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Acetylcholine in Plants: Presence, Metabolism and Mechanism of Action

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I. Abstract

Acetylcholine (ACh) has been detected in representatives of many taxonomic groups throughout the plant kingdom. The site of its synthesis in plants is probably young leaves. In some plant species choline acetyltransferase (ChAT) activity has been found. This enzyme showing properties similar to animal ChAT, probably participates in ACh synthesis from its precursors, choline and acetyl-Coenzyme A. Acetylcholinesterase (AChE) activity has also been found in many plant tissues. This enzyme decomposes ACh and exhibits properties similar to animal AChE. The presence of both ChAT and AChE in plant tissues suggests that ACh undergoes similar metabolism in plants as it does in animals. Exogenous ACh affects phytochrome-controlled plant growth and development. Mimicking red light (R), ACh stimulates adhesion of root tips to a glass surface and influences leaf movement and membrane permeability to ions. It also affects seed germination and plant growth. Moreover, ACh can modify some enzyme activity and the course of some metabolic processes in plants. Acetylcholine in the presence of calcium ions (Ca^{2+}), like R stimulates swelling of protoplast isolated from etiolated wheat leaves. It is proposed that the primary mechanism of action of ACh in plant cells is via the regulation of membrane permeability to protons (H^+), potassium ions (K^+), sodium ions (Na^+) and Ca^{2+}.

Zusammenfassung

Acetylcholin (ACh) wurde in Vertretern vieler taxonomischer Gruppen des Pflanzreiches gefunden. Es wird wahrscheinlich inden jungen Blättern synthetisiert. In einigen Pflanzen hat man daneben Cholin-Acetyltransferase (ChAT)-Activität nachweisen können; dieses Enzym ziegt ähnliche Eigenschaften wie tierische ChAT und ist offenbar an der ACh-Synthese aus sienen Vorstufen Cholin und Acetyl-Coenzym A beteiligt. Acetylcholineesterase (AChE)-Activität wurde ebenfalls in vielen Pflanzengeweben gefunden; dieses Enzym spaltet ACh und ziegt ähnliche Eigenschaften wie tierische AChE. Die Anwesenheit von ChAT und AChE in pflanzlichem Gewebe läßt vermuten, daß ACh in Pflanzen einem ähnlichen Metabolismus unterliegt wie im tierischen System.

Ähnlich wie Rotlicht stimuliert ACh die Anheftung von Wurzelspitzen an Glasoberflächen und beeinflußt Blattbewegung und Membranpermeabilität für Ionen; darüber hinaus beeinflußt es Samenkeimung und pflanzliches Wachstum. Des weiteren kann ACh Enzym-Aktivitäten modifizieren und dadurch den Ablauf einiher metabolischer Prozesse in Pflanzen. Schließlich stimuliert ACh in Gegenwart von Calcium-Ionen (Ca^{2+}), ähnlich wie Rotlicht, das Schwellen von Protoplasten etiolierter Weizenblätter. Es wird vermutet, daß die Primärwirkung von ACh in Pflanzenzellen durch Regulation der Membranpermeabilität für Protonen (H^+), Kaliumionen (K^+), Natriumionen (Na^+) und Ca^{2+} erfolgt.

II. Introduction

Before discussing the presence and mechanism of acetylcholine (ACh)[1] action in plants some basic information will be presented on the structure and action of the so-called 'cholinergic system,' which occurs at junctions between nerves and between nerves and muscles in animals (Lester, 1977).

The cholinergic system is a set of enzymes and receptors linked with the function of ACh. Acetylcholine plays the role of a synaptic mediator. The system participates in the process of transmitting information received by receptors along the neurons in the form of a code of electric impulses. It is responsible for accurate transmission of the information between the pre- and postsynaptic membranes (Dunat & Israel, 1985; Lester, 1977).

The cholinergic system is composed of:

- —Acetylcholine (ACh), an ester of acetic acid and choline released from the presynaptic membrane at the moment of its depolarization (Lester, 1977).
- —Choline acetyltransferase (ChAT), an enzyme catalyzing the synthesis of ACh from acetylcoenzyme A and choline (Israël & Manaranche, 1985).
- —Acetylcholinesterase (AChE), an enzyme occurring in the pre- and postsynaptic

[1] Abbreviations frequently used in this review. Abscisic acid, ABA; acetyl-Coenzyme A, acetyl-CoA; acetylcholine receptors, AChRs; acetylcholine, ACh; acetylcholinesterase, AChE; adenosine triphosphate, ATP; antigibberellin growth retardants: AMO-1618, CCC and Q 80; choline acetyltransferase, ChAT; cyclic adenosine monophsophate, cAMP; 2,4-dichlorophenoxyacetic acid, 2,4-D; ethyleneglycol-bis-,β-aminoethylether;-N,N,N'N',-tetraaceticacid, EGTA; far-red light, FR; fluorescein-labelled α-bungarotoxin, FITC-BTx; indole-3-acetic acid, IAA; 'muscarinic' ACh receptors, mAChRs; α-naphthaleneacetic acid, NAA; 'nicotinic' ACh receptors, nAChRs; red light, R; trifluoperazine, TFP.

membranes, responsible for hydrolysis of ACh to choline and acetic acid residues (Dunat & Israël, 1985; Israël & Manaranche, 1985).

—Acetylcholine receptors (AChRs), localized in the postsynaptic membrane. In animal cells there are two types of AChRs, so-called 'nicotinic' (nAChR) and 'muscarinic' (mAChR) receptors (Changeux et al., 1984; Dunat & Israël, 1985; Rotter, 1984; Stround & Finer-Moore, 1985; Taylor & Spivak, 1985; Venter, 1984). These are both stimulated by ACh and also by nicotine and muscarine respectively. These compounds are called 'ACh agonists.' Compounds are also known which inhibit AChRs activity, 'ACh antagonists,' such as atropine (an antagonist of mAChRs) and D-tubocurarine (an antagonist of nAChRs).

The cholinergic system is also found in non-neural tissues, e.g., in erythrocytes, sperm, and placental cells (Sastry & Sadavongvivad, 1979).

Acetylcholine is synthesized in the nerve endings and stored in synaptic vesicles. Each vesicle contains about 10,000 molecules of ACh. As a result of stimulation of the neuron ACh is removed from the cytoplasm beyond the presynaptic membrane into the synaptic space (Dunat & Israël, 1985; Israël & Manaranche, 1985) and ACh molecules unite with their receptors situated in postsynaptic membrane (Changeaux et al., 1984). The ACh-AChRs complexes, through their effect on the permeability of membranes to ions, stimulate the development of action potentials on the post-synaptic membrane (Lester, 1977). In the following step, ACh is removed from AChRs with the participation of AChE, and the choline and acetic acid resulting from its hydrolysis are reabsorbed from synaptic cleft into the synaptic ending (Dunat & Israel, 1985; Israel & Manaranche, 1985).

III. Discovery of Acetylcholine in Plants

It was about 75 years ago that acetylcholine (ACh) was discovered in non-animal cells (Ewins, 1914). Since that time it has been detected in representatives of many species of lower and higher plants as well as bacteria and fungi (Fluck & Jaffe, 1976; Hartmann & Gupta, 1989). The highest concentration of ACh has been found in some nettle species (Emellin & Feldberg, 1949; Saxena et al., 1965, 1966). Bennet-Clark (1956) was one of the first to postulate possible participation of ACh in regulation of physiological processes in plants. In 1962 Dettbarn discovered AChE activity in cells of the alga *Nitella.* In 1965 a group of Czechoslovakian researchers (Kostir et al., 1965) and somewhat later Tung and Raghavan (1968) demonstrated that exogenously administrated ACh affected germination and first growth phases of some species. At the same time Cumming and Wagner (1966) noted that in plants, just as in animals, bioelectric potentials arose in response to light stimuli. They suggested that the mechanism of control of membrane permeability by the red (R)/far-red light (FR) reversible photoreceptor phytochrome may resemble the regulation of this process by ACh in animal cells. Tanada (1968a, 1968b) discovered that the electric phenomena in the root tips of barley and bean were controlled by phytochrome. In experiments on such root tips he demonstrated that R stimulated their adhesion, and FR their release from negatively charged glass surfaces. Jaffe (1968), using a model similar to Tanada's, has revealed that adhesion and release of root tips from the glass surface are both the result of phytochrome-mediated changes in surface potentials of the root tip cells. Moreover, in 1970 Jaffe demonstrated that ACh may participate in the mechanism of phytochrome action. Subsequently, a

number of publications have confirmed Jaffe's discovery; however, data have also been published demonstrating lack of correlation between ACh and phytochrome action (for review see: Fluck & Jaffe, 1976; Hartmann & Gupta, 1989). This discrepancy in results was one of the main reasons for the decline in research on the role of ACh in plants. Recently, however, new observations have been published which give a better understanding of ACh action in plant cells.

IV. Isolation, Purification and Determination of Acetylcholine in Plants

A. CHEMICAL PROPERTIES

Acetylcholine [$CH_3COOCH_2CH_2N^+(CH_3)_3$], a quaternary amine is an ester of acetic acid and choline. Its molecular mass is 146.2. The quaternary ammonium nitrogen of ACh is positively charged over a wide pH range. All ACh salts are readily soluble in water, their solubility decreasing with decrease of the solvent's polarity, being insoluble in apolar solvents. The stability of ACh in solution depends on pH and temperature. At pH 4 maximal stability is observed and it can then be subjected to 'hot' sterilization. The stability of ACh solutions decreases with rising pH, and in basic solutions the substance hydrolyzes rapidly to choline and acetic acid. At a temperature of 25°C the half-life of decomposition of a 0.1 molar solution at pH 7.0 is about 20 days, whereas at pH 12 it is only 12 seconds (Potter, 1968). The positive charge of ACh ammonium nitrogen, besides affecting its solubility, gives it the properties of a monovalent cation. Moreover, the presence of the positive charge and of an apolar carbon chain means that the ACh molecules also have the properties of a detergent. Both these properties only manifest themselves in biological systems at an ACh concentration of 1 millimolar or higher (Fluck & Jaffe, 1976).

B. EXTRACTION, PURIFICATION AND DETERMINATION

Initially only pharmacologists investigated ACh in plants, introducing a number of methods of extraction, purification and quantitative determination.

1. Extraction

The simplest way of extracting ACh is by immersing squashed tissues in water. After centrifugation of the homogenate the presence of ACh in the supernatant can be determined by a bioassay (Devasankaraiah et al., 1974; Horton & Felippe, 1973). Other agents used for ACh extraction from plant material are: Ringer solution (Jaffe, 1970, 1972; Kopcewicz et al., 1977; Satter et al., 1972), methanol (Appel & Werle, 1959; Devasankaraiah et al., 1974, Hartmann, 1971; Tulus et al., 1961; Verbeek & Vendrig, 1977), a mixture of 80% ethanol and 2% acetic acid (Hartmann & Kilbinger, 1974a), ethanol (Tulus et al., 1961) and acetone (Saxena et al., 1966).

The following acids are most commonly used for isolation of ACh from plant material: 1 N perchloric (Hartmann & Kilbinger, 1974b; Miura & Shih, 1984; Tretyn et al., 1987), 1 N hydrochloric (Saxena et al., 1966), 1 N formic (Hoshino, 1983a, 1983b) and of a mixture of formic acid with acetone (Hoshino, 1983b; Hoshino & Oota, 1978; Tretyn et al., 1987) or acetonitrile (Jones & Stutte, 1985).

Isolation of ACh is very difficult to perform when the samples of biological material

are small. In such cases several chemical compounds are used which precipitate ACh from the solution, such as sodium tetraphenylboron (Hoshino, 1983b), J_2 + KJ (Tretyn et al., 1987) or ammonium reineckate (Miura & Shih, 1984; Tretyn et al., 1987).

2. Purification

Chromatographic methods for ACh purification from an animal source are: paper electrophoresis (Haubrich & Reid, 1974) which enables easy separation of a mixture of different choline esters (Ladinsky & Consolo, 1974); paper and thin-layer chromatography (Potter, 1968); and ion-exchange chromatography (Stein, 1981) which enables dilute ACh solutions to be isolated and concentrated on cationic resins which contain free carboxyl groups (Potter, 1968).

Only paper chromatography has been used in purification of ACh isolated from plants. Most commonly used paper for this purpose is Whatman No. 1 (Devasankaraiah et al., 1974; Jaffe, 1970; Tulus et al., 1961) or Whatman No. 3 MM (Hoshino, 1983b; Hoshino & Oota, 1978). The chromatograms are developed in one dimension using eluents composed of a mixture of acids, alcohols and water (Jaffe 1970, 1972; Hoshino, 1983b).

3. Determination

Acetylcholine isolated from biological material can be determined quantitatively in a quick and simple manner by using the method based on the reaction of ACh with hydroxylamine. However, this method has only occasionally been used for ACh determination in plant extracts (Marquardt & Falk, 1957; Roshchina & Mukhin, 1985). It is characterized by high specificity but relatively low sensitivity.

a. Bioassays

To date, the most commonly used methods of quantitative determination of ACh in plant material are bioassays, which utilize the susceptibility of isolated animal organs, tissues or whole animals to ACh. As a rule, studies concern the effect of ACh on: i) the contractions of skeletal muscles or of the heart muscle, ii) the contractions of various fragments of the alimentary canal, and iii) the reduction of arterial blood pressure.

Besides ACh, there are other pharmacologically active substances occurring in plants (Appel and Werle, 1959) which affect the sensitivity of such bioassays, such as histamine. Supplementary experiments are therefore often necessary to make sure that only ACh is active in the bioassay used. The most commonly used method is the so-called 'eserinization' of the tissue preparation (Hoshino & Oota, 1978; Jaffe, 1970; Kopcewicz et al., 1977). If the tissue is placed in a solution of eserine, an AChE inhibitor, susceptibility to ACh is increased (Jaffe, 1970). Atropine, whose action is antagonistic to ACh, causes an immediate fall in the bioassay's sensitivity (Jaffe, 1970). Inactivation of the ACh-like activity of the extract is obtained by treating it with AChE (Jaffe, 1970; Kopcewicz et al., 1977) or with a strong alkali solution (Horton & Felippe, 1973; Hoshino & Oota, 1978).

b. Gas Chromatography

For gas chromatographic determination of quaternary amines such as ACh, it is necessary to reduce them to volatile derivatives. Volatile derivatives of ACh can be obtained by pyrolysis or by N-demethylation.

The method of thermal (pyrolytic) ACh decomposition described by Szilagyi et al. (1968) is generally used for ACh determination in animal tissues. Pyrolysis is run in a special device coupled to a gas chromatograph. The sample is heated for 15 seconds to a temperature of 450°C in a stream of nitrogen which carries the volatile product, dimethylaminoethyl acetate, through the column of the chromatograph (Green & Szilagyi, 1974). Despite its simplicity and high sensitivity, the pyrolytic method has not yet been used to analyze choline esters isolated from plants.

The reduction of ACh to volatile dimethylaminoethyl acetate is carried out by N-demethylation, in which a mixture of thiophenol and sodium thiophenolate (benzenthiolate) is used. The reaction is carried out in an anaerobic atmosphere in a medium of anhydrous butanone (Jenden & Hanin, 1974) or acetone (Tretyn et al., 1987). In a subsequent step byproducts of the reaction are removed and the dimethylaminoethyl acetate is condensed and extracted into chloroform or dichloromethane (Jenden & Hanin, 1974; Tretyn et al., 1987).

Chromatographic determination of ACh in plant extracts was first introduced simultaneously by Devasankaraiah et al. (1974) and Hartmann and Kilbinger (1974a, 1974b). The authenticity of the ACh isolated from plants was demonstrated using a mass spectrograph coupled with a chromatograph (Miura & Shih, 1984).

c. Other Methods

As well as mass spectroscopy coupled with gas chromatography, the presence of ACh in extracts has been analyzed using the so-called 'field desorption mass spectroscopy' (Hoshino, 1983b). The ACh-like substance isolated from *Vigna* seedlings exhibited a similar spectroscopic spectrum to that of authentic ACh.

The presence of protons diversely bound with other atoms of the ACh molecule makes it possible to identify this compound by means of nuclear magnetic resonance (^{1}H-NMR) (Tretyn et al., 1987). The protons bound with three N-methyl groups of choline and ACh absorb radio frequencies with a chemical shift similar for the two compounds. The presence of ACh in a plant extract can be detected by observing the signal of the protons bound with the acetate group of the compound (Tretyn et al., 1987).

Besides those described, a number of other chemical methods are used for the quantitative determination of choline esters occurring in biological material. In many of them ACh is not analyzed directly, but by the compounds obtained as a result of its degradation, such as ethanol, acetic acid, and above all hydrogen peroxide (H_2O_2) (Hanin, 1974). Some of the best results are obtained when using high pressure liquid chromatography (HPLC) (Damema et al., 1985).

V. Presence of Acetylcholine throughout Plant Kingdom

Acetylcholine has been found in the tissues of more than 50 plant species belonging to all the major systematic groups (Table I).

Table I

Occurrence of acetylcholine (ACh) in higher plants

Family	Species	Site	Reference
Amaranthaceae	*Amaranthus caudatus* L.	aerial parts	Hartmann & Kilbinger (1974b)
Anacardiaceae	*Rhus copallina* L.	leaves	Miura & Shih (1984)
Aquifoliaceae	*Ilex opaca* Ait.	leaves	Miura & Shih (1984)
Betulaceae	*Betula pendula* Roth.	leaves	Miura & Shih (1984)
Caprifoliaceae	*Lonicera japonica* Thunb.	leaves	Miura & Shih (1984)
	Viburnum dilatatum Thunb.	leaves	Miura & Shih (1984)
Chenopodiaceae	*Spinacia oleracea* L.	leaves, shoots	Appel & Werle (1959) Hartmann & Kilbinger (1974b)
Compositae	*Helianthus annuus* L.	shoots, roots	Hartmann & Kilbinger (1974b)
	Porophyllum lanceolatum DC	leaves, shoots, roots	Horton & Felippe (1973) Ledeira et al. (1982b)
	Xanthium strumarium L.	shoots, roots	Ledeira et al. (1982b)
Cruciferae	*Brassica oleracea* v. *gongylodes* L.	aerial parts	Holtz & Janisch (1937)
	B. oleracea v. *napobrassica* L.	aerial parts	Holtz & Janisch (1937)
	Capsella bursa-pastoris L.	aerial parts	after Marquardt & Falk (1957)
	Sinapis alba L.	shoots, roots	Hartmann & Kilbinger (1974b) Ledeira et al. (1982a)
Cucurbitaceae	*Cucumis anguria* L.	aerial parts	Ledeira et al. (1982b)
	C. sativus L.	aerial parts	Holtz & Janisch (1937) Verbeek & Vendrig (1977)
	Cucurbita pepo L.	shoots, roots	Hartmann & Kilbinger (1974b)
Euphorbiaceae	*Codiaeum variegatum* Blume.	leaves	Miura & Shih (1984)
Gramineae	*Avena sativa* L.	aerial parts	Tretyn & Tretyn (1990)
	Stipa tenacissima L.	leaves	Antweiler & Pallade (1972)
	Zea mays L.	leaves	Miura & Smith (1984)
Hamamelidaceae	*Liquidambar styraciflua* L.	leaves	Miura & Shih (1984)
Leguminosae	*Albizia julibrissin* Durazz.	leaves, seeds	Satter et al. (1972)
	Vigna sesquipedalis (L.) Fruw.	hypocotyl	Hoshino (1983b)
	Phaseolus aureus Roxb.	shoots, roots, seeds	Jaffe (1970) Miura & Shih (1984)
	P. vulgaris L.	shoots, roots	Hartmann & Kilbinger (1974b)
	Pisum sativum L.	leaves, shoots, roots, seeds	Hartmann & Kilbinger (1974b) Jaffe (1972) Miura & Shih (1984) Roshchina & Mukhin (1985)
Lemnaceae	*Lemna gibba* G3 L.	whole plants	Hoshino & Oota (1978)
Loranthaceae	*Viscum album* L.	shoots	after Marquardt & Falk (1957)
Moraceae	*Artocarpus champeden* Merr.	leaves, seeds, fruits	Lin (1955)
	A. integra Merr.	leaves, seeds, fruits	Lin (1957)
Pinaceae	*Pinus silvestris* L.	aerial parts	Kopcewicz et al. (1977)
Plantaginaceae	*Plantago regelii* Decne.	leaves	Miura & Shih (184)
Polygonaceae	*Rumex obtusifolius* L.	aerial parts	Ledeira et al. (1982b)
Rosaceae	*Crataegus oxyacantha* L.	leaves, flowers, fruits	Fiedler et al. (1953)
	Prunus serotina Ehrh.	leaves	Miura & Shih (1984)

Table I
Continued

Family	Species	Site	Reference
Salicaceae	*Populus grandidentata* Michx.	leaves	Miura & Shih (1984)
Scrophulariaceae	*Digitalis ferruginea* L.	shoots	Tulus et al. (1961)
	D. lauta L.	leaves	Neuwald (1952)
	D. purpurea L.	leaves	Neuwald (1952)
Smilacaceae	*Smilax hispida* Muhl.	leaves	Miura & Shih (1984)
Solanaceae	*Solanum tuberosum* L.	tubers	Marquardt et al. (1952) Oury & Bacq (1938)
Umbelliferae	*Daucus carota* v. *sativa* L.	leaves	Holtz & Janisch (1937)
	Carum copticum Benth.	seeds	Devasankaraiah et al. (1974)
Urticaceae	*Girardinia heterophylla* Gandich	leaves	Saxena et al. (1966)
	Urtica dioica L.	leaves, shoots, roots	Collier & Chesher (1956) Emmelin & Feldberg (1949)
	U. parviflora Roxb.	leaves, stinging hairs	Saxena et al. (1965)
	U. urens L.	leaves, shoots, roots	Emmelin & Feldberg (1947)

VI. Distribution of Acetylcholine within the Plant

Acetylcholine has been detected in fungal hyphae (Ewins, 1914; Heirman, 1939; Oury & Bacq, 1938), in callus cultures of bryophytes (Hartmann, 1971; Hartmann & Kilbinger, 1974a), and in the aerial parts (Hartmann & Kilbinger, 1974b; Jaffe, 1970; Kopcewicz et al., 1977; Ledeira et al., 1982a; Lin, 1957; Miura & Shih, 1984; Tretyn & Tretyn, 1989), tubers (Marquardt et al., 1952) and roots (Emmelin & Feldberg, 1947, 1949; Hartmann & Kilbinger, 1974b; Jaffe, 1970, 1972; Ledeira et al., 1982b; Miura & Shih, 1984) of higher plants (Table I). In the aerial parts of angiosperms, it has been found in stems (Emmelin & Feldberg, 1947, 1949; Hartmann & Kilbinger, 1974b; Jaffe, 1970, 1972; Ledeira et al., 1982b; Miura & Shih, 1984), hypocotyls (Hoshino, 1983b; Jaffe, 1970, 1972; Verbeek & Vendrig, 1977), leaves (Antweiler & Pallade, 1972; Appel & Werle, 1959; Collier & Chesher, 1956; Emmelin & Feldberg, 1947, 1949; Fiedler et al., 1953; Hartmann & Kilbinger, 1974b; Horton & Felippe, 1973; Jaffe, 1970, 1972, Ledeira et al., 1982b; Lin, 1957; Miura & Shih, 1984; Neuwald, 1952; Satter et al., 1972; Saxena et al., 1965, 1966; Tretyn & Tretyn, 1990), apical buds (Jaffe, 1970; Lin, 1957), flowers (Fiedler et al., 1953) and seeds (Devasankaraiah et al., 1974; Ledeira et al., 1982a; Lin, 1957; Miura & Shih, 1984).

Precise analysis of ACh distribution in plants has been carried out in 4 species: *Urtica dioica* L. and *U. urens* L. (Emmelin & Feldberg, 1949), *Artocarpus integra* Merr. (Lin, 1957), and *Phaseolus aureus* Roxb. (Jaffe, 1970). In *Urtica urens* L. ACh had a similar concentration in all organs, while in *Urtica dioica* L. ACh levels differed in different plant organs. The highest ACh concentration was found, besides in the stinging hairs, in the cortex and phloem, and the lowest in the pith of the apical parts of nettle shoots (Emmelin & Feldberg, 1949).

In *Artocarpus integra* Merr (Lin, 1957) the highest ACh concentration was found in the youngest leaves. Within the leaf, a higher ACh level was noted in the nerve

(vascular tissues) than in the lamina. In the leafless shoot of *A. integra* the highest ACh concentration was observed in the pith, and the lowest in the xylem. In the root, the highest ACh level was noted in the phloem, a lower one in the cortex, and the lowest in the xylem and pith. In all the *A. integra* organs studied there was a correlation between ACh level and the age of the organs. Both in the aerial parts and in the roots the highest ACh concentration was found in the youngest parts of the organs (Lin, 1957). Lin's suggestion that an ACh concentration gradient exists in the aerial and underground parts of plants has been confirmed by Jaffe (1970). He demonstrated that in *Phaseolus aureus* the highest ACh concentration occurs in the apical buds of shoots in the root tips and in the youngest leaves.

Acetylcholine concentration in plants varies from fractions of nanomols (nanograms) (Miura & Shih, 1984) to hundreds of micromols (micrograms) (Jaffe, 1970; Lin, 1957) per gram fresh weight of the tissue under study. The plants in which the highest ACh concentration has been found are different nettle species (Collier & Chesher, 1956; Emmelin & Feldberg, 1947, 1949; Saxena et al., 1965), and the cells with the highest ACh content are the stinging hairs (Collier & Chesher, 1956; Saxena et al., 1965). Large amounts of ACh are contained in many other medicinal plants, such as *Viscum album* L., *Digitalis purpurea* L., *D. lanata* L. (Neuwald, 1952), *D. ferruginea* L. (Tulus et al., 1961) and *Carum copticum* Beuth. (Devasankaraiah et al., 1974).

VII. Metabolism of Acetylcholine in Plants

Acetylcholine isolated from plant material shows identical chemical properties to that occurring in the nerve cells.

A. SYNTHESIS OF ACETYLCHOLINE

The universal presence of ACh in plant tissues points to the existence of enzymes involved in its synthesis. In animals ACh is synthesized from acetyl-Coenzyme A (acetyl-CoA) and choline with the participation of ChAT [E.C. 2.3.1.6] (Dunat & Israel, 1985).

Choline as well as acetyl-CoA has also been detected in plants (Dasgupta, 1966; Engel, 1943; Iurisson & Iurisson, 1966; Miura & Shih, 1984; Tretyn & Tretyn, 1988; Tretyn et al., 1987). In extracts from etiolated bean seedlings the activities of choline kinase (E.C. 2.7.1.32), phosphorylcholine-cytidyl transferase (E.C. 2.7.7.15), and phosphorylcholine-diglyceride transferase (E.C. 2.7.8.2) have been found (Hock & Hartmann, 1981). These enzymes catalyze transformation of choline to phosphatidylcholine, which is one of the major phospholipids in biological membranes (Hock & Hartmann, 1981).

1. Occurrence and Characterization of Plant Choline Acetyltransferase

Preliminary attempts to detect ChAT activity in plants failed (Riov & Jaffe, 1972a). The first researchers to detect this enzyme in plant tissues were Barlow & Dixon (1973), using buds and young leaves of nettle *Urtica dioica* L., which are extremely rich in ACh (Collier & Chesher, 1956; Emmelin & Feldberg, 1949) yet devoid of AChE activity (Emmelin & Feldberg, 1947). Choline acetyltransferase activity in *Urtica dioica* L. was determined by measuring the rate of ACh synthesis from its

precursors, choline and acetyl-CoA. The concentration of the ACh developed was determined using a bioassay (Barlow & Dixon, 1973) or a radioisotope method, determining the amount of ^{3}H-acetyl-CoA incorporated in ACh (Smallman & Maneckjee, 1981). The specific activity of the enzyme isolated from *Urtica dioica* was 1067 millimols of ACh synthesized in 1 minute by 1 milligram of protein (Smallman & Maneckjee, 1981). Maximum ChAT activity was found at 40°C (Barlow & Dixon, 1973; Smallman & Maneckjee, 1981) and pH 9.0 (Barlow & Dixon, 1973). The highest enzyme activity was exhibited in extracts from the youngest parts of nettle, i.e., from buds and young leaves (Barlow & Dixon, 1973; Smallman & Maneckjee, 1981). The specific activity of the enzyme isolated from young nettle leaves was similar to that exhibited by ChAT isolated from neural tissues of insects (Smallman & Maneckjee, 1981).

Choline acetyltransferase activity has also been found in pea (*Pisum sativum*), spinach (*Spinacia oleracea* L.), sunflower (*Helianthus annuus* L.) and a blue green alga (*Oscillatoria agardhii*) (Smallman & Maneckjee, 1981), as well as in seeds of plants of the genus *Allium* (Hadačova et al., 1981). The specific activity of the enzyme isolated from these plants was about 100 times lower than that of ChAT obtained from nettle tissues (Smallman & Maneckjee, 1981).

2. *Subcellular Localization of Sites of Acetylcholine Synthesis*

There is little information concerning the sites of ACh synthesis in plant cells since only Jaffe (1976) and Hartmann (Hartmann, 1979) have studied this problem. Using vesicles obtained from endoplasmic reticulum membranes Jaffe demonstrated the different effects of R and FR on ACh synthesis. Vesicles irradiated with R were found to incorporate labelled ACh precursors at a much higher rate than those treated with FR. The rate of ACh synthesis in the presence of 0.5% Triton X-100 after R irradiation was about 100 times higher than that found in non-irradiated control vesicles (Jaffe, 1976).

Hartmann (1979) demonstrated that the incorporation of labelled choline into ACh is much slower than that of labelled acetate. Six-day-old etiolated bean seedlings incorporated labelled acetate only while irradiated with R. Glucose, a convenient precursor for ACh synthesis in animals, was virtually unused for ACh synthesis in bean seedling tissues (Hartmann, 1979). This may suggest that, as in neurons of the electric organ of *Torpedo,* the acetate residue in bean cells is used for ACh synthesis. In *Torpedo* the activity of acetyl-CoA synthetase has been detected, which catalyzes the transformation of acetate into acetyl-CoA (Israël & Manaranche, 1985). Acetyl-CoA synthase and ChAT are present in the cytosol.

In *Phaseolus aureus* root tips ACh synthesis presumably takes place in the membranes of the endoplasmic reticulum (Jaffe, 1976). However, in *Phaseolus vulgaris* L. the highest ChAT activity has been detected in the cytosol (57% of total activity), which may indicate that, at least in the bean species, as in animals (Israël & Manaranche, 1985), ACh synthesis takes place in the cytosol (Hartmann et al., 1981). However, in plants, a specific mechanism controlling ACh synthesis may function. It follows from the data obtained by Hartmann (1979) and Jaffe (1976) that both the incorporation of radioactive precursor into ACh and the rate of ACh synthesis in isolated cisternae of endoplasmic reticulum are controlled by phytochrome. Roshchina and Mukhin (1985) suggest that in pea the site of ACh synthesis may be the chloroplasts.

3. *Regulation of Acetylcholine Level*

Lin (1957), and Jaffe (1970, 1972), found the highest ACh concentration in young growing parts of plants. ACh level varies depending on the developmental phase of the plant and on environmental conditions (Jaffe, 1970; Tretyn & Tretyn, 1990). It has been found that the ACh concentration in bean tissues is much higher in green seedlings than in etiolated ones. Five-minutes R irradiation of isolated organs of this plant causes a sharp rise in ACh content. Far-red light applied immediately after R neutralizes the stimulatory effect of R, causing a rapid a fall of ACh content in bean root tips (Jaffe, 1972). Investigation of the effect of R and FR on ACh content has demonstrated that the level in bean root tips is under phytochrome control (Jaffe 1970, 1972). Hartmann (1971), and Hartmann and Kilbinger (1974a, 1974b) confirmed that phytochrome may play a role in the regulation of ACh synthesis in bryophytes and angiosperms. A similar system regulating ACh content in a representative of gymnosperms, the Scots pine, has been described by Kopcewicz et al. (1977).

White light also affects the level of endogenous ACh in plants. Miura and Shih (1984) have established that white light stimulates an increase in ACh level in leaves and a decrease in the stem of bean seedlings compared with non-irradiated plants. A similar stimulatory effect of white light on ACh concentration in etiolated and green seedlings has been described (Tretyn, 1987; Tretyn & Tretyn, 1990). These workers have also demonstrated that both white and R affect the rate of ACh synthesis in etiolated oat seedlings in a similar way (Tretyn & Tretyn, 1990).

It is presumed that the ACh level in plants is regulated by the phytochrome system by modification of AChR and ChAT activities (Jaffe, 1972, 1976; Jaffe & Fluck, 1972; Tretyn & Tretyn, 1990).

B. DEGRADATION OF ACETYLCHOLINE

In animal cells ACh can be hydrolyzed by a specific acetylcholinesterase (E.C. 3.1.1.7). Furthermore, in non-neural cells ACh may be hydrolyzed by pseudocholinesterase or by so-called butyrylcholinesterase (E.C. 3.1.1.18) (Oosterbaan & Janisz, 1965; Potter, 1968). An enzyme which decomposes ACh has also been detected in plants. It was first found in *Nitella* cells (Dettbarn, 1962) and in representatives of three families: Leguminosae, Cruciferae, and Solanaceae (Fluck & Jaffe, 1974b). Miura et al. (1982) studying 70 plant species belonging to 50 families of higher plants and 3 fern families detected cholinesterase (ChE) activity in all except 6 species. In monocotyledonous plants it was found in etiolated maize and oat seedlings (Fluck & Jaffe, 1974a; Tretyn & Tretyn, 1990), in dry seeds of 22 *Allium* species (Hadačova et al., 1981, 1983) and the caryopses of wheat (Tretyn et al., 1986). To date ChE activity has been found in the tissues of more than 100 plant species. However, the absence of ACh hydrolysis has been reported in 65 plants species (Hartmann & Gupta, 1989).

1. *Biochemical Characterization of Cholinesterases*

The standard method of extraction and purification of ChE from plant material has been described by Riov and Jaffe (1973a) for bean (*Phaseolus aureus*) roots. This method is still used with certain modifications of the biochemical techniques. The

Table II

Comparison of plant cholinesterases (ChEs) and animal acetylcholinesterases (AChEs)

Property	Plant ChE's	Animal AChE's
Localization	Membranes, cell wall	Membranes
Molecular weight (kilodaltons)	>200 or >800 or <80	Monomers? Dimers 118–160 Tetramers 290–460 Protomers 66–80
Tendency for aggregation	+	+
Hydrolysis of choline esters	Acetyl > Propionyl > Butyryl	Acetyl > Propionyl > Butyryl
Hydrolysis of acetyl-β-methylacetate	+	+
Hydrolysis of non-choline esters	+	+
K_m for acetylcholine (molar)	5.6×10^{-5}–4.6×10^{-4}	10^{-4}–3.0×10^{-4}
Optimum pH	8.0–9.0	8.0–8.3
Effects of ions	Inhibition by Mn^{2+} and Ca^{2+}, no effect of Mg^{2+}	Stimulation by Mn^{2+}, Ca^{2+}, Mg^{2+}
Effect of substrate concentration	Inhibition by excess or no inhibition	Inhibition by excess
Effect of choline	Inhibition or stimulation	Inhibition
Inhibitors:		
neostigmine	+++	+++
eserine	+	+++
ambenonium	+++	+++
BW 284 C 51	+	+++
organophosphates	+++	+++

enzyme is extracted from tissues using highly concentrated ammonium sulfate [$(NH_4)_2SO_4$] at 4°C and then purified by precipitation with the same salt and filtered through a Sephadex column.

Plant ChE can hydrolyze various choline esters, the rate of hydrolysis decreasing as the acidic chain increases in length. The model of variations in the reaction rate is as follows:

A > P > B (Riov & Jaffe, 1973a, 1973b) or

A > P >> B (Ernst & Hartmann, 1980; Gupta & Maheshwari, 1980), in *Pisum sativum* (Kasturi & Vasantharajan, 1976): where A is the choline ester of acetic acid, P is the ester of propionic and B is the ester of butyric acid.

Cholinesterase isolated from plants is also capable of decomposing non-choline esters, hydrolyzing indophenol acetate relatively actively (Kasturi & Vasantharajan, 1976; Riov & Jaffe, 1973a, 1973b) and α-naphthyl acetate to a lesser extent (Kasturi & Vasantharajan, 1976).

Optimum pH for plant ChE varies between 8.0 and 9.0 depending on the enzyme's source (Ernst & Hartmann, 1980; Fluck & Jaffe, 1975; Kasturi & Vasantharajan, 1976). The differences in optimum pH determined may be due to the rather high autocatalyzed hydrolysis of choline esters at pH above 8.0, taking place while the enzyme's activity is being determined. The optimum temperature for plant ChE is 30–36°C (Ernst & Hartmann, 1980; Kasturi & Vasantharajan, 1976). Effect of ions on plant ChE activity was studied by Ernst and Hartmann (1980). They found that

magnesium ions (Mg^{2+}) at 1–10 millimolar did not cause significant changes in the enzyme's activity, while manganese ions (Mn^{2+}) and calcium ions (Ca^{2+}) strongly inhibited it. The enzyme's affinity for the substrate is defined by the Michaelis constant (K_m). The K_m of plant ChE determined by the Lineweaver-Burk plot was from 5.6×10^{-5} molar [*Phaseolus vulgaris* roots (Mansfield et al., 1978)] to 4.6×10^{-4} molar [*Phaseolus vulgaris* hypocotyls (Ernst & Hartmann, 1980)]. The K_m for the enzyme from *Phaseolus aureus* was $7.2–8.4 \times 10^{-5}$ molar (Riov & Jaffe, 1973a), from *Cicer arietinum* 1.5×10^{-4} molar and from *Pisum sativum* 2.0×10^{-4} molar (Kasturi & Vasantharajan, 1976).

The mechanism of enzymatic hydrolysis of ACh in plant cells seems similar to that observed in animal cells (Riov & Jaffe, 1973a). In the active center there are two sites, one an anion site, which attracts the positively charged nitrogen atom of ACh, and one an ester site, at which the substrate is hydrolyzed.

Besides the inhibitory effect on plant ChE activity of excess substrate (Fluck & Jaffe, 1975; Kasturi & Vasantharajan, 1976; Mansfield et al., 1978; Riov & Jaffe, 1973a) and the ambivalent effect of choline (Ernst & Hartmann, 1980; Fluck and Jaffe, 1975; Riov & Jaffe, 1973a), there are other fairly specific inhibitors. Some are competitive inhibitors, i.e., they are treated by ChE as substrate and hydrolyzed at comparatively low concentration. One of them is neostigmine, a well known inhibitor of animal AChE. Half of the maximum inhibition (I_{50}) of plant ChE by this compound has been obtained at a concentration of 0.6×10^{-6} molar (Kasturi & Vasantharajan, 1976; Riov & Jaffe, 1973a, 1973b), which is comparable with the inhibition of animal AChE (Ott, 1985). Another classical inhibitor of animal ChE is eserine. A concentration of 10^{-5} molar, which inhibits completely (I_{100}), fails when applied to plant ChE, inhibiting activity by only several per cent (Riov & Jaffe, 1973a). Half of the maximum inhibition by eserine is obtained only if its concentration is from 10^{-4} to 6×10^{-3} molar (Ernst & Hartmann, 1980; Gupta & Maheshwari, 1980).

Riov and Jaffe (1972b, 1973a, 1973b) studied the effects of other inhibitors on the activity of ChE isolated from *Phaseolus aureus* roots. Ambenonium and BW 284 C 51, specific inhibitors of AChE, as well as ethopropazine hydrochloride, a non-specific inhibitor of ChE, only actively inhibited the plant enzymes at high concentration (10^{-2} to 10^{-3} molar). However, growth retardants Q 80 and AMO 1618 in the same concentration range were much more effective, resulting in 80% inhibition of activity.

Tests concerning plant ChE have also been carried out on organophosphate inhibitors, which constitute the base of many pesticides. Their action results from the stable phosphorylation of the enzyme at the ester site of the active center. As with animal ChE, those compounds proved to be very strong inhibitors of plant ChE. Paraoxon, for example, gives I_{50} at 10^{-5} molar and Fensulfothion at 10^{-4} molar (Kasturi and Vasantharajan, 1976). The compound Dip-F has been found to be a very effective inhibitor of plant ChE activity; at 10^{-4} molar the inhibition was complete (Mansfield et al., 1978).

2. *Localization of (Acetyl)cholinesterase in Cells*

Cholinesterases are extracted from plant tissues using high ionic strength solutions of $(NH_4)_2SO_4$ (4%) or 6×10^{-1} molar potassium chloride (KCl) (Riov & Jaffe, 1973a). This suggests that these enzymes are strongly associated with membranes. However, the $(NH_4)_2SO_4$ releases only part of the enzyme activity. The enzymes from *Solanum melongena* L. and *Zea mays* L. can be extracted with a low ionic strength buffer,

without $(NH_4)_2SO_4$ (Fluck & Jaffe, 1975). This may be evidence of structural differences of the enzymes, or of their different, subcellular localization.

Using Karnovsky and Root's (1964) cytochemical method Fluck and Jaffe (1974a) have found that in 12-day-old *Phaseolus aureus* roots the products of the ChE enzymatic reaction appear in the cell walls and the space between the cell wall and plasma membrane. A similar localization of AChE activity has been described in etiolated oat coleoptiles by Tretyn and Tretyn (1990). However, in the wheat aleurone cells the product of the enzymatic reaction was observed only on the external side of the plasma membrane, this being confirmed by scanning electron microscopy (Tretyn et al., 1986). Recently Bednarska and Tretyn (1989) have found AChE activity on the surface of *Pharbitis nil* (L.) Choisy stigmas.

No AChE activity has been found in the plasma membrane of bean protoplasts (Hock, see Hartmann & Gupta, 1989). Tretyn and Kendrick (unpubl.) also found no activity of this enzyme on the plasma membrane surface of etiolated wheat protoplasts, the activity being localized inside the proplastids (Tretyn & Kendrick, unpublished). However AChE was reported in pea chloroplasts by Roshchina and Mukhin (1985) and Roshchina (1988).

3. Comparison of Plant (Acetyl)cholinesterase and Animal Acetylcholinesterase

Plant ChEs are not identical to animal AChEs, although they have many similarities (Table II). It seems particularly interesting that in both cases the enzymes are localized with membranes. There are also a number of molecular and biochemical properties pointing to a similarity between plant ChEs and animal AChEs. The molecular mass of plant ChEs is higher than 200,000 or 800,000 daltons or lower than 80,000. These values are comparable to the molecular masses of polymers and protomers of animal AChEs (Ott, 1985). Another common feature of these enzymes is their tendency to aggregation in vitro. Plant ChEs have a high specific affinity for ACh and therefore their preference for hydrolyzing it above other choline esters. The membrane localization of plant ChEs as well as their molecular and biochemical properties indicate that these enzymes are AChEs.

The fact that plant and animal ChEs are so similar suggests they perform the same function in regulating the level of ACh in plants and animals. The difference between these enzymes can be accounted for by evolutionary variation. This suggestion is supported by the fact that enzymes showing properties intermediate between animal and plant ChE have been found in some invertebrates (Ott, 1985).

C. ACETYLCHOLINE RECEPTORS

No direct evidence has yet been obtained to confirm the presence of AChRs in plants. Fluck and Jaffe (1976) assumed that plant AChRs may have two types of biochemical function. They may show the properties of a non-enzymatic regulatory protein controlling, e.g., membrane permeability, or they may be linked with enzymes controlling specific metabolic pathways. In his preliminary studies on extracts from bean roots Jaffe (see: Fluck & Jaffe, 1976) demonstrated the presence of ACh binding sites. Hartmann et al. (1981; see also Hartmann & Gupta, 1989) found high affinity of ACh to the proteins of the cell wall fraction isolated from *Phaseolus vulgaris*. They also found AChE and ChAT activity in the extract. However, neither Jaffe nor

Table III

List of cholinergic agents, their source of origin and mechanism of action

Agent	Source	Mode of action
Eserine	Calabar bean *Physostigma venenosum* Balf.	Inhibitor of acetylcholine (ACh) esterase (AChE)
Nicotine	Tobacco *Nicotiana tabacum* L.	Agonist of nicotinic ACh receptors (nAChR's)
Muscarine	Fly agaric fungus *Amanita muscaria* (L. ex Fr.) Hooker	Agonist of muscarinic ACh receptors (mAChR's)
D-Tubocurarine	Indian arrow poison *Chondodendron tomentosum*	Specific antagonist of nAChR's
Atropine	Deadly nightshade *Atropa belladonna* L. Jimson weed *Datura stramonium* L.	Specific antagonist of mAChR's
α-Bungarotoxin	Protein snake toxin *Bungarus multicinctus*	Very specific toxin antagonist of nAChR's, binding specifically with nAChR's

Hartmann et al. have carried out a biochemical characterization of the 'ACh binding sites.'

The use of AChR antagonists (atropine, D-tubocurarine) and agonists (muscarine, nicotine) (Table III and Fig. 1) of ACh has provided indirect evidence of the existence of AChRs in plants. Atropine increases the adenosine triphosphate (ATP) level in bean buds (Kirshner et al., 1975) and the activity of choline kinase (Hartmann & Schleicher, 1977), neutralizes the stimulating effect of R and ACh on the changes in growth pattern of *Vigna* seedlings (Hoshino, 1983a), counteracts the ACh-stimulated shrinking of the cells of bean pericycle (Toriyama, 1978), nullifies the indole-3-acetic acid (IAA)-stimulated flowering in *Lemna gibba* G3 (Hoshino, 1979) and inhibits the incorporation of the radioisotope of phosphorus (^{32}P) into the phospholipids of bean hypocotyls (Hartmann et al., 1980). On the other hand, it slightly reduces the gibberellin-stimulated elongation of cucumber hypocotyls (Verbeek & Vendrig, 1977), has no effect on auxin-stimulated elongation of oat coleoptile sections (Evans, 1972), no effect on the synthesis of flavonoids in barley tissues (Saunders & McClure, 1973) and no effect on the germination of photoblastic seeds of *Rumex obtusifolius* L. and *Cucumis anguria* L. (Ledeira et al., 1982a). It has also been found that D-tubocurarine inhibits R-stimulated uptake of sodium acetate by isolated bean root tips (Jaffe & Thoma, 1973), while nicotine induces morphogenesis of tobacco roots (Peters et al., 1974). Muscarine and atropine at concentrations equal to or higher than 1 nanomolar inhibited by up to 50% the rate of nicotinimide adenine dinucleotide phosphate ($NADP^+$) photoreduction and of noncyclic phosphorylation in pea chloroplasts (Roshchina, 1987). The inhibition of both these processes by D-tubocurarine was much weaker. Muscarine, like ACh, stimulated 15- and 2-fold efflux of sodium ions (Na^+) and potassium ions (K^+) from chloroplasts respectively, while atropine and D-tubocurarine slightly affected the transport of both cations across the membranes of isolated chloroplasts (Roshchina, 1987). Roshchina (1987) suggests that AChRs in pea chloroplast membranes regulate their permeability to ions. She proposes that these AChRs are different from those found in animals, having molecular and bio-

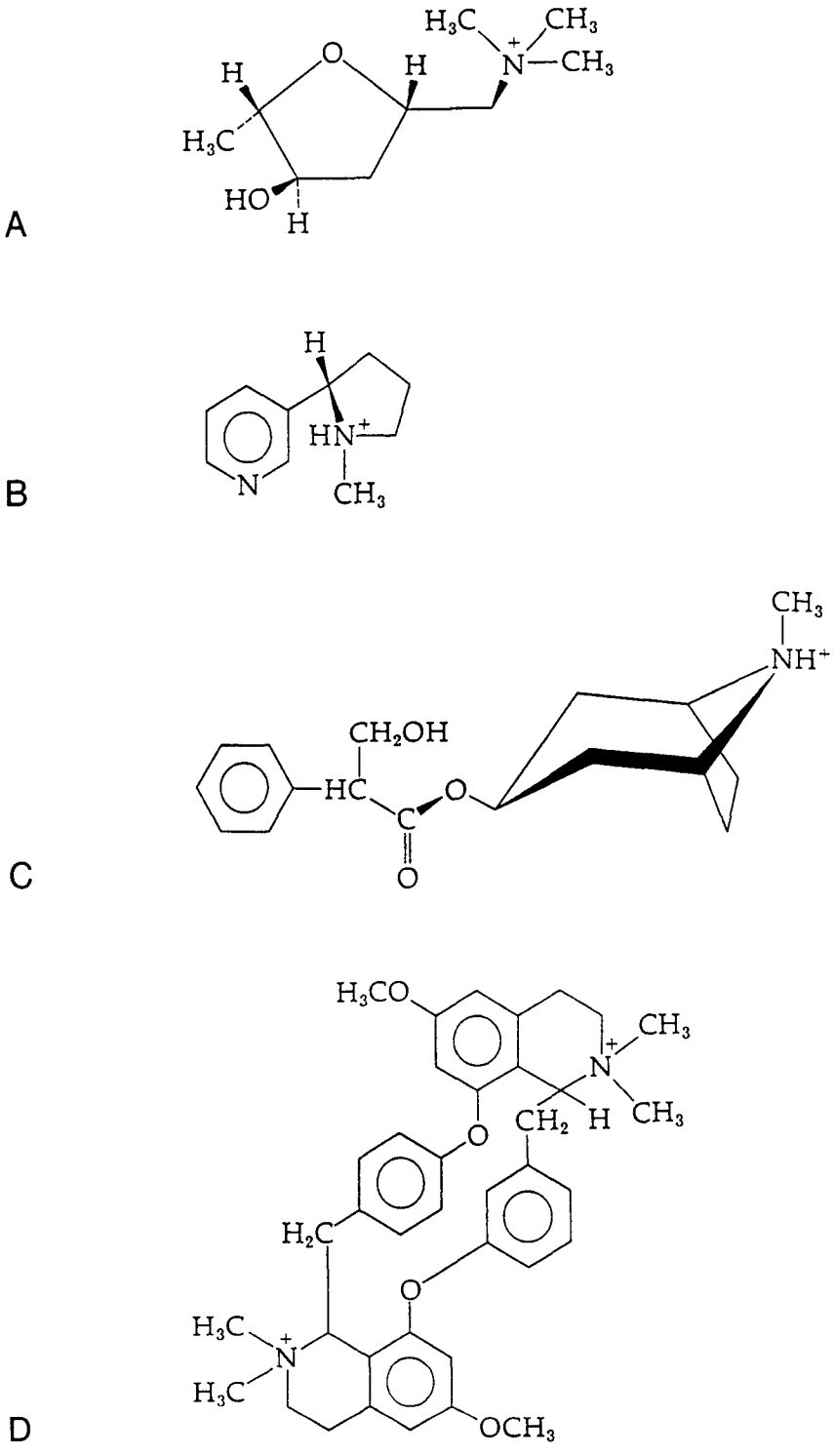

Fig. 1. Chemical structure of acetylcholine receptor (AChR) agonists (A, muscarine; B, nicotine) and antagonists (C, atropine; D, D-tubocurarine).

chemical properties intermediate between those of nAChRs and mAChRs (Roshchina, 1987). The existence of AChRs in plants has also been postulated by Raineri and Modenesi (1986). They suggest that ACh may have regulated membrane permeability in the ancestral organisms from which primitive plants and animals evolved.

VIII. Effect of Acetylcholine on Metabolism, Growth and Development

In recent years a number of investigations have been carried out on the effect of ACh on many growth and development processes in plants, especially those processes controlled by phytochrome. In most cases experiments have been carried out on whole plants or isolated plant organs. Only a few studies have been carried out with isolated cells or organelles. Attention has been paid to the occurrence and distribution of ACh in the particular tissues, as well as to the enzymes involved in its synthesis and degradation.

A. SEED GERMINATION

Koštir et al. (1965) were the first to note the effect of choline and ACh on seed germination. They demonstrated that ACh modifies the germination and the early growth in several species of cultivated plants.

In the seventies Lees and Thompson (1975), Lees et al. (1978), Kasturi (1978, 1979), and Kasturi and Vasantharajan (1976) obtained evidence pointing to the involvement of the ACh-AChE system in the control of germination and growth of bean and pea seedlings. Lees and Thompson (1975) detected AChE activity in the cotyledons of germinating bean seed. The total AChE activity increased between the second and third day during germination, remained at the same level until the sixth day, and then decreased with ageing of the cotyledons (Lees & Thompson, 1975). This variation in AChE activity was accompanied by changes in ACh level. In the first two days of the experiment a low ACh concentration was observed. The ACh content increased rapidly reaching a maximum on the fourth day, followed by a rapid decline to a nearly undetectable level (Lees et al., 1978). Prolonged treatment with a 19 millimolar neostigmine (an inhibitor of AChE) clearly inhibited growth of seedlings and produced a 10-fold increase in ACh content in the cotyledons, compared to those of control plants (Lees et al., 1978). Lees et al. (1978) suggested that the ACh present in the cotyledons may participate in controlling transport of reserve substances from the cotyledons to the rapidly developing parts of the bean seedlings.

In dry pea seeds, AChE activity has also been detected (Kasturi & Vasantharajan, 1976, Kasturi, 1978). During the first two days of germination a complete loss of AChE activity was observed. On the third day, AChE activity was detected again, as a result of *de novo* synthesis (Kasturi, 1978). This synthesis of AChE was controlled by phytochrome, being inhibited by R and the effect of R reversed by FR (Kasturi, 1979). There was no effect of R on AChE activity. According to Kasturi and Vasantharajan (1976) AChE in pea seeds and seedlings participates in the regulation of the endogenous ACh level.

There are conflicting reports concerning the effect of ACh on the germination of photoblastic (light sensitive) seeds. Holm and Miller (1972) have demonstrated that 0.1 millimolar ACh, like R, accelerates or inhibits germination in *Agropyron repens* (L.), *Echinochloa crusgalli* L., *Chenopodium album* L., *Brassica kaber* (DC.) Wheeler,

Setaria viridis (L.) Beauv. Eserine (0.1 millimolar) sensitized seeds to application of ACh (Holm & Miller, 1972). Investigations carried out by Gupta and Briggs (Marmé, 1977, and reference therein) failed to confirm ACh participation in the photoregulation of seed germination of photoblastic seeds. No effect of ACh and atropine on the germination of the positively photoblastic seeds of *Rumex obtusifolius* and of the negatively photoblastic seeds of *Cucumis anguria* was reported by Ledeira et al. (1982b). Eserine inhibited germination in *R. obtusifolius* but had no effect on *C. anguria* (Ledeira et al., 1982b).

Recently Tretyn et al. (1988) studied the effect of choline, ACh, carbamylcholine [$NH_2COOCH_2CH_2N^+(CH_3)_3$] and AChE inhibitors (eserine and neostigmine) on the germination of positively, neutral and negatively photosensitive seeds. None of these substances, even at very high concentrations, affected the germination of photoblastically neutral wheat (*Triticum vulgare* L.) and oat (*Avena sativa* L.) caryopses in darkness or under continuous white light. In the light ACh accelerated the germination rate of the positively photoblastic seeds of *Rumex obtusifolius* L. and inhibited germination of the negatively photoblastic seeds of *Plantago lanceolata* L. Choline had no effect on germination in these cases irrespective of light conditions. The remaining substances, i.e., carbamylcholine, eserine and neostigmine acted similarly to ACh, but not as effectively (Tretyn et al., 1988). Tretyn et al. (1988) found that ACh only demonstrates its activity when light is present. These authors propose that light participates in the inhibition of AChE which has been shown to be present in all of the seeds studied (Tretyn et al., 1986). The fact that endogenous ACh has been found in the seeds of a number of plant species (see Table 1) indicates that ACh could be a factor involved in the regulation of germination.

B. GROWTH

It has been known for a long time that quaternary amines, such as AMO-1618, are strong inhibitors of plant growth (Marth et al., 1953). A derivative of the quaternary amine choline, 2-chloroethyl-3-methylamine chloride, known as chlorocholine chloride (CCC), also strongly inhibits growth of many plants (Cathey, 1964). The similarity in chemical structure of CCC, choline and ACh inspired studies on the effect of these substances on plant growth (Tung & Raghavan, 1968).

It has been shown that ACh mimics the effect of R in inhibiting development of secondary roots in bean seedlings (Jaffe, 1970). The analogue of ACh, carbamylcholine slightly enhances the FR-stimulated development of secondary roots in mustard seedlings, without affecting the elongation of the roots and hypocotyl growth (Kasemir & Mohr, 1972), Dekhuijzen (1973) demonstrated that CCC (0.1 molar) treatment of leaves and roots inhibits growth of wheat seedlings. This inhibitory effect of CCC was reduced by adding 10 millimolar ACh to the medium on which the plants were grown. At the same concentration ACh added to the medium in the absence of CCC, caused about a 30% stimulation of growth of wheat seedlings and a similar percentage increase in dry weight. The effect of ACh on both of the above processes was most pronounced at pH 6 (Dekhuijzen, 1973).

Acetylcholine affects the growth of isolated plant organs and tissues in different ways. At a concentration of 10 micromolar it increases the elongation rate of isolated segments of oat coleoptiles (Evans, 1972). In the case of explants, ACh is particularly effective if the auxin-stimulated elongation growth is inhibited by adding a high Ca^{2+} concentration to the incubation solution (Evans, 1972). The growth rate of segments

of cucumber hypocotyls is also increased by ACh. Its effect was most pronounced when the segments were devoid of cotyledons (Verbeek & Vendrig, 1977).

Under natural lighting conditions (day/night), ACh at 10 millimolar clearly stimulated growth of isolated soybean hypocotyls (Mukherjee, 1980). However its effect on the elongation of *Vigna sesquipedalis* (L.) Fruw. hypocotyls was different (Hoshino, 1983a). Growth of seedlings of this species is regulated by phytochrome. Red light causes an increase in growth rate of the epicotyl and inhibition of growth of the hypocotyl compared to seedlings grown in darkness (Hoshino, 1983a). At concentrations > 10 micromolar, ACh applied in darkness to *Vigna* seedlings also inhibited growth of the hypocotyl and stimulated elongation of the epicotyl. In addition, choline had no effect on the growth of the organs under study. Neostigmine had a similar effect to ACh and atropine a converse effect on the growth of the *Vigna* hypocotyl and epicotyl. Atropine also neutralized the effect of R on the growth pattern of *Vigna* seedlings (Hoshino, 1983a).

The experiments reported above were conducted on widely different plant species, different plant organs and under different experimental conditions. However, the mechanism of ACh activity in plants seems to be related to the light environment under which the experiments were conducted, and its highest activity was observed in the acid pH range. The inhibitory effect of ACh on growth has been accounted for by its properties as a growth retardant. Jaffe (1972) has found that ACh, like AMO-1618, inhibits growth of etiolated cucumber seedlings. In seedlings treated with AMO-1618, Jaffe detected a 50% increase in endogenous ACh content compared to the controls. The effect of AMO-1618 on plant growth was probably a result of the inhibitory effect of this substance on AChE activity (Riov & Jaffe, 1972b, 1973b). Organophosphate pesticides (Gupta & Maheshwari, 1980; Kasturi & Vasantharajan, 1976) have similar properties to AMO-1618. A number of naturally occurring glycosides in plants, such as eserine (physostigmine) and neostigmine, as well as some synthetic herbicides, pesticides and insecticides inhibit the activity of AChE isolated from plant tissues (Kasturi & Vasantharajan, 1976; Miura et. al., 1982; Riov & Jaffe, 1973a). The highest AChE level is observed in young growing plant organs (Fluck & Jaffe 1974a, 1976; Kasturi, 1978), i.e., at the main sites of ACh synthesis (Jaffe, 1970, 1972; Lin, 1957). All factors modifying AChE activity may affect the growth rate in plants through their regulation of ACh concentration.

It has also been demonstrated in many experiments that ACh can stimulate growth. In such cases the effect of ACh was accounted for by its interaction with different growth regulators, such as auxin (Evans, 1972), gibberellins (Lawson et al., 1978; Mukherjee 1980; Verbeek & Vendrig, 1977), or ethylene (Mukherjee, 1980). It has also been suggested that ACh may control plant growth through the regulation of membrane permeability to ions (Dekhuijzen, 1973; Evans, 1972).

C. GENERATIVE DEVELOPMENT

Mimicking the action of blue light, ACh in the presence of 5 micromolar eserine induced the production of conidial spores in *Trichoderma viridis* growing in darkness (Gressel et al., 1971). However, in long-day spinach, induction of flowering is associated with phytochrome-controlled changes in peroxidase activity (Penel & Greppin, 1973). Acetylcholine, imitating the action of R, inhibited the FR-stimulated peroxidase activity. The light- and ACh-regulated variations in peroxidase activity may constitute the first stage of flower induction in spinach (Penel & Greppin, 1973, 1974).

Kandeler (1972) and Hoshino and Oota (Hoshino, 1979; Oota, 1977; Oota & Hoshino, 1974) have observed that ACh affects flowering in duckweeds. Under continuous irradiation (24 hours light/0 hour dark) 10 micromolar ACh inhibited flowering in *Lemna gibba* G1 and stimulated it in *Lemna perpusilla* Torr. Acetylcholine affected flowering in both duckweed species only when ascorbic acid was present in the medium (Kandeler, 1972). Flowering of the long-day *Lemna gibba* G3 grown under continuous irradiation at a temperature of 26°C is inhibited as a result of a drop in ambient temperature to 21°C between 12 and 48 hour from the start of the first light cycle. This duckweed growing under a long photoperiod (16 hours light/8 hours dark) was sensitive to chilling only during the dark period (Oota & Hoshino, 1974). Plants kept under continuous light in a medium containing 10 micromolar ACh showed a similar sensitivity to chilling to that of those grown under a long photoperiod. Oota and Hoshino (1974) have also found that when the temperature is lowered from 26 to 21°C during the second light cycle (24 hours light/0 hours dark), flowering in control plants is inhibited while no effect is observed on plants grown in a medium with ACh. In addition, ACh or eserine (at 10 micromolar) inhibited flowering in *Lemna* by about 40% when grown under continuous light at a constant temperature of 26°C. The inhibitory effect of ACh on flowering in duckweeds was neutralized by the cyclic adenosine monophosphate (cAMP) analog N^6,2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate (Oota, 1977). A similar effect on flowering as that of ACh was exhibited by IAA. Synthetic auxins, such as α-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D), were inactive in this process (Oota, 1977). Atropine inhibited, while D-tubocurarine had no effect at all on flowering in *Lemna gibba* G3 grown under continuous irradiation and subjected to 12 hours chilling. Furthermore, atropine neutralized the stimulating effect of ACh and IAA on flowering of *Lemna* growing under the above light and temperature conditions (Hoshino, 1979). The effect of ACh and IAA on flowering of duckweeds may result from its effect on muscarine-like ACh receptors which were proposed by Hoshino (1979) to occur in the membranes of this plant.

Hoshino's suggestions are in line with earlier findings of Greppin et al (1973) and Greppin and Horowitz (1975), who demonstrated that the effect of ACh on the flowering process may result from its action on the permeability of plant membranes to ions. They demonstrated that ACh affects the bioelectric potentials of leaves associated with photoperiodic induction of flowering in *Spinacia oleracea* and *Perilla nankinensis* Decaisne. Changes in membrane potentials also accompanied phytochrome-regulated induction of flowering in *Lemna paucicostata* Hegelm. (Löppert et al., 1978) and *Lemna gibba* G1 (Kandeler et al., 1980), i.e., in those duckweed species whose flowering was induced and inhibited by ACh, respectively (Kandeler, 1972). It has been proposed that the primary mechanism of ACh action on flowering is due to its effect on membrane permeability to ions (Greppin et al., 1973; Greppin & Horowitz 1975; Hoshino, 1979; Kandeler, 1972; Penel & Greppin, 1974). However, the plant's transition from the vegetative to the generative phase need not necessarily be associated with significant changes in ACh level (Ledeira et al., 1982a).

D. INTERACTIONS BETWEEN SPECIES AND BETWEEN CELLS

Acetylcholine may take part in interactions between bacteria and higher plants. It has a chemotactic effect on the bacterium *Pseudomonas fluorescens* (Fitch, 1963a, 1963b), and it may perform a certain, but not clearly understood, function in the symbiosis of *Rhizobium* with leguminous plants (Fluck & Jaffe, 1976). Acetylcho-

linesterase activity has been detected in root papillae of *Glycine max* Merr. infected with *Rhizobium*. The peak in AChE activity coincided with the period of the most intensive nitrogen assimilation by these structures (Fluck & Jaffe, 1976).

Raineri and Modenesi (1986) have provided evidence of possible ACh participation in interspecific and intercellular reactions. Using histo- and biochemical methods they detected AChE activity in the thalli of the lichen *Parmelia caperota*. The enzyme probably occurred on the surface of cell membranes of both symbionts composing the lichen. Its activity increased during production of soredia, the structures serving for asexual reproduction. Activity of AChE was first observed in the cells of the algae producing aplanospores, and then in the apical portions of terminally growing mycelial hyphae surrounding the dividing algae. The newly developed soredia are made up of 'nuclei' composed of the splitting algae, and an 'envelope' made up of mycelial hyphae. Intensive AChE activity was found in both layers of soredia, and in particular in their contact zone. Raineri and Modenesi (1986) presume that the AChE-ACh system may participate in controlling the intercellular reactions of the two symbionts composing *Parmelia*. The operation of the proposed system concerns the control of membrane permeability to ions and is regulated by the light environment (Raineri & Modenesi, 1986).

The AChE-ACh system may also participate in the regulation of pistil-pollen interaction. Using an electron microscope, Bednarska and Tretyn (1989) found AChE activity on the surface of the pistil stigma in *Pharbitis nil*. The presence of this enzyme has also been found in pollen grains and on the tips of growing pollen tubes of *Pharbitis* (E. Bednarska, unpubl.). Furthermore, it has been found that ACh agonists and antagonists and AChE inhibitors affect the germination of pollen grains and growth of pollen tubes of *Pharbitis* and several other plant species (E. Bednarska, unpubl.).

Earlier, Martin (1972) had demonstrated that eserine inhibits growth of pollen tubes of *Crinum asiaticum* L. Eserine and ACh inhibited (Gharyal, unpubl.; see Hartmann & Gupta, 1989), while neostigmine had no effect (Fluck & Jaffe, 1976) on growth of pollen tubes of *Lathyrus sativus* L. and *L. latifolia*. The growth of *Arachis hypogaea* L. pollen tubes is controlled by phytochrome and the blue light-receptor (Chabra & Malik, 1978). Red light stimulates, while blue light inhibits, this process. Acetylcholine at 55 micromolar imitated R action (Chabra & Malik, 1978). Neither R nor ACh had any effect on the germination of pollen grains in *Pisum sativum, Cajanus cajan* Millsp. and *Lathyrus odoratus* (Gharyal, unpublished; see Hartmann & Gupta, 1989).

E. INTERACTION WITH GROWTH REGULATORS

In many cases exogenously applied ACh affects the hormonal control of growth and development in plants. Like IAA, ACh stimulated growth of oat (Evans, 1972) and wheat (Lawson et al., 1978) coleoptile sections, and inhibited root growth in *Lens culinaris* Med. (Penel et al., 1976). Hoshino (1979) noted an inhibitory effect of ACh and IAA on flowering in *Lemna gibba* C3 growing under continuous light conditions.

Parpus (1976) has pointed out that there is interaction between ACh and IAA and ethylene metabolism. Using sections of etiolated bean hypocotyls he found that IAA (1 micromolar to 1 millimolar) stimulates ethylene synthesis while inhibiting hook

opening. At concentrations from 0.1 to 1 millimolar ACh had no effect on either of these processes, but when applied in conjunction with IAA it reduced the auxin-stimulated ethylene synthesis by more that 50% and neutralized its inhibitory effect on the opening of the bean hook (Parpus, 1976). Acetylcholine and ethylene also interacted in the growth regulation of protonema cells of the fern *Athyrium filix-femina* Roth. (Bähre, 1975, 1977). Ethylene at low concentrations stimulated elongation growth of protonema cells, while ACh added to the medium neutralized the effect (Bähre, 1975). High ACh concentrations caused a pronounced lowering of the cell growth rate, while ACh enhanced protonemal cell growth in *Athyrium* at supra-optimal ethylene concentrations (Bähre, 1975). In conjunction with the anti-auxin (PCIB), ACh reduced the sensitivity of protonemal cells to the effect of treatment with ethylene alone. Anti-auxin applied jointly with ACh or pilocarpine (an AChR agonist) accelerated the R-stimulated alternation of growth pattern of *Athyrium* cells from filamental to two-dimensional (Bähre, 1977).

Acetylcholine inhibited ethylene synthesis in tissues isolated from soybean leaves (Jones & Stutte, 1986). Acetylcholine (11 millimolar) and neostigmine (5.5 millimolar) applied in darkness acted like R, lowering the rate of ethylene synthesis. Atropine raised ethylene synthesis to three times the control level, while ACh and R reduced its stimulatory effect (Jones & Stutte, 1986).

Acetylcholine may interact with gibberellin in plants. It reduced the inhibitory effect of the antigibberellin (CCC) on growth in wheat seedlings (Dekhuijzen, 1973) and partially replaced gibberellin in inducing growth of cucumber hypocotyls (Verbeek & Vendrig, 1977). Extensive studies on interaction of ACh and gibberellin in Scots pine seedlings have been carried out by Kopcewicz et al. (1977, 1979) and Kopcewicz and Cymerski (1980). These authors have demonstrated that irradiation of etiolated seedlings with R is associated with an increase in ACh and free gibberellin content (Kopcewicz et al., 1977). Acetylcholine treated plants contained more free gibberellins than controls (Kopcewicz et al., 1979). The stimulatory effect of ACh on gibberellin content was not reversed by FR (Kopcewicz et al., 1979), but was neutralized by atropine (Kopcewicz & Cymerski, 1980).

F. ENZYME ACTIVITY

Penel et al., (1976) demonstrated that growth inhibition of roots of *Lens culinaris* by high ACh concentrations is associated with changes in isoperoxidase activity. Acetylcholine enhanced the activity of 11 isoperoxidases, and reduced the activity of two others (Penel et al., 1976).

In barley tissues R stimulates the activity of phenylalanine ammonia-lyase (PAL) and the synthesis of flavonoids (Saunders & McClure, 1973). Incubation of such tissues in 0.1 millimolar ACh solution did not affect PAL activity and flavonoid level, but partially neutralized the stimulating effect of R (Saunders & McClure, 1973).

In pea roots ACh slightly inhibited the *de novo* synthesis of AChE (Kasturi, 1979). However, it had no effect on the phytochrome-regulated synthesis of anthocyanins in the hypocotyl of *Sinapis alba* L. seedlings (Kasemir & Mohr, 1972), or on the blue light-stimulated biosynthesis of carotenoids in *Fusarium aqueductum* (Rau, 1980). In pea seedlings Jones and Sheard (1975) noted no effect of ACh on the phytochrome-regulated activity of nitrate reductase.

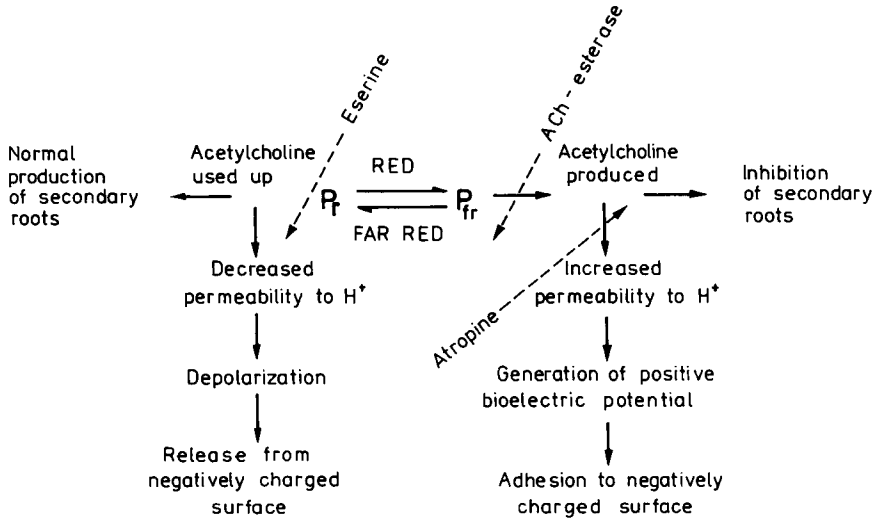

Fig. 2. Summary of the effects of phytochrome and acetylcholine (ACh) on bean root tips. Pr and Pfr = red light- and far-red light-absorbing forms of phytochrome, respectively (Modified after Jaffe, 1970).

G. PROCESSES ASSOCIATED WITH MEMBRANE PERMEABILITY

1. The Tanada Effect

The first phenomenon know to be modified by exogenous ACh was the so-called 'Tanada effect' (Tanada, 1968a, 1968b). Adhesion to and release of isolated barley and bean root tips from a glass surface is controlled by phytochrome (Tanada, 1968a, 1968b; Jaffe, 1968; Yughans & Jaffe, 1970). Red light stimulates adhesion of root tips to a negatively charged glass surface, while FR reverses the effect of R by releasing them to the medium. Acetylcholine at 0.1 millimolar, imitates the action of R, inducing adhesion of isolated bean root tips in darkness (Jaffe, 1970). At the same time, its presence in the medium inhibited their FR-stimulated release, atropine and AMO-1618 applied in darkness imitated R and ACh action. Eserine raised the tissues' susceptibility to R and ACh and inhibited FR-stimulated release (Jaffe, 1972). In order for the Tanada effect to proceed, Ca^{2+}, as well as a number of other specific agents, are necessary (Tanada, 1968b; Yughans & Jaffe, 1970).

Using paper chromatography and various bioassays, Jaffe (1970, 1972) has demonstrated the presence of endogenous ACh in bean root tips. He has also found that R stimulates and FR inhibits ACh synthesis in root tissues and its release into the medium (Jaffe, 1970). Furthermore, besides affecting the Tanada effect, ACh in bean seedlings may affect three other phytochrome-regulated processes. Acetylcholine, like R, inhibits the development of secondary roots, stimulated proton (H^+) efflux into the medium and induces the development of positive surface biopotentials in root tissues (Jaffe, 1970, 1972). Based on his studies, Jaffe (1970) proposed the model illustrated in Figure 2 for the effects of phytochrome and ACh in bean root tips.

In this model Jaffe assumes that irradiation of roots with R is associated with the formation of P_{fr} (active far-red light absorbing form of phytochrome), which stim-

ulates ACh synthesis. The rise in ACh level in the tissues increases H^+ efflux from the root cell and this induces the generation of a positive charge on their surface, which in turn makes the roots adhere to the glass surface. The increase in endogenous ACh content also leads to an inhibition of the development of secondary roots. Conversely, FR irradiation of roots stimulates the transformation of P_{fr} to the R-absorbing form of phytochrome (P_r) form, which, by increasing the rate of ACh decomposition, causes the root tips to be released from the glass and the secondary roots to undergo normal organogenesis. In Figure 2 the dashed arrows mark the assumed sites for inhibitory action of eserine, atropine and AChE on the particular steps of the proposed chain of events.

Other authors, besides Jaffe, confirmed the involvement of phytochrome in the control of the Tanada effect (Racusen & Etherton, 1975; Racusen & Miller, 1972). It has been revealed that phytochrome regulates electrical phenomena at the cell membrane level. However, it has not been confirmed that ACh plays a regulatory role in controlling the Tanada effect. Tanada has demonstrated that Na^+ at a concentration lower than that of ACh imitates the action of R. On the other hand, K^+ acts similarly to FR, stimulating the release of root tips (Tanada, 1972). It has also been found that IAA and abscisic acid (ABA) may be involved in phytochrome-regulated responses of root tips. At concentrations lower than 1 micromolar, IAA and ABA stimulated the adhesion and the release of root tips from glass surfaces respectively (Tanada, 1973).

2. Leaf Movements

Nyctinastic movement of leaves in *Mimosa pudica* L., *Albizia julibrissin* Durazz and *Samanea samen* Merr. are phytochrome-controlled. These leaf movements are initiated by a change in the membrane potential. The signal is perceived in pulvinules and pulvini, which act as motor organs at the base of leaflets and leaves, respectively. Light stimuli cause large changes in ion fluxes, leading to turgor changes in the motor tissues (Hartmann & Gupta, 1989).

Seeing a similarity of this process to the rapid changes in ion transport known in neurons, Toriyama and Jaffe (1972) suggested a possible involvement of ACh in the regulation of leaf movement in *Mimosa.* Studies carried out by Satter et al. (1972), however, argued against the possibility of ACh involvement in the regulation of leaf movement in *Mimosa pudica* L., *Albizia julibrissin* Durazz., *Samanea samen* Merr. and *Phaseolus multiflorus* (Willd.) Lam. Using a bioassay, the authors demonstrated that in *Albizia* there was no difference in ACh content between the lamina and the pulvinule. They also detected no changes in ACh level in the pulvinules during phytochrome-mediated leaflet movements. Treatment of pulvinules with exogenous ACh (from 10 micromolar to 1 millimolar) had no effect on leaflet movement when irradiated with either R or FR, or stored in darkness (Satter et al., 1972). In contrast, Kumaraval et al. (1979) found that ACh could be associated with leaf movements of *Samanea samen* Merr. These authors found that ACh levels in leaves in the closed state (during the dark) was higher than in the open state (during the day).

3. Membrane Permeability to Ions

In 1970 Jaffe suggested that the primary mechanism of ACh action in plants consisted in regulating the permeability of membranes to ions. He demonstrated that

ACh stimulates efflux of H^+ from bean root tips cells. In spinach leaves Greppin et al. (1973) demonstrated that ACh induced changes in bioelectric potentials. Hartmann (1975) found that blue light induced hyperpolarization of the bioelectric potentials in the bean hypocotyl hook. Acetylcholine does not appear to mimic the blue light effects on biopotentials. However, an increase in ACh concentration inhibited the blue light-stimulated hyperpolarization of potentials and also inhibited K^+ uptake by the hook tissues (Hartmann, 1977).

Evans (1972) and Jaffe (1972) postulated an influence by ACh on membrane transport of Ca^{2+}. Tretyn (1987) showed that ACh, like R, increases the rate of $^{45}Ca^{2+}$ uptake by etiolated oat coleoptile sections. Pre-incubation of tissues in eserine raised their sensitivity to ACh. Treatment of sections with Ca^{2+} channel blockers: lanthanum ions (La^{3+}) and Verapamil, neutralized the stimulatory effect of R and ACh on $^{45}Ca^{2+}$ uptake (Tretyn, 1987).

Recently, Roshchina (1987) carried out studies on the effect of ACh, its agonists and antagonists on Na^+ and K^+ transport in isolated chloroplasts of pea leaves. This author presumes that the ACh stimulated efflux of Na^+ and K^+ into the medium is controlled by the AChRs.

The possibility of membrane permeability control by AChRs has also been pointed out by Bähre (1975); Dekhuijzen (1973); Greppin et al. (1973); Hoshino (1979); Mukherjee (1980); Parpus (1976) and Raineri and Modenesi (1986).

4. Phospholipid Metabolism

In animals, ACh regulates membrane permeability to ions by means of two different processes. In neurons, ACh binds to receptors and stimulates the activity of ion channels (Changeaux et al., 1984). In non-neural cells ACh changes membrane permeability to ions, leading to activation of enzymes involved in the membrane phospholipids pathway (Hokin, 1985). The enzymes stimulate the formation of diacylglycerol (DG) and inositol triphosphate (IP_3), which act as second messengers (Berridge, 1987).

Fluck & Jaffe (1976) were first to suggest that ACh may affect phospholipid metabolism in plants. Hartmann et al. (1980) revealed that under aerobic conditions a 10 micromolar ACh solution inhibits radioisotopic inorganic phosphate ($^{32}P_i$) incorporation into phospholipid molecules isolated from sections of etiolated bean seedlings. Under anaerobic conditions ACh had a stimulatory effect on this process. Atropine had a similar effect to ACh. Eserine and neostigmine had no effect at all on the phospholipid metabolism in sections isolated from etiolated bean hypocotyls (Hartmann et al., 1980). Detailed investigations have demonstrated that under aerobic conditions ACh inhibits $^{32}P_i$ incorporation into the phospholipid fraction containing phosphatidyl-ethanol-amine (PC) and phosphatidyl-choline (PC), while stimulating its incorporation into the fraction enriched in phosphatidyl-inositol (PI) (Hartmann & Gupta, 1989). Studies carried out by Hartmann and coworkers (Hartmann & Schleicher, 1977; Hartmann et al., 1980; Hock & Hartmann, 1981) have confirmed the similarity between phospholipid metabolism in plants and in animals.

H. RESPIRATORY PROCESSES

One of the compounds necessary for the Tanada effect is ATP (Tanada, 1968b; Yughans & Jaffe, 1970). Using a Warburg apparatus and a Clark oxygen (O_2) elec-

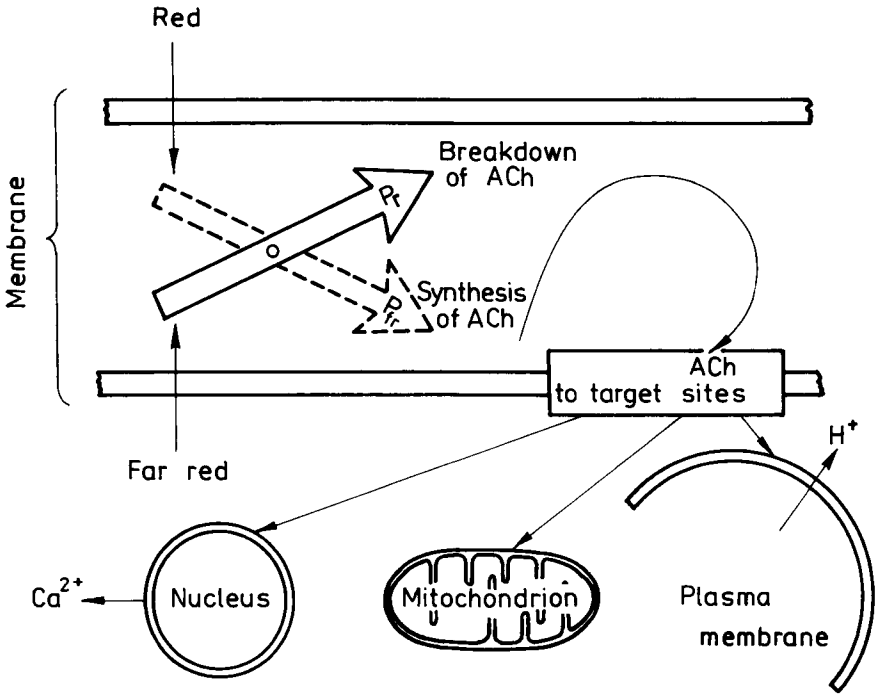

Fig. 3. Model of proposed mechanism of interaction of phytochrome and acetylcholine (ACh). Pr and Pfr = red light- and far-red light-absorbing forms of phytochrome, respectively (Modified after Jaffe, 1972).

trode, it has been revealed that ACh and R both increase the consumption of O_2 by bean root tips tissues, while FR reverses the R effect (Yughans & Jaffe, 1970, 1972). Mitochondria isolated from the roots responded in a similar way, but the ACh concentration necessary to obtain an effect similar to that obtained in root tips was lower. In isolated bean root tips R and ACh caused a 10-fold decrease in ATP level and a 14-fold increase in P_i content. The drop in ATP level was not due to an increase in ATPase activity, since both R and ACh had no effect on its activity (Yughans & Jaffe, 1972), but may be due to the uncoupling effect of ACh and R on oxidative phosphorylation with the transport of electrons through the respiratory chain, or to an increased rate of ATP consumption. The effect of uncouplers of oxidative phosphorylation on the Tanada effect support the former alternative. Substances such as gramicidin, oligomycin, digitoxin and valinomycin acted similarly to ACh, i.e., they had no effect whatsoever on R-induced adhesion of root tips to glass and they inhibited FR-stimulated release (Yughans & Jaffe, 1972). Based on the above data, as well as on the results of studies pointing to the effect of ACh on H^+ extrusion (Jaffe, 1970) and on the intracellular distribution of Ca^{2+} (Jaffe, 1972), Jaffe proposed the model of ACh action in bean root cells shown in Figure 3.

In this model a differential effect of R and FR on endogenous ACh level is postulated. The active P_{fr} form of phytochrome enhances ACh synthesis. As its intracellular concentration rises, the ACh reaches its target sites of activity. What follows is an increase in H^+ extrusion across the plasma membrane, a rise in O_2 consumption

and in ATP catabolism. Inside the mitochondria these processes are associated with an enhancement of membrane transport of univalent cations, and the release of Ca^{2+} from the nuclear envelope which activates many other intracellular processes (Jaffe, 1972).

In the subsequent years Hartmann (1974), Bürcky and Kauss (1974), White and Pike (1974) and Kirshner et al. (1975) questioned the results obtained by Yughans and Jaffe (1970, 1972). Bürcky and Kauss (1974) have demonstrated that although a 4-minute irradiation of the bean root tips with R is associated with their adhesion to glass, their ATP content increases, contrary to what was claimed by Yughans and Jaffe (1972).

Using the callus of a hybrid obtained by crossing two moss species (*Physcomitrium piriforme* × *Funaria hygrometrica*), Hartmann (1974) discovered that R stimulated O_2 uptake and the effect of R was neutralized by FR. Whereas ACh applied in darkness had no effect, ACh was only active when applied during or just after R or FR irradiation. In this system, light and the presence of ascorbic acid and ATP were essential for ACh activity (Hartmann, 1974).

In agreement with the results of Bürcky and Kauss (1974), White and Pike (1974) and Kirshner et al. (1975) found that R stimulates an increase in ATP level in buds of etiolated seedlings of *Phaseolus vulgaris*. The highest ATP concentration was recorded in the 4th minute of irradiation (Kirshner et al., 1975) or 1 minute after completing a 5-minute R pulse (White & Pike, 1974). The R effect could be reversed by FR. Treatment of buds for 6 minutes with ACh (at 10 picomolar to 1 millimolar) induced a ca. 70% drop in ATP content compared to the control (Kirshner et al., 1975; White & Pike, 1974). Acetylcholine applied simultaneously with R reduced its stimulatory effect. Atropine (0.1 millimolar) raised the ATP concentration in bean buds several fold. The substance was much more effective when applied in darkness than with simultaneous R treatment. On the contrary, AMO-1618, which like atropine raised the ATP level, was more active when the tissues were simultaneously irradiated with R (Kirshner et al., 1975).

I. CHLOROPLASTS

Roshchina and Mukhin (1985) and Roshchina (1988) observed AChE activity in isolated pea chloroplasts. This enzyme occurred in both whole and osmotically broken chloroplasts (Roshchina & Mukhin, 1985). Its activity was inhibited by high concentrations of the substrate and neostigmine. Acetylcholine has also been detected in pea leaves (Roshchina & Mukhin, 1985). Exogenous ACh at 10 millimolar inhibited ATP synthesis by 80–100%, without affecting electron transport. Moreover, it has been found that ACh, dependent upon its concentration, either stimulates (<0.1 micromolar) or inhibits (>0.1 micromolar) non-cyclic phosphorylation without affecting $NADP^+$ photoreduction (Roshchina, 1987). Neostigmine likewise inhibited ATP synthesis, without affecting electron transport from water to cytochrome *f* or to $NADP^+$ (Roshchina & Mukhin, 1985). Muscarine and atropine also inhibited $NADP^+$ photoreduction and non-cyclic phosphorylation (Roshchina, 1987). As well as affecting ATP synthesis (Roshchina & Mukhin, 1985) and non-cyclic phosphorylation (Roshchina, 1987), ACh, depending on its concentration, increased or reduced O_2 consumption by isolated pea chloroplasts, inhibited light-dependent swelling of chloroplasts (Roshchina & Mukhin, 1985) and stimulated efflux of Na^+ and K^+ (Roshchina, 1987). Roshchina & Mukhin (1985) assume that the ACh accumulated

and hydrolyzed inside pea chloroplasts may regulate membrane permeability of these structures and affect coupling between electron transport and ATP synthesis.

K. UPTAKE OF ACETYLCHOLINE

While extensive research has been carried out on the effect of ACh on processes in plants, little attention has been given to ACh uptake by plants. Lack of effect of exogenous ACh on many of these processes has been accounted for by its poor penetration into plant tissues (Kasemir & Mohr, 1972; Saunders & McClure, 1973, Tretyn et al., 1988).

The only author to carry out extensive research on ACh uptake by plants was Hartmann (1978). Using ACh labelled with tritium (^3H) and the radioisotope of carbon (^{14}C) he demonstrated that ACh uptake by isolated segments of bean (*Phaseolus vulgaris* L.) hypocotyl hooks is under phytochrome control. Red light stimulated, while FR after R inhibited, the rate of ACh uptake. In darkness, about 90% of ACh taken up by this tissue was hydrolyzed. The studies failed to determine whether phytochrome regulated the uptake rate or controlled the metabolism (synthesis) and/or hydrolysis of ACh inside the hypocotyl cells. The rate of ACh uptake by the tissues is also dependent on the pH of the medium. Determining the content of labelled ACh in the incubation medium, Hartmann (1978) demonstrated that the optimum pH for ACh uptake by segments of bean hypocotyls lies within the range of 7.1 to 8.5, i.e., in a pH range at which the rate of ACh hydrolysis in the tissues is highest. It was possible to limit its degradation by irradiating the tissues with R or by adding eserine to the medium (Hartmann, 1987). However, in the latter case, particularly in long-term experiments, it is impossible to eliminate the inhibitors' non-specific effect on the processes under study, since besides acting on ChE it may also inhibit the activity of other plant enzymes (Hartmann & Schleicher, 1977).

L. SUMMARY OF STUDIES

Data on the mechanism of ACh action in plant cells are controversial. The lack of an effect of exogenous ACh can be accounted for by its poor penetration into the plants' tissues and/or its rapid hydrolysis. The rate of ACh uptake by the tissues of etiolated bean hypocotyls is regulated by phytochrome and the pH of incubation medium (Hartmann, 1987). Experiments in which ACh is used must therefore be conducted under controlled light conditions and at a pH ensuring maximum ACh uptake from the medium. Furthermore, Hartmann (1978) postulated that in order that an effect of ACh on a given physiological process in plants might be recognized three conditions should be fulfilled: a) the ACh concentrations must be low (>1 micromolar); b) ACh must be applied for a comparatively short time, and c) in all experiments, especially those of long duration, choline and acetic acid must be used as controls.

In most experiments carried out so far these conditions have not been satisfied. Except for studies concerning ACh effects on ATP levels in plants and on the processes taking place inside isolated mitochondria and chloroplasts (see above), in order to obtain a detectable effect on the process under study ACh was applied at concentrations (>100 micromolar) which lead to non-specific effects. Taking this into account, Hartmann and Gupta (1989) proposed the latest model of ACh action in plant cells (Fig. 4), in which R induces photoconversion of P_r to the active P_{fr} form of phyto-

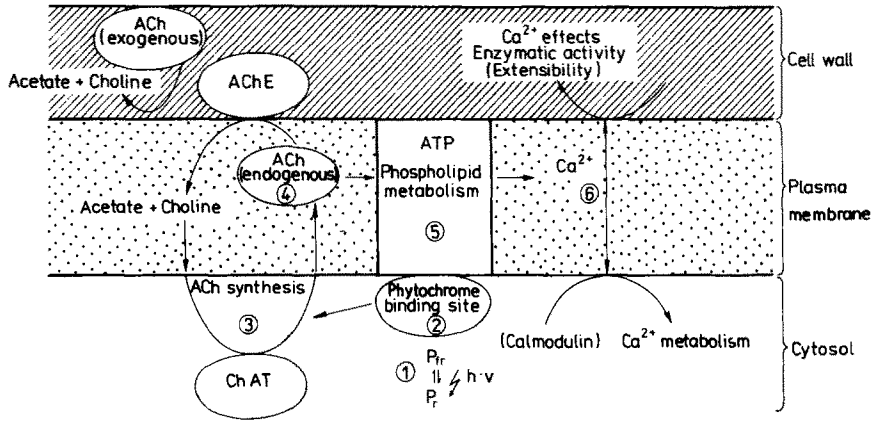

Fig. 4. Summary of the Hartmann and Gupta model of acetylcholine (ACh) metabolism and action in plants. Pr and Pfr = red light- and far-red light-absorbing forms of phytochrome, respectively. AChE = acetylcholinesterase. ChAT = choline acetyltransferase. See text for details (Modified after Hartmann and Gupta, 1989).

chrome, which binds to a submembrane P_{fr} receptor. The P_{fr}-receptor complexes are proposed to stimulate ChAT activity, which leads to a rise in ACh level in the cell. After reaching a certain intracellular concentration, ACh stimulates respiratory processes and the decomposition of membrane phospholipids, which by regulating the activity of Ca^{2+} channels, affect cell growth and a number of physiological processes. In their model, Hartmann and Gupta (1989) assume that exogenous ACh is subject to decomposition by extra-plasma membrane AChE. However, it can be presumed that the application of high concentration of ACh and/or inhibition of AChE activity (e.g., with eserine or probably by R) leads to the penetration of exogenous ACh inside the cell, where it can initiate the same processes that are controlled by endogenous ACh. Hartmann and Gupta's model explains the relationship between ACh and phytochrome action, as well as the mechanism of ACh action in plants. However, the model says little about the way endogenous ACh affects the processes it controls.

IX. Plant Protoplasts as an Approach to the Study of Acetylcholine Action

It follows from the above review of literature that ACh can mimic the action of R in the regulation of some aspects of photomorphogenesis in plants. In addition, ACh action in plants may concern the control of membrane permeability to K^+, Na^+ and Ca^{2+}. However, the mechanism of ACh action on membrane permeability remains unclear.

Considering this, studies were undertaken on the effect of ACh on the swelling of protoplasts isolated from etiolated wheat leaves. Swelling of these protoplasts is under phytochrome control (Bossen et al., 1988). In an isotonic solution of sorbitol a 1 minute R pulse causes an increase in protoplast volume. The stimulatory effect of R is manifested only in a medium containing Ca^{2+}, and is nullified by a subsequent 3 minute FR. The protoplasts start swelling immediately after being irradiated with

R and reach their maximum volume after 15–30 minutes in darkness (Bossen et al., 1988).

As with R, the Ca^{2+} ionophore (A 23187) (Bossen et al., 1988) and the agonist of animal Ca^{2+} channels, Bay K-8644 (Tretyn et al., 1990b), also stimulated swelling of protoplasts incubated in darkness in a sorbitol solution containing Ca^{2+}. The Ca^{2+} channels blockers: La^{3+}, Verapamil (Bossen et al., 1988), nifedipine (Tretyn et al., 1990b) as well as lithium ions (Li^{+}) (an inhibitor of the membrane phospholipid pathway) (Bossen et al., 1990) and 'calmodulin' inhibitors (A. Tretyn & R. E. Kendrick, unpubl.) neutralized the stimulatory effect of R on protoplast volume. It has also been shown that so-called 'G-protein' (G_p guanosine triphosphate (GTP)-binding protein (Hokin, 1985)) and protein kinase C may participate in phytochrome-controlled swelling of wheat protoplasts (Bossen 1990, Bossen et al., 1990).

A. PROTOPLAST SWELLING

Protoplasts placed in a sorbitol solution containing 0.5 millimolar calcium chloride ($CaCl_2$) or 0.1 millimolar KCl or 0.1 millimolar sodium chloride (NaCl) did not change in volume after a 3 minute FR pulse (Tretyn et al., 1990b). However, protoplasts irradiated with FR and then treated with ACh swelled in darkness to a comparable volume of those irradiated with 1 minute R. In contrast to the phytochrome-controlled swelling response (Bossen et al., 1988; Tretyn et al., 1990a), ACh was not only active in the presence of Ca^{2+} but also in that of K^{+} or Na^{+} (Tretyn et al., 1990a; 1990c). Maximum protoplast swelling was noted in a 1 micromolar ACh solution (Tretyn et al., 1990a).

Addition of the AChE inhibitor eserine (Table III), to the medium enhanced sensitivity of protoplast swelling in response to ACh. In a medium containing eserine (10^{-5} molar), in the presence of Ca^{2+}, protoplasts reached their maximum volume at 0.1 micromolar ACh (Tretyn et al., 1990a).

The specificity of the ACh effect on the swelling of protoplasts was studied with the use of several choline derivatives. Protoplasts incubated in a medium containing either K^{+}, Na^{+}, or Ca^{2+} with 1 micromolar choline, propionylcholine or butyrylcholine failed to result in any volume change. Besides ACh, only carbamylcholine (carbachol) stimulated protoplast swelling (Tretyn et al., 1990a, 1990c). Experiments have demonstrated: i) ACh is active at concentrations lower than 1 micromolar; ii) its effect is observed immediately after its application; iii) besides ACh, only carbamylcholine stimulated the swelling response. Based on these findings it is concluded that ACh specifically induces swelling of etiolated wheat mesophyll protoplasts.

B. MECHANISM OF ACTION

Using substances blocking ACh activity in animals (Table III) (Changeux et al., 1984; Dunat & Israël, 1985; Hokin, 1985) research has been carried out on the mechanism by which ACh influences the swelling of etiolated wheat protoplasts. Calcium channel-blockers, nifedipine and La^{3+} inhibited ACh-stimulated Ca^{2+}-dependent protoplast swelling (Tretyn et al., 1990a). So too did Li^{+}, 'calmodulin' inhibitors (Tretyn et al., 1990a) and guanosine-5'-0-(2-thiodiphosphate) (GDP-β-S) (a G_p inhibitor) (Bossen, 1990a). None of these substances, except the 'calmodulin' inhibitors, had any effect on the ACh-induced K^{+}/Na^{+} dependent swelling response (Tretyn et al., 1990a; 1990c).

C. ACETYLCHOLINE RECEPTOR AGONISTS AND ANTAGONISTS

In order to test the possible involvement of AChRs in the control of protoplast swelling, studies were undertaken with AChR agonists muscarine, nicotine and antagonists atropine, D-tubocurarine (Table III). The experiments were conducted in a medium containing Ca^{2+}, K^+ or Na^+ and one of the above substances.

Nicotine (an nAChRs agonist) stimulated swelling of protoplasts in an incubation medium containing K^+ or Na^+. The optimum nicotine concentration was 0.1 micromolar. Protoplasts incubated in a medium containing Ca^{2+} and treated with nicotine showed no change in volume (Tretyn et al., 1990c). The opposite results were obtained when muscarine (an mAChR agonist) was used. It stimulated protoplast swelling in the presence of Ca^{2+}, while having no effect in a medium containing K^+ or Na^+ (Tretyn et al., 1990c).

The effect of AChRs antagonists on the protoplast swelling response was also dependent on the ionic composition of the incubation medium. Atropine (an mAChR antagonist) at concentrations from 1 to 10 micromolar, in a medium containing K^+ or Na^+, had no affect on ACh-stimulated protoplast swelling. However, in the presence of atropine Ca^{2+} neutralized the stimulatory effect of ACh (Tretyn et al., 1990c). D-Tubocurarine (an nAChR antagonist) in the presence of Ca^{2+} only slightly inhibited protoplast swelling, while completely blocking the ACh effect in a medium containing K^+ or Na^+ (Tretyn et al., 1990c).

D. LOCALIZATION OF ACETYLCHOLINE RECEPTORS

The results outlined above suggest that ACh receptors similar to those in animal cells may be present in the protoplasts isolated from etiolated wheat leaves.

Their preliminary cytochemical localization was carried out using fluorescein-labelled α-bungarotoxin (FITC-BTx, an nAChR antagonist, see Table III). This antagonist completely blocked the ACh-stimulated K^+/Na^+-dependent protoplast swelling. It had no effect at all on the ACh-induced Ca^{2+}-dependent swelling response (Tretyn & Kendrick, unpubl.).

Using the procedure for localization of animal AChRs (Ross et al., 1988) distribution of these receptors has been studied in wheat protoplasts. Incubation of etiolated protoplasts in FITC-BTx solution (1 μg/ml) resulted in labelling of the protoplasts with this toxin. All protoplasts became labelled, and fluorescence of the FITC-BTx complex was mainly observed on their surface. Pre-incubation of protoplasts with unlabelled BTx blocked the binding of FITC-BTx with protoplasts (Tretyn & Kendrick, unpubl.). These data are the first direct evidence of the occurrence of AChRs in plant cells. Recent experiments with gold-labelled α-bungarotoxin may lead to a better understanding of the intracellular localization of nAChRs in wheat protoplasts.

E. COMPARISON OF THE EFFECT OF PHYTOCHROME AND ACETYLCHOLINE

It has been demonstrated that ACh can mimic the effect of R-induced phytochrome-controlled processes of plant growth and development. The results of experiments with protoplasts lend partial support to this conclusion. However, a number of observations contradict the possibility that ACh is a component of the phytochrome-induced transduction chain leading to the swelling response.

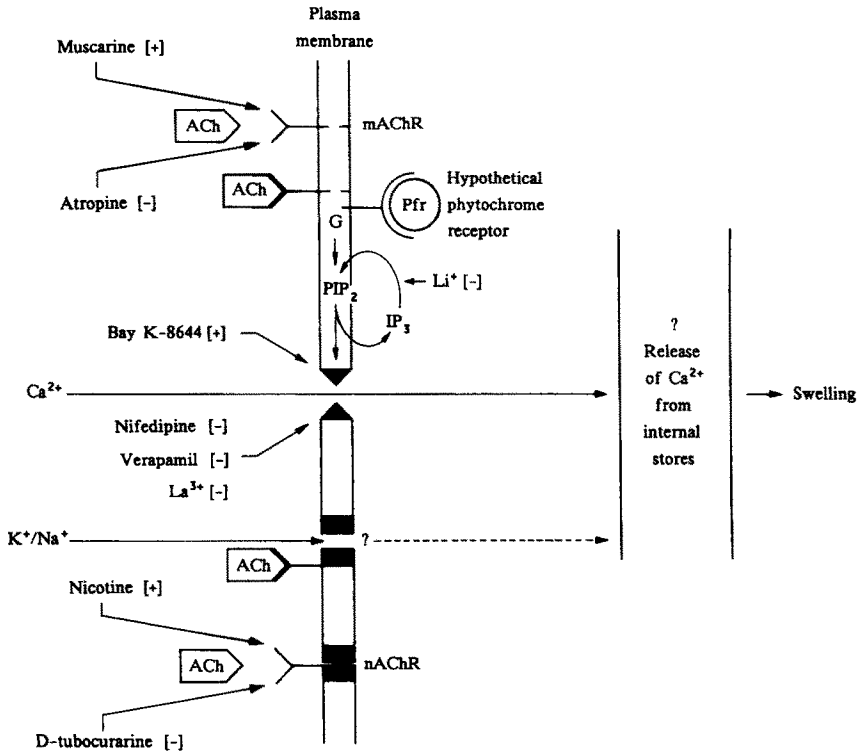

Fig. 5. Proposed model of acetylcholine (ACh) and phytochrome action on protoplasts and leaf sections from dark-grown primary wheat leaves. The release of Ca^{2+} from internal stores is proposed to trigger the transduction chain which leads to protoplast swelling and leaf unrolling. [+] = activator or agonist; [−] = inhibitor or antagonist; mAChR = muscarinic ACh receptor; nAChR = nicotinic ACh receptor; G = G-protein; Pfr = red light-absorbing form of phytochrome; PIP_2 = phosphatidyl-inositol 4,5-biphosphate; IP_3 = inositol 1,4,5-triphosphate. See text for details.

In summary it can be stated:

1) Far-red light reverses the inductive effect of R, but this effect is lost after pretreatment of protoplasts with ACh (Tretyn et al., 1990a).
2) While ACh is active in a medium containing either Ca^{2+}, K^+ or Na^+ (Tretyn et al., 1990a; 1990c), R-induced swelling response only occurs in a medium containing Ca^{2+} (Bossen et al., 1988; Tretyn et al., 1990b).
3) Atropine and D-tubocurarine inhibit ACh-induced Ca^{2+}-and K^+/Na^+-dependent protoplast swelling, but do not neutralize the stimulating effect of R (Tretyn et al., 1990c).
4) Nifedipine, La^{3+} (Bossen et al., 1988, 1990; Tretyn et al., 1990a; 1990c) and GDP-β-S (Bossen, 1990) inhibit both the ACh- and the R-induced Ca^{2+}-dependent protoplast swelling. None of these compounds block the ACh-induced K^+/Na^+-dependent response (Bossen 1990; Tretyn et al., 1990c).
5) α-Bungarotoxin inhibits the ACh-induced K^+/Na^+-dependent protoplast swelling and has no effect on either ACh- or R-induced Ca^{2+}-dependent protoplast swelling (Tretyn & Kendrick, unpubl).

X. Acetylcholine and Leaf Unrolling

Besides inducing the swelling of etiolated wheat mesophyll protoplast exogenous ACh also mimics another phytochrome-controlled process (Viner et al., 1988): leaf unrolling of primary leaf sections from 8-day-old dark-grown wheat seedlings (Tretyn & Kendrick, 1990). Acetylcholine (1 micromolar) stimulated unrolling of leaf sections prewashed in ethyleneglycol-bis-(β-aminoethylether)-N,N,N'N',-tetraacetic acid (EGTA) in darkness, if 1 millimolar $CaCl_2$ was present in the medium during a 30 minute treatment period after which the sections were returned to darkness for 24 hours before measurement. Acetylcholine also induced leaf unrolling in the absence of Ca^{2+}, when 100 micromolar NaCl was present in the medium. Apart from ACh, only carbamylcholine out of the choline derivatives tested was active in induction of leaf unrolling in the presence of 1 millimolar $CaCl_2$. The AChR antagonists, atropine (10 micromolar) and D-tubocurarine (10 micromolar), nullified the ACh-induced Ca^{2+}- and Na^+-dependent leaf unrolling respectively. The ACh agonists muscarine and nicotine (1 micromolar) stimulated leaf unrolling in the presence of Ca^{2+} and Na^+ respectively. The ACh-induced Ca^{2-}dependent leaf unrolling was reduced by 1 micromolar Nifedipine, 10 micromolar Li^+ and 10 micromolar 'calmodulin' inhibitor, trifluoperazine (TFP), whereas only TFP was active in the reduction of the ACh-induced Na^+-dependent leaf unrolling response.

XI. Conclusions

Based on results of our studies concerning the effect of ACh both on the wheat protoplast swelling and unrolling of primary wheat leaf sections we proposed the following model of ACh action on these processes (Fig. 5). We proposed that ACh influences both protoplast swelling and leaf unrolling via mAChRs and nAChRs. Just as for animal cells (Lester, 1977; Sastry & Sadavongvivad, 1979; Stround & Finer-Moore, 1985) mAChRs appear to be associated with a phosphatidyl-inositol-dependent pathway (Hokin, 1985) which leads to the opening of Ca^{2+} channels, whereas the nAChRs are phosphatidyl-inositol independent (Changeux et al., 1984) and possibly associated with K^+/Na^+ channels. Similarity between the action of ACh via mAChRs and R via phytochrome in protoplast swelling and leaf unrolling suggests they share a common signal transduction pathway (Fig. 5). In proposing this model we hope to stimulate further research which will ultimately lead to a full understanding of the fascinating roles played by ACh in plants.

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