THE CONJUNCTIVAL SAC

AChE Act.

PUPIL DIAM.

ACh LEVELS

Ch LEVELS

MINUTES

MINUTES
Figure 7
MULTIPLE EFFECTS OF DFP ON CHOLINERGIC SYNAPSES

DFP \rightarrow AChE

\[ \text{Ch} \rightarrow \text{ACh} \]

\[ \text{ACh} \rightarrow \text{Ch} \]

\[ \text{ACh or Ch levels} \]