PROBING ION CHANNELS WITH NATURAL AND SYNTHETIC HETEROCYCLES

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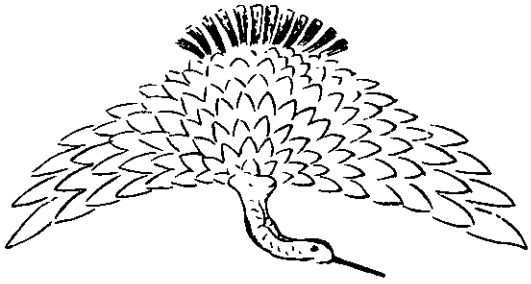
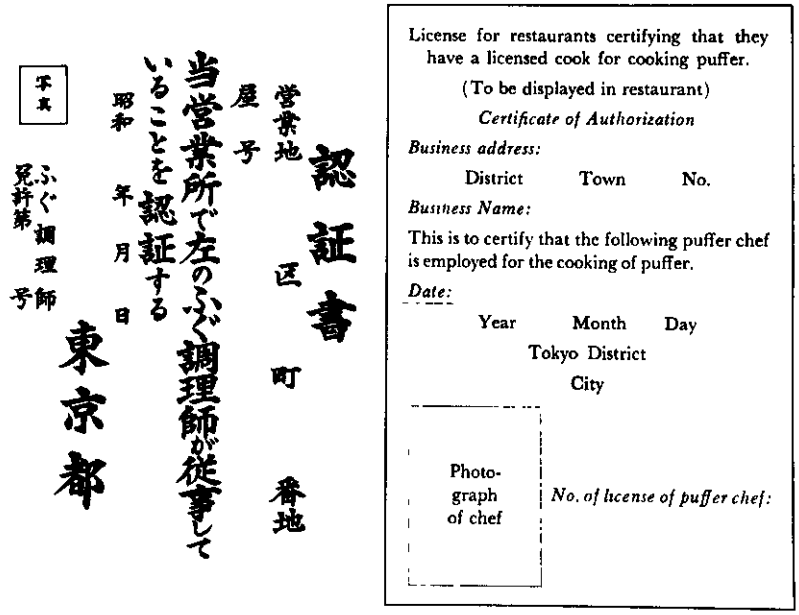
Abstract - Professor Tsuda's pioneering contributions to the crystallization and elucidation of tetrodotoxin serve as the starting point of a review on natural and synthetic heterocycles that have helped to study the topography, characteristics and dynamics of ion channels and membrane pores serving the influx and efflux of sodium, potassium and calcium.



INTRODUCTION

The crane, symbol of good fortune as an introductory vignette, serves here three purposes: first as a harbinger of good years still to come for Professor Tsuda, then as a reminder of his pioneering contributions to that part of the pufferfish that should not be eaten (see below) and, finally, as a memory of the unforgettable - ICHI GO ICHI E 一期一会 - Third International Symposium on the Chemistry of Natural Products held in Kyoto in April 1964[1] where so ably Professor Sugasawa reviewed the history of natural products isolated from animals in Japan: Takamine found adrenaline in adrenal glands in 1901, Munio Kotake characterized cinobufagin from toads in 1928, another student of my teacher Heinrich Wieland, Shimizu, located ursodeoxycholic acid, a new bile acid, in bears, while Kyosuke Tsuda, for whom this review is written, crystallized tetrodotoxin from puffer fish[2] with which we Westerners ("MI-KAI-JIN") then made our first hesitant acquaintance in the licensed sushi bars of Kyoto.



認証書

営業地 区 町 番地

屋号

当営業所で左のふぐ調理師が従事していることを認証する

昭和 年 月 日

東京都

写真

ふぐ調理師免許第 号

License for restaurants certifying that they have a licensed cook for cooking puffer.

(To be displayed in restaurant)

Certificate of Authorization

Business address:

District Town No.

Business Name:

This is to certify that the following puffer chef is employed for the cooking of puffer.

Date:

Year Month Day

Tokyo District

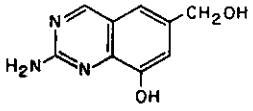
City

Photo-graph of chef

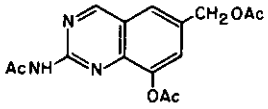
No. of license of puffer chef:

TETRODOTOXIN AND SAXITOXIN

The group of Professor Tsuda reported on the first major alkaline degradation product, the



I



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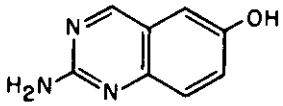
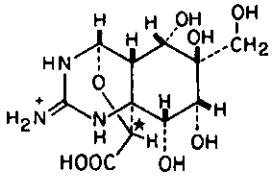
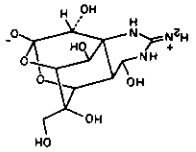


III

qinazolone (I)[3] which he synthesized and which Woodward obtained as the N,O,O-triacetyl derivative (II), the so-called "Singer acetate."[4] What seemed remarkable at the time was the isolation of the 6-hydroxyqinazoline (III) by Hirata's group as the result of the action of concentrated sulfuric acid on tetrodotoxin.[5] An important stepping stone ("tobi-ishi") to the complete



IV

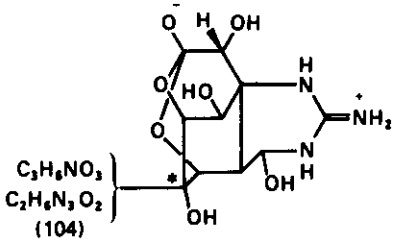


V

structure of tetrodotoxin (V) was Tsuda's tetrodoic acid hydrobromide (IV).[6]

Woodward ended his lecture in Kyoto[4] by raising two questions: "This is not the place to speculate upon the relationship of tetrodotoxin, and the striking physiological activity of the poison, nor upon the biogenesis of the molecule; though in the latter respect, the wild surmise might be briefly made that the molecule could be constructed by a variant of the familiar polyacetate scheme, with added branching carbon, along lines somewhat similar to those used in the construction of sclerotiorin and its relatives."

Well, after all these years, the biosynthesis of tetrodotoxin is still a mystery.[7] Feeding universal precursors, such as radioactive acetate, as adumbrated by Woodward, gave no incorporation in the local puffer fish, Spheroides maculatus, nor in the newts Taricha torosa and Taricha granulosa.[7]



CHIRIQUITOXIN

VI

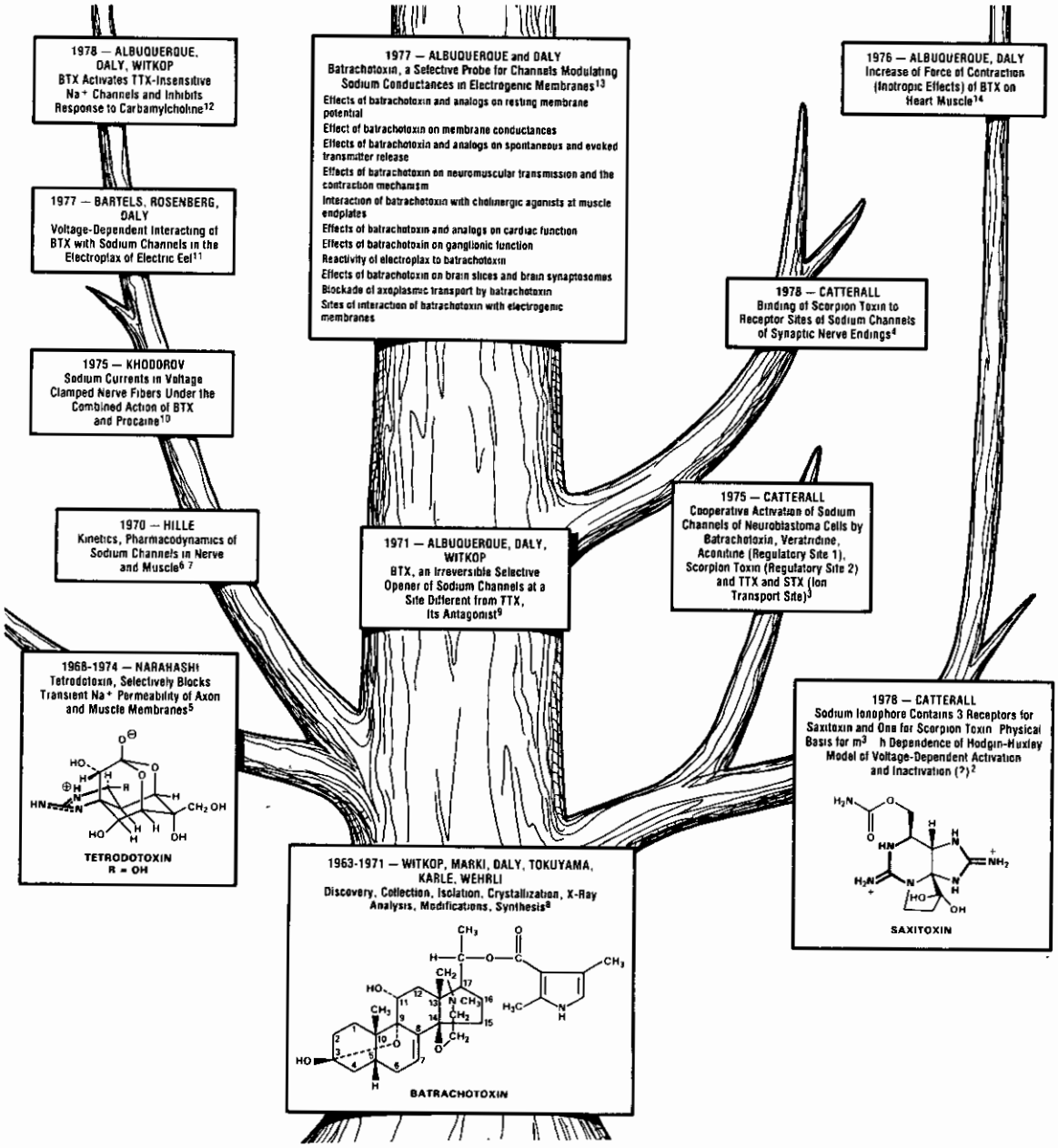
The second question, structure-activity relationships, have been discussed frequently, especially in terms of the essential guanidinium group of which the related saxitoxin (Chart I) possesses two, and in the attempt to equate the dimension of the hydrated sodium ion with that of the "prosthetic" guanidinium group which acts as a competitor in blocking the entrance to the sodium channel. This explanation has recently been challenged by a congener of tetrodotoxin, chiriquitoxin (VI), isolated from the Costa Rican frog Atelopus chiriquensis.[8] Unlike tetrodotoxin chiriquitoxin also blocks potassium channels, an action which is reversed by tetrodotoxin. The new theory postulates that both tetrodotoxin and chiriquitoxin occupy a receptor outside of the channel perhaps at a site overlapping the channel entrance. We see how important it is to have ever new tools for mapping ionic channels.

The history of "fugu" or puffer fish poison goes back to pictures in Egyptian tombs almost 5000 years old (Fig. 1) and in Japan to feeding experiments with human prisoners by Fukushima in the 16th century. Thanks to the chemical contributions, especially of K. Tsuda, the mechanism of

Fig. 1. The puffer fish Tetraodon stellatus representation from Egyptian tomb of 2700 B.C.

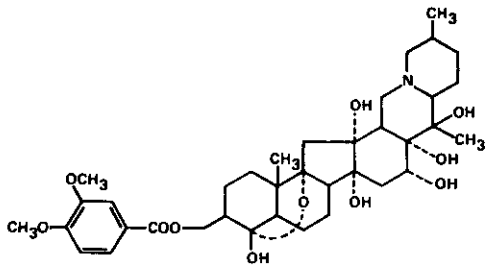
action could be explored in the mid 1960's by noted electrophysiologists, such as Narahashi and Ritchie.[9] Thus, a new chapter in neurobiology and cytopharmacology was started and we learned about sodium currents which propagate electric impulses in excitable membranes.[10]

Again, the International Symposium in Kyoto in 1964 marked another event: historically and topographically tetrodotoxin, in the reports of Sugasawa and Woodward, and the novel frog venom batrachotoxin (Chart I), in the lecture of Klaus Biemann (ref. 1), for the first time were placed side by side in the same book. Much later the two venoms were linked phylogenetically by their occurrence in neotropical frogs: tetrodotoxin in Atelopus from Costa Rica, and batrachotoxin in the skin of the Colombian poison arrow frog Phyllobates aurotaenia[11] and in the newly discovered species P. terribilis.[12] In its lethal action batrachotoxin surpasses tetrodotoxin almost by a factor of 10. While the hydrophilic tetrodotoxin blocks sodium channels at the outside in a reversible manner, the lipophilic batrachotoxin blocks the sodium channel in the "modified open position" more or less irreversibly.[13] Thanks to the availability of its antagonist tetrodotoxin, batrachotoxin became a valuable selective probe for the channels that modulate conductance in electrogenic membranes of nerve and muscle (Chart I). This picture is being refined continuously: Khodorov considers as receptors for the axonal sodium channels sites allosterically linked to the function of the participating subunits, such as the voltage sensor, the gating system responsible for the voltage-dependent opening and closing steps of the sodium ionophore, and the selectivity filter, i.e., the structure which defines the cationic specificity of the channel.[14] Regulatory sites can be distinguished from ion transport sites by specific ligands, such as veratridine (VII),



Concept of the Na⁺-Channel Aided by Selective Toxins

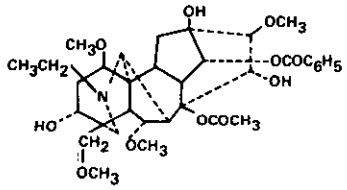
CHART I



Veratridine

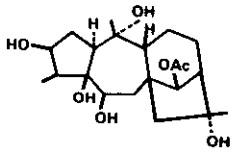
VII

aconitine (VIII), grayanatoxin (IX) and batrachotoxin (Chart I) which all bind to regulatory



Aconitine

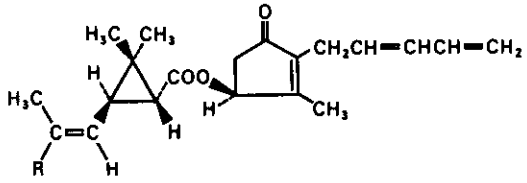
VIII



Grayanotoxin

IX

site 1, whereas the synergistic toxins from scorpion or sea anemone bind to regulatory site 2. The ion transport site binds inhibitors, such as tetrodo- and saxi-toxin as well as the insecticidal pyrethroids (X), the latter possibly at another site.[15] These binding studies have now been



PYRETHRIN I (R = CH_3)
PYRETHRIN II (R = $COOCH_3$)

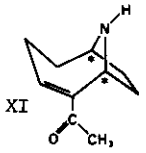
X

carried to a point at which the voltage-dependent sodium channel acquires the connotation of a "drug receptor"[16] in the sense of Paul Ehrlich.[17] Selective oxidation of the primary alcohol group at C-11 of tetrodotoxin provided an aldehyde that was coupled with (tritiated) glycine, lysine or photosensitizable groups, e.g. N-(2-nitro-4-azidophenyl)lysine. In this way ligands with high specific activity, ∿ 45 Ci/mmol, or photoactivatable derivatives were obtained which on irradiation irreversibly blocked the sodium channel.[16] A note of caution may be sounded: modifications in position 11 may alter ion specificity, as the example of chriquitoxin demonstrates.

The absence of blocking effects for potassium channels would have to be established in each case.

While sodium, potassium and calcium are transported in separate and discrete channels in electrogenic tissue of nerve and muscle, the ionophore attached to, or coupled with, the nicotinic acetylcholine receptor provides passage both for sodium and potassium through the same pore. The mechanism of nicotinic channel activation and blockade, therefore, is subject to different rules and requires neurotoxins of novel structure and specificity.[18] The acetylcholine receptor consists of a recognition site which responds to agonists, such as acetyl- or carbamyl- choline, and an ion conductance modulator which controls the opening and closing of an ionophore for the (rapid) influx of sodium and the (slower) efflux of potassium ions.

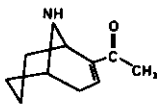
A novel toxin from a filamentous freshwater blue-green alga, Anabaena flos-aquae anatoxin-a (XI),



XI

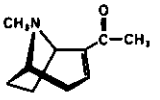
ANATOXIN-A

proved to be an ideal agonist for the nicotinic receptor.[19] Anatoxin-a is not an ester and therefore not a substrate for acetylcholine esterase. When it acts, it keeps the single channel open for an average halflife time that, at various transmembrane potentials, is indistinguishable from that of acetylcholine. Anatoxin-a and its derivatives, e.g., the much less active isoanatoxin-a (XII) or derivatives of the plant alkaloid ferruginine (XIII), promise to shed light on the second level of agonist mechanism by



D,L-ISOANATOXIN-A

XII



FERRUGININE
(Isomer of Anatoxin-a)

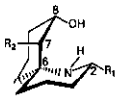
XIII

correlating the ultimate binding mechanism with the molecular details of receptor activation and desensitization as a function of well-defined rigid stereochemical parameters.

The classical inhibitor for the AcCH recognition site is bungarotoxin, the venom of the Formosan snake Bungarus multicinctus. Selective ligands to the ionic channel, as a rule, do not compete with the binding of (tritiated) bungarotoxin and vice versa. Anatoxin-a binds to the acetylcholine recognition site with a K_D=0.1 ~ 0.2 M, as determined by competitive inhibition of specific binding of tritiated acetylcholine, the natural agonist, and tritiated tubocurarine, a cholinergic blocker. Anatoxin-a stimulates binding of ion channel blockers, such as tritiated histrionicotoxin, phencyclidine and its methiodide at ED_{50}-values ranging from 0.14 to 0.28 M.[20]

The first selective ligands shown to act on both open and closed conformations of nicotinic channels were the unique venoms of the Southamerican frog Dendrobates histrionicus, viz. histrionicotoxin, dihydroisohistrionicotoxin, octahydrohistrionicotoxin and the synthetic perhydrohistrionicotoxin (Figure 2).[21]

Figure 2



2S, 6R, 7S, 8S (1 ~ 3)

2R, 6R, 7S, 8S (4 ~ 6a)

6R, 7S, 8S (7a)

1 Histrionicotoxin $R_1 = CH_2CH=CH-C\equiv CH$; $R_2 = CH=CH-C\equiv CH$

2 Isodihydrohistrionicotoxin $R_1 = CH_2CH_2CH=C=CH_2$; $R_2 = CH=CH-C\equiv CH$

3 Neodihydrohistrionicotoxin $R_1 = CH_2CH=CH-C\equiv CH$; $R_2 = CH=CH-CH=CH_2$

4 Allodihydrohistrionicotoxin $R_1 = CH_2CH_2CH_2C\equiv CH$; $R_2 = CH=CH-C\equiv CH$

5 Octahydrohistrionicotoxin $R_1 = CH_2CH_2CH_2CH=CH_2$; $R_2 = CH_2CH_2CH=CH_2$

6a Perhydrohistrionicotoxin $R_1 = n\text{-}C_5H_{11}$; $R_2 = n\text{-}C_4H_9$

7a Desamylperhydrohistrionicotoxin $R_1 = H$; $R_2 = n\text{-}C_4H_9$

a - Series = (-) - Series

These exciting findings stimulated many clever approaches to the total synthesis of this novel spirocyclohexyl-piperidine system.[22] However, gram amounts of racemic as well as optically active natural and antipodal histrionicotoxin derivatives did not become available, until Kimio Takahashi, guided by Arnold Brossi, translated some of the published procedures into a "pilot plant synthesis." Table 1 summarizes some of the electrophysiological activities. of these new synthetic derivatives in their capacity as blockers of the nicotinic channel.[23]

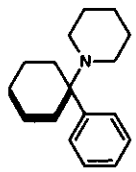
TABLE 1

EFFECTS OF 2-DESAMYLPERHYDROHISTRIONICOTOXIN ON THE SIATIC NERVE SARTORIUS MUSCLE PREPARATION OF THE FROG.COMPOUNDS USED IN μM CONCENTRATION

Compound	% Block of Muscle Twitch at Time Shown						Maximum Transient Potentiation of Muscle Twitch*	
	Indirect (min)			Direct (min)			Indirect	Direct
	15	30	60	15	30	60	%	%
(±)-2-Desamyl-PTX.HBr	85 ± 6	96 ± 1	100 ± 5	35 ± 16	52 ± 8	52 ± 8	–	–
(-)-H_{12}HTX.HCl	90 ± 1	100	100	32 ± 8	47 ± 3	45 ± 5	–	–

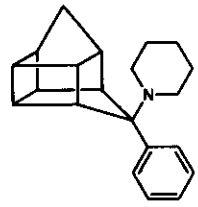
*Muscles were exposed to toxins for 60 min. The values shown are mean ± S.D. from two to five muscles. Transient potentiation developed following addition of the toxin into the bath and lasted approximately 5-15 min. There was no contracture at this concentration. Stock solution (10-500 mM) were made in 95% EtOH.

Before this program was started, a search for substitutes for the histrionicotoxins provided a number of suitable inhibitors, possibly binding to different sites within the channel. Phencyclidine (XIV), a local anesthetic and hallucinogen ("angel dust"), is very effective in blocking efflux of potassium ions (delayed rectification) and thus in prolonging the action potential.[24] Tritiated phencyclidine or its methiodide[25,26] or cage analogs[27] (XV) available through photosynthesis and derivatization,[27] hold promise



PHENCYCLIDINE

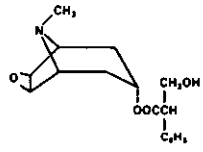
XIV



CAGE ANALOG OF PHENCYCLIDINE

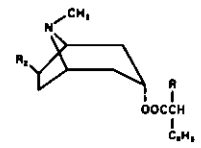
XV

as useful probes for the nicotinic channel. Antimuscarinic drugs, such as scopolamine (XVI), atropine (XVII), homatropine (XVIII) and 6-hydroxyatropine (XIX) interact



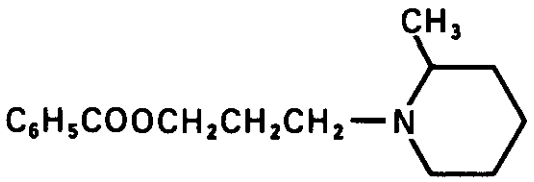
SCOPOLAMINE

XVI



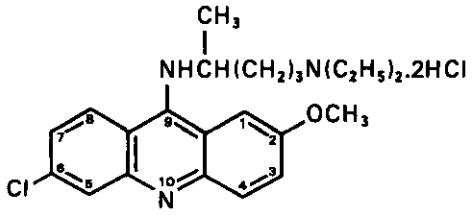
R = OH HOMATROPINE (R_2 = H) XVIII
R = CH_2OH ATROPINE (R_2 = H) XVII
R = CH_2OH 6-HYDROXYATROPINE (R_2 = OH) XIX

preferentially with the open conformation of the end-plate channel in the same way as the morphine antagonist naltrexone. A second binding site may be indicated for drugs that in addition prevent the ion channel from opening. Precise localization of binding sites is made difficult by the subtle allosteric interactions that regulate the events between recognition and ion transport sites. Such inhibitors are piperocaine (XX), quinacrine (XXI), amantadine, lidocaine, and tetraethylammonium ion. The wide spectrum of pharmacological properties, sedative, hypnotic, hallucinogenic, general and local anesthetic, morphine antagonist and antimuscarinic is suggestive of a "unified receptor-mediated modulation theory" of ion transport



D,L-PIPEROCAINE

XX

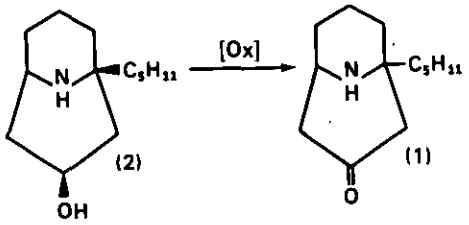


D,L—QUINACRINE·HCl

XXI

phenomena involving the essential ions Na^+, K^+ and Ca^{++}.

The nicotinic and muscarinic receptors share the same natural agonist, acetylcholine which is the tenuous bridge that, at least conceptually, spans the abyss between two different receptor worlds. Anatoxin-a has little effect on the muscarinic receptor. To the chemist it is a rewarding challenge to devise more common agonists or to elucidate the structural requirements and modalities that turn muscarinic into nicotinic agonists. There are promising leads: Adaline (XXII), the toxin of the lady bug, as the dihydro-derivative (XXIII), obtained by synthesis,[28] competes with other cholinergic ligands at the muscarinic receptor of neural cell lines.[29] The structural relationship with scopolamine and anatoxin-a bids fair to find common agonists among the tropanes.[30] It is in this area that discrepancies between biochemical binding studies and biophysical and electrophysiological data and between muscarinic receptors and nicotinic ionic channel binding sites, will have to be reconciled.[31]



XXIII DIHYDRO-ADALINE ADALINE XXII

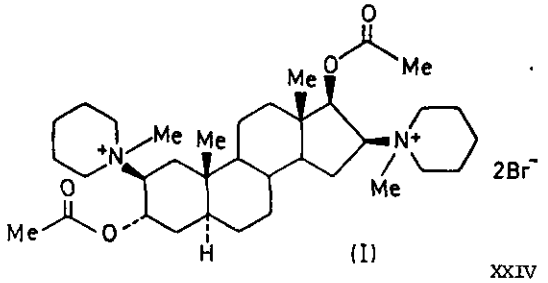
The dualism of research on membrane pores[32] and ion channels rests on the two approaches: the electrophysiological data try to relate alterations in voltage potentials and ionic currents to *movements of gating particles*, while the biochemist is concerned with binding kinetics, conformational changes, identification and isolation of protein subunits.[33] The novel neurotoxins, their structural modifications and syntheses help both the electrophysiologist as well as the biochemist to achieve and correlate these aims. The subtle allosteric effects, the multiplicity of binding sites, the interdependence of the three major ions and their common or individual channels will vary and bring out new vistas depending on the model system that so far started with frog muscle end plates, proceeded to electric organs, squid axons, nodes of Ranvier, electrically excitable strains of neuroblastoma cells, chick embryo fibroblasts and, recently, to bovine adrenal medulla cells in culture.[34] Only in this way was it possible to discover the dynamics of slow tetrodotoxin-insensitive sodium channels changing to fast tetrodotoxin-sensitive channels as a function of time and vice versa. There are observations pointing to more than one closed conformation of the sodium channel, namely, resting or inactivated, as well as to several conformations of the open channel. There is even an unexpected crossover of batrachotoxin into the nicotinic ionophore of the frog muscle endplate resulting in depolarization in the presence of cholinergic agonists. The tentative explanation of this surprising phenomenon was that batrachotoxin and veratridine interact with tetrodotoxin-insensitve sodium-specific channels at the endplate region of the neuromuscular junction that are functionally coupled to the activators of the acetylcholine receptor ion-conductance complex.[35] More recent observations on the inhibition of catecholamine secretion from adrenal medulla cells by neurotoxins point to the need for a reassessment of this explanation.[36]

Inhibition of potassium flux in nicotinic channels in the central nervous system, as recent electrophysiological and behavioral studies have shown,[26] provide a model for schizophrenia and,

eventually, an explanation, as we may hope. Antidepressants, such as amitryptiline or nortryptiline, also bind to the nicotinic channel, presumably to the activated but non-conducting conformation, a possible clue for the autonomic, central and motor disturbances and side effects seen in patients treated with tricyclic antidepressants for prolonged periods of time.[37]

In summary, tetrodotoxin has been invaluable as a tool for the exploration not only of the movement of sodium but, above all, calcium ions through ionophores and membrane pores by suppressing the transport of sodium ions.[38]

A discussion of the detailed structural and electrophysiological events at the activation or m-gate and at the inactivation or h-gate, the latter presumably blocked by pancuronium bromide (XXIV), of the channel receptor gating particles and of the selectivity filters[39] is beyond the



(I) XXIV

scope of this brief survey, whose purpose is to convey to the reader the impression that both ions and ideas are in a lively flux: 万物流転 [BAN-BUTSU RU-TEN] or as Heraclitos (535-475 B.C.) phrased it: πάντα ῥεῖ.

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