THE USE OF HISTOCHEMISTRY IN PHARMACOLOGICAL STUDIES*

GEORGE B. KOELLE

Department of Pharmacology, Schools of Medicine University of Pennsylvania, Philadelphia 4, Pa., U.S.A.

THERE are two general approaches to the cytological localization of sites of drug action: (1) demonstration of the distribution of the active drug, or a close analog, at the cellular level following its systemic administration, and (2) the demonstration at discrete loci of changes in the concentration of a specific group or compound with which the drug is known to react to produce its characteristic pharmacological effects.

Several types of microscopic techniques which either have been employed or have potential usefulness for the first approach are listed in Table I (I). Some of these will be discussed in detail in the subsequent papers of this symposium. All require carefully controlled conditions to eliminate or minimize artifactual localization due to diffusion of the drug, during the preparation of sections, from its location *in vivo* at the time of sacrifice. However, the major reservation to the interpretation of results obtained with these methods is that in general they can indicate only potential sites of drug action. On the basis of several factors, including aqueous- and lipoid-solubility, ionic or induced charge, and molecular size and shape, a drug may be distributed at several cellular sites, or even tissues or organs, in addition to those where it combines with specific receptors or enzyme systems to produce its major pharmacological effects. An obvious example is the concentration of ether and other central nervous system depressants in the subcutaneous fat.

The second approach avoids the latter disadvantage, but is limited by the fact that the reactive groups or compounds with which most drugs combine are either unknown or not readily subject to demonstration or quantification. A few active groups and enzymes, for which there is reasonable assurance that they represent the major sites of

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action of certain drugs, are listed in Table I (II); at present, it would be difficult to add many more, other than a few classes of toxic agents. The characterization of pharmacophore-receptor groups, enzymic or otherwise, is clearly one of the major fields for future development in biochemical pharmacology.

TABLE I

General approaches to the cytological localization of sites of drug action

I. Demonstration of distribution of drug or close analog.
   A. Direct microscopic visualization of chromophoric compounds or reaction products.
   B. Fluorescence microscopy.
   C. Fluorescent antibody technique with antigenic compounds.
   D. Radioautography.
   E. Electron microscopic localization of electron-dense compounds.
II. Demonstration at discrete loci of changes in concentration of (A) specific group or (B) specific compound with which drug reacts to produce its characteristic pharmacological effects.
   A. Specific groups
      — SH groups of renal tubules and mercurial diuretics.
   B. Specific compounds
      1. Receptor proteins
      1. Enzymes
         a. Carbonic anhydrase
         b. Monoamine oxidase (MAO)
         c. Acetylcholinesterase (AChE)

As an example of the cytological approach to pharmacology, the anticholinesterase (anti-ChE) agents represent a particularly favorable class of drugs. There is convincing evidence that the primary mechanism of action of many of the potent agents of this group is the inhibition or inactivation of acetylcholinesterase (AChE). The distribution and concentration of the enzyme at the cytological level can be determined by three types of procedures (Table II). The first, ultramicro-analysis, permits quantification of AChE activity in single cells, or even their

TABLE II

General procedures for cytological localization of acetylcholinesterase

I. Ultramicro-analytical methods
   A. Colorimetric
   B. Microgasometric (Cartesian diver)
II. Centrifugal fractionation of homogenates
III. Microscopic histochemistry
   A. Subjective evaluation
   B. Quantitative applications

individual components; techniques of this type are discussed by Lowry and Giacobini in their papers which follow. The value of centrifugal fractionation procedures is limited generally to the determination of the cellular consitituents with which the enzyme is associated normally in relatively homogeneous tissues. Microscopic histochemistry, while allowing only rough approximations of the relative concentrations of AChE

TABLE III

Microscopic histochemical methods for localization of acetylcholinesterase

A. Long-chain fatty acid methods
B. Thiocholine ester methods
C. α or β-naphthyl acetate-diazonium coupling methods
D. Indoxyl acetate → indigo blue methods
E. Thiolacetic acid methods

at various sites, has the advantages of both greater sensitivity and greater accuracy of localization with respect to detailed cellular structure. Five histochemical techniques have been used for the microscopic histochemical localization of AChE (Table III); the technical details, advantages and limitations of each have been discussed in several monographs[1,2,3]. The thiocholine method (B), when properly controlled, is both highly specific and exteremely sensitive. As developed originally[4], it consists in incubating fresh frozen sections in a medium containing acetylthiocholine (AThCh) and copper glycinate; as the substrate is hydrolysed by AChE and non-specific cholinesterase (BuChE, pseudo-ChE), the thiocholine liberated is precipitated as a mercaptide salt, e.g., copper thiocholine sulfate[5]; subsequent immersion of sections in ammonium sulfide solution converts the latter to a dark brown precipitate of copper sulfide, the visualization of which can be improved further by gold-toning.

$$(CH_3)_3\overset{+}{N}CH_2CH_2SCOCH_3 \xrightarrow[+H_2O]{(ChE)} HSCH_2CH_2\overset{+}{N}(CH_3)_3$$

Acetylthiocholine ion

Thiocholine ion (plus copper glycinate)

$$CuS \xleftarrow{(NH_4)_2S} [CuSCH_2CH_2\overset{+}{N}(CH_3)_3]SO_4$$

Copper thiocholine sulfate

By employing the proper concentration of diisopropyl phosphorofluoridate (DFP) as a selective inhibitor of BuChE, with AThCh as substrate, or by substituting butyrylthiocholine for AThCh as the substrate, it is

possible to localize selectively AChE or BuChE, respectively[6]. The specificity of the method can be assured by the inclusion of several control solutions, containing additional selective inhibitors of AChE, BuChE, or both[7]. Artifacts of localization due to diffusion of the enzymes or the reaction products are minimized by the incorporation of appropriate high concentrations of sodium sulfate in the incubation media[8]. With the foregoing modifications, it was found that high concentrations of AChE are present throughout the entire lengths of cholinergic neurons; in contrast, nono-cholinergic neurons (e.g., adrenergic, primary afferent) in all species studied contain distinctly lower concentrations, varying from moderate amounts to little or none. This situation is illustrated by the neurons of some autonomic ganglia of the cat in Fig. 1, which were stained for AChE by the foregoing technique[9]. In the ciliary ganglion, all the neurons of which give rise to cholinergic postganglionic parasympathetic fibers, the neurons and their processes are all heavily stained (Fig. 1C). Most of the neurons of the stellate ganglion (Fig. 1A) are very faintly stained; these are presumably the neurons which give rise to the adrenergic postganglionic sympathetic fibers. It has been assumed that the small number of heavily stained neurons in this ganglion represent the cells of origin of the cholinergic fibers to the sweat glands or the vasodilator fibers[8]; evidence in support of the former interpretation has just been presented by Sjöqvist and Fredricsson[10]. Most of the heavily stained fibers in the stellate ganglion are preganglionic fibers and their terminations, as indicated by their disappearance after chronic preganglionic denervation (Fig. 1B). This raises the question of the significance of the intermediate intensity of staining shown by a small number of neurons in the stellate and other sympathetic ganglia of the cat. In other species, such as the rabbit and rhesus monkey, the majority of the sympathetic neurons are lightly but distinctly stained for AChE by the same procedure[7].

A second question raised by related histochemical studies concerns the relative distributions of AChE at the pre- and postjunctional membranes at various sites of cholinergic transmission. At the neuromuscular junction, Couteaux and Taxi[11] have shown by a modification of the AThCh method that most of the enzyme is concentrated at the postjunctional or modified sarcolemmal membrane (Fig. 2), as indeed Couteaux had concluded from earlier denervation studies[12]. The situation in autonomic ganglia is somewhat more complicated. Both the ganglion cells and the terminals of the preganglionic fibers are closely invested by glial cells, which contain BuChE. The active groups of the AChE of the presynaptic terminals are probably oriented externally with respect to the axonal membrane, inasmuch as the enzyme at this site is readily inhibited by quaternary anti-ChE agents. On the other hand, the AChE of the sympathetic ganglion cells appears to be enclosed almost entirely

within a lipid-containing membrane since it is not generally accessible to inhibition by compounds of this class; in the parasympathetic ganglia studied (ciliary, sphenopalatine) the AChE is located both externally and internally to the neuronal membranes of the ganglion cells[13,9]. Synaptic transmission in the superior cervical ganglion of the cat was found to be modified by inactivation of only the external, and not by inactivation

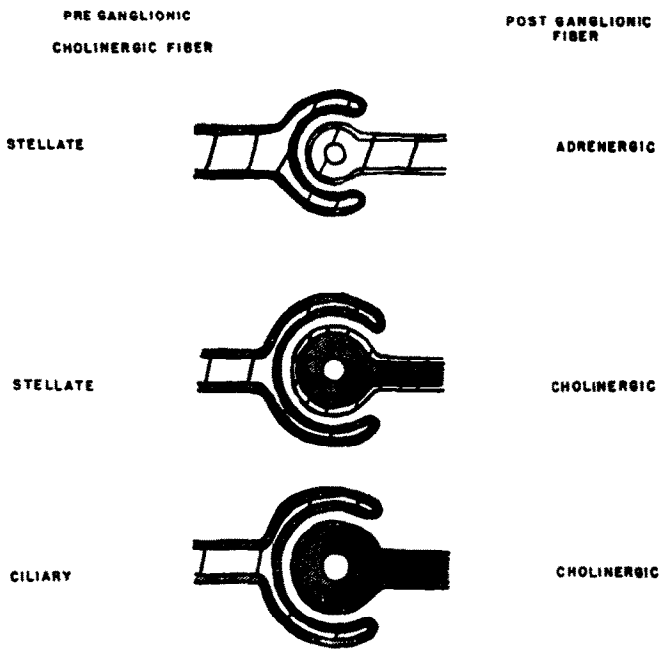

FIG. 3. Diagrammatic representation of distribution of functional (external) and reserve (internal) AChE at synapses of autonomic ganglia. Density of cross-hatching indicates relative concentration of enzymatic activity. From Koelle and Koelle[9].

of the internal, AChE; hence the terms "functional" and "reserve" seem appropriate for these respective fractions. Their ganglionic distributions, as described above, are depicted diagrammatically in Fig. 3[9]. It is apparent that the relative distribution of functional AChE at the pre- and postjunctional sites in the sympathetic ganglia is the reverse of that at the neuromuscular junction, with the ciliary ganglion occupying an intermediate position. What is the physiological basis of these differences?

These two questions, the significance of the low or intermediate concentrations of AChE in presumably non-cholinergic neurons, and the physiological functions of AChE at various junctional sites in view of the differences in its relative pre- and postjunctional distributions,

have been investigated by a combination of physiological and histochemical techniques. Tentative answers have been provided by a working hypothesis, according to which ACh acts not only trans-synaptically, as a neurohumoral agent at sites of cholinergic transmission, but in addition at presynaptic terminals of both cholinergic and certain non-cholinergic axons[15].

The physiological function of the AChE of the cat superior cervical ganglion was studied by my colleague, R. L. Volle[16], by determining the effects of the anti-ChE agent, DFP, on the activity of the post-ganglionic trunk. This was studied both in resting ganglia, and during activation by preganglionic stimulation or by the intra-arterial injection of ACh or its hydrolysis-resistant analog, carbachol (Car). Earlier reports, that under certain conditions anti-ChE agents increase the postganglionic response to preganglionic stimulation [17-20], were confirmed. In addition it was found that high doses of DFP, which caused nearly complete inactivation of the ganglionic AChE, resulted in persistent spontaneous postganglionic firing; this indicated that ACh is liberated continually during the resting stage, as had been shown previously for the neuromuscular junction[21]. The most provocative results were those derived from determinations of the intra-arterial threshold doses of ACh and Car for activation of postganglionic firing, in normal and chronically denervated ganglia, both before and after the intra-arterial injection of DFP. In a large series of cats, there was no significant difference between the mean threshold doses of ACh in normal and in denervated ganglia. However, the threshold dose of Car was 26-fold higher in denervated ganglia. Following DFP, the threshold dose of ACh in normal ganglia was reduced nearly 40-fold, but that of Car was reduced only slightly. The threshold doses of ACh and Car were 5-fold and 3-fold higher, respectively, in DFP-treated denervated, as compared with DFP-treated normal ganglia. Finally, DEP caused an 11-fold decrease in the threshold doses of both ACh and Car in denervated ganglia. Several possible interpretations of these, in many respects unexpected, results were discussed in the original paper[16]. However, the major conclusion drawn was that in normal ganglia, threshold doses of Car, and possibly of ACh, act at the presynaptic terminals, causing them to liberate sufficient quantities of ACh to activate the ganglion cells. With the loss of the former site consequent to denervation, the considerably greater threshold dose of Car would reflect the amount necessary for the direct activation of the ganglion cells. The same primary site of action of ACh in normal ganglia might be prevented by the protective sheath of functional AChE surrounding the presynaptic terminals; this is indicated both by the marked lowering of its threshold dose in normal ganglia following DFP, and by its higher threshold dose in DFP-treated denervated than in DFP-treated normal ganglia. In addition, the results indicated that DFP

causes sensitization of the ganglion cells to both ACh and Car by some mechanism other than AChE-inactivation.

As an answer to the second question raised above, it was proposed that the primary function of the predominantly presynaptically localized AChE of the cat superior cervical ganglion is to protect the more ACh-sensitive site, the axonal terminals, from continuous re-excitation (and consequent postsynaptic activation) by the ACh which they liberate both following activation, and in smaller quantities during the resting stage. This would explain all the immediately foregoing observations[16], including the persistent spontaneous postganglionic firing which followed high doses of DFP. At other sites, such as the neuromuscular junction, the necessity for more precise temporal limitation of the action of the transmitter at the postjunctional membrane may be the basis for the relatively greater concentration of AChE there. A tentative answer to the first question raised has been provided by extension of this concept and its application to several types of neurons generally considered to be non-cholinergic[15], including the hypothalamico-neurohypophyseal tract, the adrenergic sympathetic fibers, and vagal afferent fibers, each of which will be considered briefly.

On the basis of the effects of anti-ChE agents, Pickford and associates[22,23] have shown that it is likely that cholinergic fibers are involved in the secretion of oxytocin and vasopressin by the hypothalamico-neurophypophyseal tract. However, in the region of the hypothalamus of the dog from which the tract originates, the only significant staining for AChE noted was in the neurons of the paraventricular and supraoptic nuclei, which represent the cell bodies of the tract[24]. It was found subsequently that the fibers of the tract exhibit light to moderate staining for AChE in the neurohypophysis of the cat[25]. Electron microscopic studies of the neurohypophysis of the rat[26] and toad[27] have revealed that the terminations of the neurohypophyseal secretory fibers contain two distinct populations of vesicles, which may represent the endocrine secretions and some neurohumoral agent, such as ACh, respectively. From these observations, it has been suggested[24,25,27] that impulses conducted along the tract may liberate first ACh at the terminals, and that this in turn liberates oxytocin and vasopressin from the same axonal terminations. This sequence is very similar to one of the proposals made by Burn and Rand[28] on the basis of findings indicating that ACh is involved in the liberation of norepinephrine by adrenergic sympathetic postganglionic fibers: namely, that the fiber liberates first ACh, and that it in turn activates the release of the catecholamine from the same site. The suggestion that a similar mechanism may be involved in central transmission by the afferent fibers of the vagus comes from a recent study by Matsumura[29] in our laboratory. The actions of eserine and a wide variety of ganglionic

stimulating and blocking agents were measured on a series of cat superior cervical ganglia which had been denervated preganglionically, and reinnervated by the central afferent fibers of the vagal nodose ganglia, as performed originally by De Castro[30]. The results indicated that the transmission of impulses from the central vagal afferent terminals, which showed light staining for AChE, to the sympathetic ganglion cells was effected by ACh or a similarly acting agent. However, by the usual criteria the vagal afferent fibers are generally considered non-cholinergic. Accordingly, it was suggested that under normal circumstances, synaptic transmission by the vagal afferent fibers might be brought about by an unidentified neurohumoral agent which is liberated from the terminals by prior release of ACh.

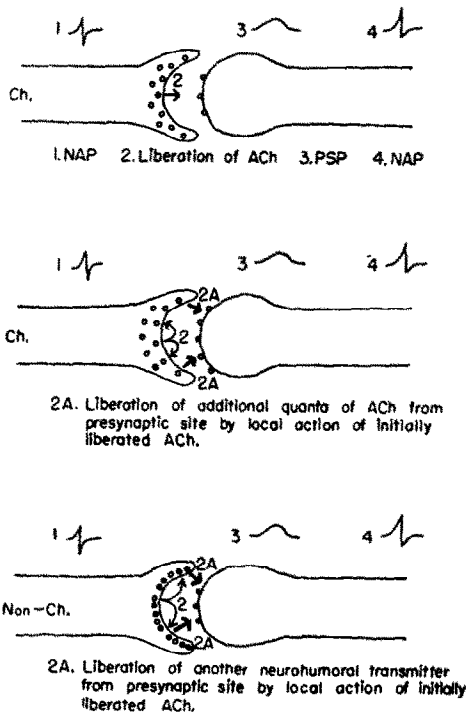

FIG. 4. Proposed dual neurohumoral role of ACh. See text for description. From Koelle[31].

The mediation of ACh in the release of other neurohumoral transmitters by non-cholinergic neurons in the foregoing three situations, would explain the function of low to moderate concentrations of AChE in such neurons, i.e., for the regulation of the initial process. To carry the working hypothesis one step further, the neuronal concentrations of AChE may reflect quantitatively the relative amounts of ACh involved in this proposed mechanism.

Fig. 4[31] depicts the steps involved in the standard concept of cholinergic (Ch) transmission (upper diagram), in which the nerve action potential (NAP) brings about the release of ACh, which diffuses across the synaptic cleft, combines with receptors in the postsynaptic membrane, and initiates the postsynaptic potential (PSP); the latter then initiates electrogenically a NAP in the second neuron. The proposed intermediate participation of ACh (step 2A) in cholinergic (Ch) and certain non-cholinergic (Non-Ch) neurons in shown in the middle and lower diagrams, respectively. It should be emphasized that this represents only a working hypothesis to explain the foregoing results of a combined histochemical and physiological approach to the investigation of the pharmacology of the anti-ChE agents at the cellular level.

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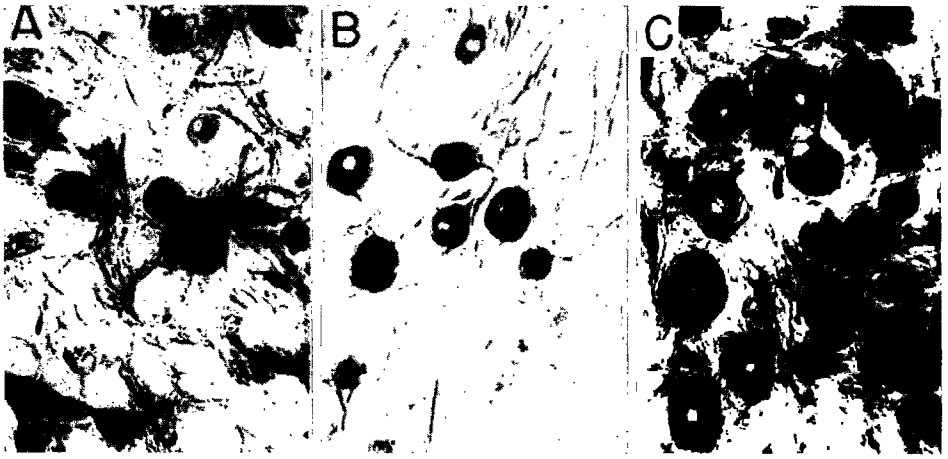

FIG. 1. Autonomic ganglia, cat, stained for acetylcholinesterase activity. Sections (10 μ) incubated 80 min in AThCh medium following selective inhibition of non-specific ChE by DFP. Magnification × 200. From Koelle and Koelle[9].

A. Stellate ganglion, normal.
B. Stellate ganglion, preganglionically denervated.
C. Ciliary ganglion.

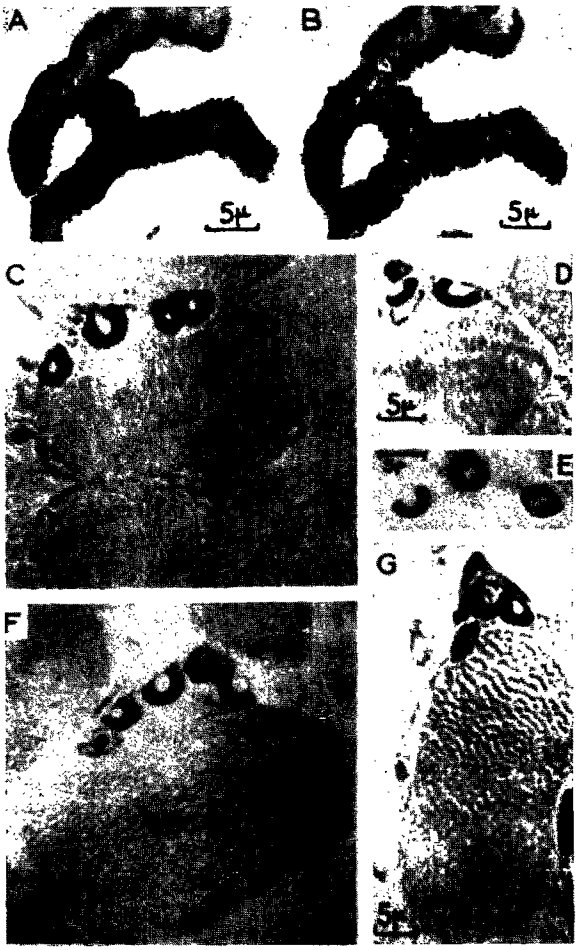

FIG. 2. Localization of ChE activity at the level of the motor endplate of mouse intercostal muscle. (Formalin fixation; 45 min incubation with AThCh at pH 4.7).

A and B. Front view of motor endplate. A, focussed at the border of the synaptic gutters and showing the leveling of the subneural apparatus at the surface; B, focussed at the base. Magnification × 1750.

C to G. Cross sections of muscle fibers of different types, showing the endplates at the level of the synaptic junction. In C, the condenser has been adjusted to render the muscle fibers colorless; in the remainder, secondary staining due to carmine is detectable. Magnification × 1500. From Couteaux and Taxi[11].