REVIEW

Application of Physiologically Active Substances Isolated from Natural Resources to Pharmacological Studies

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Received December 26, 1996

ABSTRACT—Numerous neurotoxins that alter Na^+ -channel function have been shown to be useful tools for characterizing Na^+ channels. Polypeptide blockers of voltage-dependent K^+ channels (dendrotoxins, etc.) and Ca^{2+} -activated K^+ channels (apamine, etc.) have been studied extensively by numerous investigators. Peptide toxins, calciseptine and ω -conotoxins have been attracting much attention as inhibitors of L-type and N-type Ca^{2+} channels, respectively, while ω -conotoxins-MVIIC and ω -agatoxin IVA have been used as new types of Ca^{2+} -channel blockers. Ryanodine and bromoeudistomin D analogues have been extensively used to elucidate Ca^{2+} -release-channel functions and to purify its target protein. Polypeptide toxins (myotoxin α , etc.) and macrolides (FK 506, etc.) are useful Ca^{2+} -releasers with a novel mechanism, while natural products such as thapsigargin and gingerol have been used as modulators of Ca^{2+} -pumping ATPase. Some modulators of the function of myosin (purealin, etc.) and actin (goniodomin A, etc.) have been demonstrated to be important chemical probes for understanding the physiological roles of the contractile proteins in structural changes and their interaction in muscle contraction. A large number of protein kinase inhibitors (staurosporine, etc.) and phosphatase inhibitors (okadaic acid, etc.) are widely used as first-choice reagents for studying protein phosphorylation. These natural products have become essential tools for studying the regulatory mechanism of cellular ion movements, muscle contraction and protein phosphorylation.

Keywords: Physiologically active substance, Pharmacological tool, Ion channel, Excitation-contraction coupling, Protein phosphorylation

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I. Introduction

The physiological basis of cell excitability is the voltage-dependent and ion-selective permeability of the cell membrane. Experimental evidence indicates that the two properties do not apply to the entire membrane surface, but are restricted to specific sites known as channels. These are proteic structures spanning the entire lipid bilayer and randomly distributed within it. Ion channels are specialized integral membrane proteins that undergo conformational changes to allow transmembrane movement of ions. The increases in membrane permeability to Na^+ , K^+ and Ca^{2+} are mediated by voltage-dependent Na^+ , K^+ and Ca^{2+} channels, respectively. Neurotoxins that selectively block the voltage-sensitive Na^+ , K^+ or Ca^{2+} channels are essential tools for understanding the molecular basis of electrical excitability. From this viewpoint, a variety of natural toxins have been extensively studied by pharmacologists, physiologists and biochemists because they interact with specific channels on excitable membranes (1).

Ca^{2+} is critically involved in numerous cellular regulatory processes including excitation-contraction coupling, excitation-secretion coupling, neuronal excitability, ionic membrane permeability and cell growth and differentiation. In muscle biology, especially lacking is knowledge of the mechanism whereby the sarcoplasmic reticulum (SR) releases Ca^{2+} that has been accumulated during muscle relaxation (2). In striated muscle contraction, Ca^{2+} activation of force development is triggered by Ca^{2+} binding to myofilaments, which causes a conformational change in the contractile machinery to stimulate the actin-myosin interaction. It is generally accepted that reversible protein phosphorylation plays an essential role in controlling a wide range of cellular events such as smooth muscle contraction, cell division, cell signaling, differentiation and metabolism (3). Selective modulators (inhibitors and/or activators) are extremely useful probes for characterizing target proteins.

This review focuses on research that has given insight into the cellular and molecular mechanisms of action of natural products isolated from marine organisms, terrestrial plants and microorganisms. These studies have revealed the molecular regulatory mechanisms of ion channels, excitation-contraction coupling and protein phosphorylation through the use of these compounds

as chemical probes.

II. Elucidation of Molecular Properties of Ion Channels

II-1. Natural products that affect Na^+ -channel function

Voltage-dependent Na^+ channels are found in virtually all multicellular animals, where they play a key role in the initiation of electrical activity. The Na^+ channel from rat brain consists of a large α -subunit (260-kDa) that is associated with smaller β_1 (36-kDa) and β_2 (33-kDa) subunits. The β_2 -subunit is linked to the α -subunit by disulfide bonds. Na^+ -channel α -subunit cDNAs have been isolated from a variety of sources including rat brain, skeletal muscle and cardiac muscle. Each cDNA encodes a protein consisting of four homologous domains (I–IV), each consisting of six predicted transmembrane α -helices (S1–S6).

Characterization of neurotoxin receptor sites 1 to 5

Numerous neurotoxins that alter Na^+ -channel function bind at five separate receptor sites in the molecule (Table 1) (1). The heterocyclic guanidine derivatives, tetrodotoxin (TTX) from puffer fish of the suborder *Gymnodontes* and saxitoxin from the dinoflagellate *Gonyaulax catenella* (Fig. 1), specifically inhibit the ion permeability of

Table 1. Neurotoxin receptor sites associated with the Na^+ channel

Receptor site	Ligands	Physiological effect
1	Tetrodotoxin Saxitoxin Geographutoxin (μ -Conotoxin)	Inhibit Na^+ transport
2	Veratridine Batrachotoxin Aconitine Grayanotoxin	Alter Na^+ channel activation and inactivation. Cause persistent activation of Na^+ channels
3	α -Scorpion toxins Sea anemone toxin Goniopodatoxin	Inhibit Na^+ channel inactivation. Enhance persistent activation of Na^+ channels
4	β -Scorpion toxins γ -Scorpion toxins	Alter Na^+ channel activation rather than inactivation. Repetitive firing
5	Brevetoxin Ciguatoxin	Alter Na^+ channel activation. Repetitive firing

Abbreviations used are (in alphabetical order): BED, 7-bromoeudistomin D; $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentration; ω -CgTX, ω -conotoxin; ChTX, charybdotoxin; CTX, ciguatoxin; DBHC, 4,6-dibromo-3-hydroxycarbazole; DHS-1, dehydrosaponin-1; DTX, dendrotoxin; FKBP, FK506-binding 12-kDa protein; GTX, geographutoxin; HSR, heavy fraction of the fragmented sarcoplasmic reticulum; IbTX, iberiotoxin; KTX, kaliotoxin; MBED, 9-methyl-7-bromoeudistomin D; MCD, mast cell-degranulating; MgTX, margatoxin; MTX, maitotoxin; MYTX, myotoxin α ; PAL, puff-adder lectin; PKC, protein kinase C; Ser/Thr, serine/threonine; SH, sulfhydryl; SR, sarcoplasmic reticulum; StTX, striatotoxin; TC, terminal cisternae; TTX, tetrodotoxin; XQN, xestotoxin; ZT-A, zoanthellatoxin-A.

Fig. 1. The chemical structures of natural toxins that bind to receptor site 1 associated with Na⁺ channels.

Na⁺ channels without affecting voltage-dependent K⁺ channels and Ca²⁺ channels. The isolation, structure determination and general pharmacology of the toxins have been reviewed in detail. Several lines of experimental evidence suggest that these positively charged toxins are bound close to the extracellular mouth of the channel in a region containing negatively charged groups (SS1–SS2 region) (4–6). Recently, it has been shown that a single mutation in rat Na⁺ channel II that reduces net negative charge renders the channel insensitive to TTX and saxitoxin and resulted in a Na⁺ channel with a reduction in its single-channel conductance without preventing gating function. Ligand-binding experiments with [³H]TTX and [³H]saxitoxin show that both toxins bind to a common receptor site (receptor site 1) associated with Na⁺ channels. These results suggest that receptor site 1 is located at the extracellular vestibule or inside the ion-conducting pore of the channel. Both toxins have been employed as selective ligands for purification and subsequent structural characterization of Na⁺-channel protein. The second receptor site (receptor site 2) binds the lipid-soluble toxins such as batrachotoxin (steroidal alkaloid from the Colombian frog *Phylllobates aurotaenia*), aconitine (aconitum alkaloid from the plant *Aconitum napellus*), veratridine (veratrum alkaloid from liliaceous plants of the species *Veratreae*) and grayanotoxin (terpene possessing the andromedane skeleton from plants of the family Ericaceae). These toxins shift the voltage-dependence of

activation to more negative membrane potentials and block Na⁺-channel inactivation, suggesting that this site may be located on a region of the Na⁺ channels important for both of these voltage-dependent processes. The third receptor site (receptor site 3) binds some polypeptides, α -scorpion toxins from the North African scorpion *Leiurus quinquestratus* and sea anemone toxin from the sea anemone *Anemonia sulcata*; the α -scorpion toxins bind to receptor site 3 on the extracellular surface of the membrane in a voltage-dependent manner to slow inactivation of Na⁺ channels, suggesting that the binding site for these molecules represents an extracellular site that undergoes voltage-dependent conformational changes that are required for channel inactivation. Photolabeling of the brain Na⁺ channel with a photoreactive scorpion toxin derivative resulted in labeling of the α - and β_1 -subunits. However, actions of α -scorpion toxins are normal on channels resulting from expression of the α -subunit alone, indicating that the physiologically relevant sites are on that subunit. The sites on the α -subunit that were photolabeled were identified by proteolytic digestion of the labeled protein followed by identification of the labeled fragment with site-directed antibodies. This site is located on the extracellular loop connecting transmembrane segments S5 and S6. A new class of scorpion toxins (β -scorpion toxins) isolated from the American scorpion *Centruroides sculpturatus* binds at receptor site 4 in Na⁺ channels. These toxins modify Na⁺-channel activation

Fig. 2. The chemical structure of dinoflagellate toxins that bind to receptor site 5 associated with Na^+ channels.

rather than the inactivation process. β -Scorpion toxin binding is not voltage-dependent in contrast to α -scorpion toxin binding.

The oxygenated polyether compounds such as brevetoxin and ciguatoxin (CTX) (7) isolated from marine dinoflagellates (Fig. 2) bind at a new common receptor site (receptor site 5) associated with Na^+ channels to cause repetitive firing and an increase in the frequency of action potentials.

CTX causes a type of food poisoning known as ciguatera and is produced by the benthic dinoflagellate *Gambierdiscus toxicus* and transmitted to a variety of reef fish through the food chain. CTX has been reported to cause depolarization of the plasma membrane (8) and contraction of the vas deferens (9, 10). It also has a concentration-dependent inotropic action on the papillary muscles (11) and left atria (11, 12), and these effects of CTX are abolished by TTX. These pharmacological studies have clearly indicated that the inotropic response to lower concentrations of CTX is primarily the result of an indirect action mediated through noradrenaline release, whereas that induced by higher concentrations occurs mainly through a direct action on cardiac muscle. Furthermore, our detailed electrophysiological studies have re-

vealed that in cardiac myocytes, CTX shifts the voltage dependence of Na^+ -channel activation toward more hyperpolarized potentials without affecting the time course of inactivation or the peak current amplitude (12).

Characterization of Na^+ channel by a novel type of marine toxins

Cone shells have a well-developed venom apparatus to catch their prey, such as fishes, shells and worms (13). *Conus geographus* is highly toxic and has been responsible for human injury. The crude venom of *C. geographus* has an inhibitory effect on the contractile response of stimulated skeletal muscle (14, 15). Geographotoxins I and II (GTX I and II) composed of 22 amino acid residues have been isolated from the venom by monitoring the pharmacological activity (16) and are classified as μ -conotoxin GIIIA and GIIIB, respectively. The primary structures of the two peptides are very similar to each other (see Fig. 1) (17).

GTX II was shown to inhibit twitch responses of the isolated mouse diaphragm to direct stimulation, and the IC_{50} value of GTX II is a little higher than that of TTX. The toxin blocks the action potential of the guinea pig skeletal muscle without affecting the resting membrane

potential (18). Voltage clamp experiments on bullfrog skeletal muscle fiber have indicated that Na^+ currents are abolished by GTX II. However, action potentials of the crayfish giant axon, mouse neuroblastoma NIE-115 cell and guinea pig cardiac muscle are not affected by GTX II even at high concentrations (19). These findings have indicated that GTX II selectively blocks skeletal muscle Na^+ channels in much the same way as TTX. GTX II has been shown to distinguish two different types of voltage-sensitive Na^+ currents: GTX II-sensitive and GTX II-insensitive currents, which correspond respectively to currents with high or low TTX sensitivity (20, 21). In addition, GTX II at higher concentrations causes blockade of neuromuscular transmission in skeletal and smooth muscle preparations (22, 23).

GTX II has been demonstrated to inhibit [^3H]saxitoxin binding to Na^+ channels in skeletal muscle homogenates and T-tubular membranes at concentrations similar to those that block muscle contraction. The K_d value is increased by GTX II, while the B_{max} value is not modified, indicating a competitive mode of inhibition (24, 25). However, [^3H]saxitoxin binding to membranes of the superior cervical ganglion is not modified by GTX II even at high concentrations. These results suggest that GTX II interacts competitively with saxitoxin in binding at neurotoxin receptor site 1 associated with the Na^+ channel in a highly tissue-specific manner. GTX II is the first reagent that discriminates at this site between nerve and muscle Na^+ channels. GTX II is used to identify these tissue-specific regions of the Na^+ -channel structure at neurotoxin receptor site 1. It has become a useful chemical probe for classifying Na^+ -channel subtypes.

Various analogues of GTX II have been synthesized to elucidate the molecular nature of its active center for blocking skeletal Na^+ channels. Analysis of NMR or CD spectral data of these analogues, including GTX II, indicates the similarity of their conformations (26). The arginine residue at the 13th position and basicity of the molecule are important for the inhibitory activity. The structure-activity relationship studies lead to the conclusion that arginine is a key residue for peptide toxins to interact with receptor site 1 in Na^+ channels.

Striatoxin (StTX), a glycoprotein (molecular weight of 25,000), has been isolated from *Conus striatus*, a piscivorous Conidae, as a cardiotonic component (27). StTX causes an inotropic effect on isolated guinea pig left atria (28) and a rhythmic contraction of the ileum followed by relaxation (29). StTX provoked action potentials with a plateau phase of long duration. These effects of StTX are reversed by TTX. Whole cell patch-clamp experiments have showed that StTX slows Na^+ -channel inactivation without affecting the time course of channel activation (30). The binding of saxitoxin or α -scorpion toxin to

nerve Na^+ channels is not significantly affected by StTX. A possible explanation of these results has been that StTX binds to a new receptor site associated with Na^+ channels, at which specific effects on channel inactivation can occur. These observations suggest that the inotropic effect of StTX is caused by increasing Na^+ permeability of Na^+ channels due to slowed inactivation of channel kinetics and that this may result in an increase in Ca^{2+} availability in cardiac muscle cells.

II-2. Natural products that affect K^+ -channel function

K^+ channels comprise a family of proteins that have been classified according to their biophysical and pharmacological characteristics. These channels modulate a number of cellular events such as muscle contraction, neuro-endocrine secretion, frequency and duration of action potentials, electrolyte homeostasis and resting membrane potential. The biochemical characterization of K^+ channels is underdeveloped due to the paucity of selective high affinity probes.

Characterization of voltage-dependent K^+ channels by peptide toxins

Various snake venoms that affect K^+ -channel function have been extensively studied by many investigators; and dendrotoxins (DTXs), polypeptide blockers of several types of Ca^{2+} -independent, voltage-dependent K^+ channels, were isolated from the Eastern green mamba snake, *Dendroaspis angusticeps* (Fig. 3) (31). DTXs have been [^{125}I]-radiolabeled for use in ligand-binding assays. DTX-I, isolated from *D. polylepis* venom, has been used to purify DTX-sensitive K^+ channels (32–34). Furthermore, DTX-I affinity chromatography has been employed for the purification of a DTX-receptor protein, a voltage-dependent K^+ channel. Several of the other DTX have become important tools for purification and characterization of various voltage-dependent K^+ channels.

Mast cell-degranulating (MCD) peptide, a disulfide-rich basic peptide has been purified from bee venom. It is composed of 22 amino acid residues and contains two disulfide bridges (Fig. 3). The monoiodinated derivative of MCD peptide has been prepared by us to identify binding sites in rat brain membranes (35). It has been demonstrated that these binding sites are evenly distributed throughout the brain and copurify with synaptic membranes. The binding experiment indicates a single population of sites with a concentration of 200 fmol/mg membrane protein in partially fractionated, lysed brain membranes and the dissociation constant of 140 pM. These binding sites may be associated with the neurotoxication of MCD peptide in the central nervous system.

It has been reported that MCD peptide releases hista-

Glx-Pro-Arg-Arg-Lys-Leu-Cys-Ile-Leu-His-Arg-Asn-Pro-Gly-Arg-Cys-Tyr-Asp-Lys-Ile-Pro-Ala-Phe-Tyr-Tyr-Asn-Gly-Lys-Lys-Lys-Gln-Cys-Glu-Arg-Phe-Asp-Trp-Ser-Gly-Cys-Gly-Gly-Asn-Ser-Asn-Arg-Phe-Lys-Thr-Ile-Glu-Glu-Cys-Arg-Arg-Thr-Cys-Ile-Gly

Dendrotoxin

Ile-Lys-Cys-Asn-Cys-Lys-Arg-His-Val-Ile-Lys-Pro-His-Ile-Cys-Arg-Lys-Ile-Cys-Gly-Lys-Asn-NH₂

Mast cell-degranulating peptide

Thr-Ile-Ile-Asn-Val-Lys-Cys-Thr-Ser-Pro-Lys-Gln-Cys-Leu-Pro-Pro-Cys-Lys-Ala-Gln-Phe-Gly-Gln-Ser-Ala-Gly-Ala-Lys-Cys-Met-Asn-Gly-Lys-Cys-Lys-Cys-Tyr-Pro-His

Margatoxin

Glx-Phe-Thr-Asn-Val-Ser-Cys-Thr-Thr-Ser-Lys-Glu-Cys-Trp-Ser-Val-Cys-Gln-Arg-Leu-His-Asn-Thr-Ser-Arg-Gly-Lys-Cys-Met-Asn-Lys-Lys-Cys-Arg-Cys-Tyr-Ser

Charybdotoxin

Cys-Asn-Cys-Lys-Ala-Pro-Glu-Thr-Ala-Leu-Cys-Ala-Arg-Arg-Cys-Gln-Gln-His

Apamin

Ala-Phe-Cys-Asn-Leu-Arg-Met-Cys-Gln-Leu-Ser-Cys-Arg-Ser-Leu-Gly-Leu-Leu-Gly-Lys-Cys-Ile-Gly-Asp-Lys-Cys-Glu-Cys-Val-Lys-His

Leiurotoxin

Glu-Phe-Thr-Asp-Val-Asp-cys-Ser-Val-Ser-Lys-Glu-Cys-Trp-Ser-Val-Cys-Lys-Asp-Leu-Phe-Gly-Val-Asp-Arg-Gly-Lys-Cys-Met-Gly-Lys-Lys-cys-Arg-Cys-Tyr-Gln

Iberiotoxin

Gly-Val-Glu-Ile-Asn-Val-Lys-Cys-Ser-Gly-Ser-Pro-Gln-Cys-Leu-Lys-Pro-Cys-Lys-Asp-Ala-Gly-Met-Arg-Phe-Gly-Lys-Cys-Met-Asn-Arg-Lys-Cys-His-Cys-Thr-Pro

Kaliotoxin

Fig. 3. The chemical structures of natural products that modulate K⁺ channels.

mine from mast cells. It produces arousal at low concentrations and convulsions at higher doses. These effects are mediated through high-affinity binding sites that are concentrated in cortical structures, notably the hippocampus (36). This structure appears to be the source of changes in the electrocorticogram that follow injections of MCD peptide into the cerebral ventricle and which induce a

quasi-permanent hippocampal theta rhythm in the motionless rat alternating with epileptiform spike waves. MCD peptide causes a long-term potentiation after application to the CA1 region of hippocampal slices. This potentiation is indistinguishable from the classical long-term potentiation produced by trains of high-frequency electrical stimulation and is probably related to memory.

Interestingly, an endogenous peptide equivalent of MCD peptide has been found in brain extracts using binding experiments and radioimmunoassay techniques. An attractive interpretation is that a MCD-like peptide plays an important role in long-term potentiation.

A novel peptidyl inhibitor of voltage-dependent K^+ channels named margatoxin (MgTX) has been isolated from the venom of the new world scorpion *Centruroides margaritatus* and the primary structure of the 39 amino acid peptide named margatoxin has been determined by amino acid compositional analysis and peptide sequencing (Fig. 3). MgTX potently inhibits binding of radio-labeled charybdotoxin (ChTX) to voltage-dependent K^+ channels in brain synaptic plasma membranes (37). Like ChTX, MgTX blocks the N-type current of human T-lymphocytes ($K_v1.3$ channel). MgTX is 20-fold more potent than ChTX in K^+ -channel blocking activity ($EC_{50} = 50$ pM). Recombinant MgTX has been expressed in *Escherichia coli* as part of a fusion protein. After cleavage and folding, purified recombinant MgTX has been shown to display the same properties as native peptide. Replacement of the COOH-terminal histidine residue of MgTX with asparagine reduces its potency by tenfold, suggesting that the COOH-terminal amino acid may play an important role in the binding of MgTX to the channels. MgTX displays significant sequence homology with previously identified K^+ -channel inhibitors (e.g., ChTX, iberiotoxin, noxiustoxin). MgTX has become a useful tool for investigating the physiological role of $K_v1.3$ channels because of its potency and unique selectivity.

ChTX is a 37 amino acid basic peptide purified from the venom of the scorpion *Leiurus quinquestriatus var. hebraeus* (Fig. 3) (38). It was first reported to be a potent blocker of the Ca^{2+} -activated K^+ channels (38). Subsequently, ChTX has been shown to block other types of K^+ channels (39, 40). ChTX seems to bind to the outer face of the channel to block K^+ permeability through an electrostatic mechanism whereby positive charges on the toxin molecule interact with negatively charged residues located within or near the ion-transporting pore of the channel (41). This electrostatic mechanism is also considered to be involved in the interaction of ChTX with voltage-dependent K^+ channels in human T-lymphocytes. Scatchard analysis of [^{125}I]ChTX binding to brain synaptic plasma membrane clearly indicates the presence of a single class of binding sites for [^{125}I]ChTX with the K_d value of 29 pM and B_{max} value of 0.3 pmol/mg protein. An interesting finding is that three different rat brain cDNA clones that express voltage-dependent K^+ channels in *Xenopus* oocytes are sensitive to ChTX (39, 42), although the molecular properties of the rat brain ChTX receptor are yet to be revealed.

Characterization of Ca^{2+} -activated K^+ channels by peptide toxins

Small-conductance Ca^{2+} -activated K^+ channels are defined as those having single-channel conductances of less than 20 pS. These channels are widely distributed in both excitable and nonexcitable cells. Apamin has been isolated as a minor component of the venom of the honeybee *Apis mellifera*. It is an 18 amino acid polypeptide neurotoxin (molecular weight of 2000) containing two disulfide linkages (Fig. 3). Apamin is the first K^+ channel toxin to be isolated and characterized. Apamin-sensitive Ca^{2+} -activated K^+ channels are known to underlie neurotransmitter and hormone-induced increase in K^+ permeability in a variety of cells. It has been demonstrated that the ionic currents through these channels are responsible for maintaining the slow after-hyperpolarizing potential that follows bursts of action potentials. It has been used as a pharmacological tool to characterize ionic currents flowing through K^+ channels. [^{125}I]Apamin has been used to characterize specific high affinity binding sites for this toxin in brain synaptosomes (43), embryonic neurons (44) and hepatocytes (45). Apamin binding has been correlated with the inhibition of a specific class of Ca^{2+} -activated K^+ channels in certain neurons.

Leiurotoxin I, an inhibitor of apamin binding, has been purified to homogeneity in three chromatographic steps from the venom of the scorpion *Leiurus quinquestriatus hebraeus* (Fig. 3). It is a 3.4-kDa peptide with little structural homology to apamin, although it has some homology to other scorpion toxins such as ChTX and noxiustoxin. Both the toxins cause a contraction of taenia coli previously relaxed with epinephrine, and they block the after-hyperpolarization due to Ca^{2+} -activated K^+ -channel activity in cultured muscle cells. Like apamin, leiurotoxin I blocks the epinephrine-induced relaxation of guinea pig teniae coli ($ED_{50} = 6.5$ nM) without affecting the rate or force of contraction in guinea pig atria or rabbit portal vein preparation. Thus, leiurotoxin I and apamin demonstrate similar pharmacological properties in a variety of tissues in spite of structurally unrelated peptides (46). Both the toxins appear to be useful tools for elucidating the physiological role of the small conductance, Ca^{2+} -activated K^+ channels in different tissues.

Leiurotoxin I completely inhibits [^{125}I]apamin binding to rat synaptosomal membranes with a K_i of 75 μ M, indicating that it is 10–20-fold less potent than apamin. Leiurotoxin I is not a strictly competitive inhibitor of this binding. The total synthesis of this scorpion neurotoxin as well as some aspects of its structure-function relationships have been studied. The analog [Tyr 2] leiurotoxin I (scyllatoxin) has been monoiodinated at high specific radioactivity (2,000 Ci/mmol) and has served for the characterization of the properties of [^{125}I]-[Tyr 2] leiurotox-

in I binding sites ($K_d = 80$ pM) (47). Binding experiments have revealed molecular masses of 27 and 57 kDa for the two polypeptides in the leiurotoxin I binding protein. Despite having a different chemical structure, apamin competitively inhibits ^{125}I -[Tyr²] leiurotoxin I binding.

Iberitoxin (IbTX), a peptide inhibitor of the high conductance Ca^{2+} -activated K^+ channel, has been purified to homogeneity from the venom of the scorpion *Buthus tamulus* (48) (Fig. 3). IbTX consists of a single 4.3-kDa polypeptide chain and its complete amino acid sequence has been determined. It has been demonstrated that IbTX reversibly blocks Ca^{2+} -activated K^+ channels in excised membrane patches from bovine aortic smooth muscle and interacts at the outer face of the channel (48). Its blockage of channel activity appears distinct from that of ChTX since IbTX decreases both the probability of channel opening and the channel mean open time. It is noticeable that IbTX is a selective inhibitor of high conductance Ca^{2+} -activated K^+ channels without affecting other types of voltage-dependent ion channels including other types of K^+ channels that are sensitive to ChTX. Furthermore, it has been demonstrated that IbTX is a partial inhibitor of ^{125}I ChTX binding in bovine aortic sarcolemmal membrane vesicles ($K_i = 250$ pM). The Scatchard analysis suggests that IbTX functions as a noncompetitive inhibitor of ChTX binding. These data suggest that IbTX interacts at a distinct site on the channel and modulates ChTX binding by an allosteric mechanism. Therefore, IbTX defines a new class of peptidyl inhibitor of Ca^{2+} -activated K^+ channels.

An important finding is that like ChTX, IbTX is only able to block the skeletal muscle membrane Ca^{2+} -activated K^+ channels incorporated into neutral planar bilayers when applied to the external side (49). From single-channel recording, it is possible to determine that IbTX binds to the Ca^{2+} -activated K^+ channel in a bimolecular reaction. A detailed electrophysiological analysis gives an apparent equilibrium dissociation constant of 1.16 nM. This constant is tenfold lower than that of ChTX. Tetraethylammonium competitively inhibits the IbTX binding to the channel, suggesting that this toxin binds to the channel external vestibule. Increasing the external K^+ concentration decreases the association rate constant without affecting the dissociation reaction. This indicates that the surface charges located in the external mouth of the channel play an important role in modulating toxin binding.

Kaliotoxin (KTX), a peptidyl inhibitor of the high conductance Ca^{2+} -activated K^+ channels, has been isolated from the venom of the scorpion *Androctonus mauretanicus mauretanicus* and has been shown to be a single 4-kDa polypeptide chain (Fig. 3). Electrophysiological experiments have revealed that KTX specifically inhibits the

whole cell Ca^{2+} -activated K^+ current in nerve cells (50). KTX has no effect on voltage gated K^+ currents (delayed rectifier and fast transient A current) or on L-type Ca^{2+} currents. KTX interacts in a one-to-one way with Ca^{2+} -activated K^+ channels with a K_d of 20 nM. In single channel experiments on high conductance Ca^{2+} -activated K^+ channels, KTX acts at the outer face of the channel to cause a transient period of fast-flicker block followed by a persistent channel blockade. The blocking action of KTX is not voltage-dependent, suggesting differences in the blockade of Ca^{2+} -activated K^+ channels by KTX and ChTX. An interesting observation is that comparison of KTX and ChTX sequences leads to the identification of a short amino acid sequence that may be implicated in the toxin-channel interaction. Therefore, KTX should be useful for elucidating the molecular mechanism of the high conductance Ca^{2+} -activated K^+ channel.

Characterization of Ca^{2+} -activated K^+ channels by indole alkaloids

Tremogenic indole alkaloids induced neurological disorders including stagges syndromes in ruminants (51, 52). Binding of ^{125}I ChTX to high conductance Ca^{2+} -activated K^+ channels in the sarcolemmal membrane of aortic smooth muscle is inhibited by paspalitrem A, paspalitrem C, aftrem, penitrem A (Fig. 3) and paspalinine, but enhanced by three structurally related compounds, paxiline, verruculogen and paspalicine. On the basis of binding studies, it is probable that covalent incorporation of ^{125}I ChTX into the 31-kDa subunit of the channel is blocked by compounds that inhibit ^{125}I ChTX binding and increased by compounds that stimulate ^{125}I ChTX binding. Modulation of ^{125}I ChTX binding was due to allosteric mechanisms. Despite their different effects on the binding of ^{125}I ChTX, all these compounds potently inhibited high conductance Ca^{2+} -activated K^+ channels with no effect on other types of K^+ channels in electrophysiological experiments. Taken together, these data suggest that the indole diterpenes are the most potent nonpeptidyl inhibitors of high conductance Ca^{2+} -activated K^+ channels. It is also suggested that some of their pharmacological properties could be explained by inhibition of the channels, although tremorgenicity may be unrelated to channel block.

Application of openers of Ca^{2+} -activated K^+ channels

A large number of synthetic openers of Ca^{2+} -activated K^+ channels have now been developed by pharmaceutical industries for the treatment of cardiovascular diseases. Recently dehydrosaponin-1 (DHS-1), a triterpenoid glycoside that was isolated from the medicinal herb *Desmodium adscendens*, has been demonstrated to be an opener of Ca^{2+} -activated K^+ channels (Fig. 3) (53). DHS-1 is effec-

tive only when it is applied intracellularly because of the poor membrane permeability of this compound. DHS-1 has been attracting much attention since it is the first compound that has been found to open K^+ channels via an interaction with the β -subunit of Ca^{2+} -activated K^+ channels. Maxikdiol, another opener of Ca^{2+} -activated K^+ channels, has been isolated from a fermentation broth (Fig. 3) and identified by its ability to displace labeled ChTX from its binding site (54). [^{125}I]ChTX binding to Ca^{2+} -activated K^+ channels in aortic sarcolemma is inhibited by maxikdiol, whereas that to voltage-dependent K^+ channels in brain synaptic membranes is not affected by it even at high concentrations, indicating specificity. The biological data indicate that the pharmacological profile of maxikdiol is very similar to that of structurally dissimilar DHS-1. Both DHS-1 and maxikdiol should be essential tools for investigating the regulation of channel gating of Ca^{2+} -activated K^+ channels.

II-3. Natural products that affect Ca^{2+} -channel function

There has been a dramatically growing awareness that Ca^{2+} movements in the cell can play a central role in many cellular functions. From this point of view, substances that influence cellular Ca^{2+} movements provide valuable probes for the elucidation of various cellular functions. The plasma membrane separates extracellular mM Ca^{2+} from intracellular μM Ca^{2+} concentrations. Ca^{2+} entry occurs through Ca^{2+} channels. Six classes of voltage-dependent Ca^{2+} channels (termed L, N, P, T, R and Q) have been defined on the basis of their physiological and pharmacological properties (55). Molecular cloning has also revealed the existence of six high-voltage-dependent Ca^{2+} channel subtypes. Expression studies have indicated that basic high-voltage-dependent channel function, which is characteristic of the L (skeletal muscle, cardiac muscle and neuroendocrine tissue)-, N-, P-, Q- and R-type channels is carried by the corresponding α_1 -subunits (56). Auxiliary subunits, such as α_2/δ and β , modulate the kinetics of activation, inactivation, current density and drug binding, thereby creating considerable potential for multiple Ca^{2+} channel functions.

Characterization of L-type channels by calciseptine

The L-type Ca^{2+} channel has been extensively studied by numerous pharmacologists. Blockers of this type of channel can abolish contractions in cardiac and smooth muscle cells. A high number of relatively simple organic molecules including 1,4-dihydropyridines, phenylallylamines and benzothiazepines have been extensively studied because these drugs have been used clinically for their antianginal, vasodilator, and antiarrhythmic properties. Calciseptine has been isolated as a specific Ca^{2+} -channel

blocker from the venom of the black mamba *Dendroaspis polylepis polylepis*. It contains 60 amino acids and has been fully sequenced (Fig. 4). Calciseptine causes a relaxation of smooth muscle and an inhibition of cardiac muscle. The pharmacological properties of the peptide have been shown to be similar to that of drugs such as the 1,4-dihydropyridines, which are important in the treatment of cardiovascular diseases (57). Calciseptine, like the 1,4-dihydropyridines, selectively blocks L-type Ca^{2+} channels without altering the activity of N-type and T-type channels (57). It is the only natural polypeptide that has been shown to be a specific inhibitor of L-type Ca^{2+} channels.

Characterization of N-type channels by ω -conotoxin (ω -CgTX)

It has been reported that the N-type Ca^{2+} channel is present in neuronal cells and is blocked by a polypeptide toxin isolated from *C. geographus*, ω -CgTX (Fig. 4). The peptide toxins have been shown to block individual Ca^{2+} channel subtypes. ω -CgTX was initially reported to block both the N- and L-type channels in nerve preparations (58). Detailed electrophysiological experiments, however, have shown that the toxin selectively blocks N-type channels without affecting the L-type channels (59–61). Voltage-dependent Ca^{2+} channels in the nervous tissues can be classified pharmacologically into three subclasses: ω -CgTX-sensitive, dihydropyridine-sensitive, and both ω -CgTX and dihydropyridine-insensitive.

Purification and elucidation of ω -CgTX target proteins have been extensively studied by numerous investigators. Cross-linking experiments with labeled ω -CgTX reveal that a 210-kDa (62) or 170-kDa (63) protein is a target molecule in synaptosomal membranes of chick brain. In rat brain, bands of 310, 240 and 34 kDa (64) or 220 and 33 kDa (63) are labeled. Recently, the ω -CgTX receptor (N-type Ca^{2+} channel) of rabbit brain membranes has been purified by heparin chromatography, immunoaffinity chromatography and density gradient centrifugation (65). The channel is composed of four subunits with molecular weights of 230 (α_1B), 160 ($\alpha_2\delta$), 57 (β_3) and 95 kDa (glycoprotein subunit). Reconstitution experiments with the purified ω -CgTX receptor indicated that the complex forms functional Ca^{2+} release channels with the same pharmacological properties as those of native ω -CgTX-sensitive Ca^{2+} channels. Recent biochemical studies suggest that the subunit composition of ω -CgTX receptor is similar to, but distinct from, that of the dihydropyridine receptor (66). Furthermore, molecular biological studies have made it possible to express the complementary DNA encoding the human neuronal α_1 -subunit producing ω -CgTX-sensitive current (67).

Characterization of ω -CgTX-insensitive Ca^{2+} channels in neurons by a novel conus toxin

Voltage-dependent Ca^{2+} channels that control neurotransmitter release are blocked by ω -CgTX-GVIA from *C. geographus*, the most widely used inhibitor of neurotransmitter release. However, many mammalian synapses are ω -CgTX-GVIA insensitive. ω -CgTX-MVIIC from the cone shell *C. mgus* has been shown to inhibit ω -CgTX-GVIA-resistant synaptic transmission, depolarization-induced $^{45}\text{Ca}^{2+}$ uptake in rat synaptosome preparations, "P" currents in cerebellar Purkinje cells and currents in CA1 hippocampal pyramidal cells (68). Furthermore a peptide toxin named ω -agatoxin-IVA from funnel web spider venom is a potent inhibitor of both Ca^{2+} entry

into rat brain synaptosomes and P-type Ca^{2+} channels in rat Purkinje neurons (69). ω -Agatoxin-IVA will facilitate characterization of brain Ca^{2+} channels resistant to existing channel blockers and may assist in the design of neuroprotective drugs.

Characterization of a novel type of Ca^{2+} -permeable channels by marine toxins

Maitotoxin (MTX), the most potent marine toxin known, has been isolated from poisonous fish *Ctenochaetus striatus* and the dinoflagellate *Gambierdiscus toxicus* (Fig. 5) (70). We have shown for the first time that MTX causes Ca^{2+} -dependent release of noradrenaline from a rat pheochromocytoma cell line (71, 72),

Arg-Ile-Cys-Tyr-Ile-His-Lys-Ala-Ser-Leu-Pro-Arg-Ala-Thr-Lys-Thr-Cys-Val-Glu-Asn-Thr-
Cys-Tyr-Lys-Met-Phe-Ile-Arg-Thr-Gln-Arg-Glu-Tyr-Ile-Ser-Glu-Arg-Gly-Cys-Gly-Cys-
Pro-Thr-Ala-Met-Trp-Pro-Tyr-Gln-Thr-Glu-Cys-Cys-Lys-Gly-Asp-Arg-Cys-Asn-Lys

Calciseptine

Cys-Lys-Ser-Hyp-Gly-Ser-Ser-Cys-Ser-Hyp-Thr-Ser-Tyr-Asn-Cys-Cys-Arg-Ser-
Cys-Asn-Hyp-Tyr-Thr-Lys-Arg-Cys-Tyr

ω -Conotoxin GVIA

Cys-Lys-Ser-Hyp-Gly-Ser-Ser-Cys-Ser-Hyp-Thr-Ser-Tyr-Asn-Cys-Cys-Arg-Ser-
Cys-Asn-Hyp-Tyr-Thr-Lys-Arg-Cys-Tyr-Gly

ω -Conotoxin GVIB

Cys-Lys-Ser-Hyp-Gly-Ser-Ser-Cys-Ser-Hyp-Thr-Ser-Tyr-Asn-Cys-Cys-Arg-Ser-
Cys-Asn-Hyp-Tyr-Thr-Lys-Arg-Cys

ω -Conotoxin GVIC

Cys-Lys-Ser-Hyp-Gly-Thr-Hyp-Cys-Ser-Arg-Gly-Met-Arg-Asp-Cys-Cys-Thr-
Ser-Cys-Leu-Leu-Tyr-Ser-Asn-Lys-Cys-Arg-Arg-Tyr

ω -Conotoxin GVIIA

Cys-Lys-Ser-Hyp-Gly-Thr-Hyp-Cys-Ser-Arg-Gly-Met-Arg-Asp-Cys-Cys-Thr-
Ser-Cys-Leu-Ser-Tyr-Ser-Asn-Lys-Cys-Arg-Arg-Tyr

ω -Conotoxin GVIBB

Cys-Lys-Gly-Lys-Gly-Ala-Lys-Cys-Ser-Arg-Leu-Met-Tyr-Asp-Cys-Cys-Thr-
Gly-Ser-Cys-Arg-Ser-Gly-Lys-Cys

ω -ConotoxinMVIIA

Fig. 4. Amino acid sequences of peptide toxins that inhibit Ca^{2+} channels.

Fig. 5. The chemical structures of dinoflagellate toxins maitotoxin and zoosaxanthellatoxin-A.

Ca^{2+} -dependent contraction of smooth muscle (73–75), and positive inotropic (76) and cardiotoxic effects (77–79). Furthermore MTX has been shown to cause Ca^{2+} -dependent excitatory effects on pituitary cells (80) skeletal muscle (81) and platelets (82) and to increase the tissue Ca^{2+} content, $^{45}\text{Ca}^{2+}$ uptake or intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) of these cells or tissues. All these actions of MTX are markedly inhibited by Ca^{2+} -antagonists, polyvalent cations or Ca^{2+} -free medium,

suggesting that enhanced Ca^{2+} influx plays a dominant role in the excitatory effects of MTX. In cardiac myocytes, MTX causes a sustained Ca^{2+} inward current without affecting the voltage-dependent Ca^{2+} channel current. Most significant is the finding that the voltage-dependence of the MTX-induced current has a linear current-voltage relationship and is clearly different from that of the usual Ca^{2+} channel current. A probable explanation for these observations is that the MTX-induced

steady current is different from the voltage-dependent Ca^{2+} channel current and that this is possibly a current that flows through a new type of Ca^{2+} -permeable channels (83). This channel may account for the mechanism of enhanced Ca^{2+} influx through the cell membrane induced by MTX. Since Ca^{2+} plays an important role in the regulation of many cellular functions, MTX has been widely used as a Ca^{2+} agonist by many researchers.

Recently, it has been reported that MTX increases formation of inositol phosphates from phosphoinositides and arachidonic acid from phospholipids in a variety of cells (84). However, MTX-induced phosphoinositide breakdown is not modified by Ca^{2+} antagonists and is stimulated at lower concentrations of MTX than those required to elevate neurotransmitter release from PC12 cells. The inositol phosphate formation by MTX may be a secondary response to Ca^{2+} influx. The mechanism of action of MTX on phosphoinositide metabolism remains to be elucidated in detail.

Recently, we have purified zooxanthellatoxin-A (ZT-A), a novel 62-membered lactone, from cultured zooxanthella (*Symbiodinium* sp.) isolated from the flatworm *Amphiscolops* sp. (Fig. 5) (85). ZT-A, like MTX, causes concentration-dependent aggregation of rabbit platelets, accompanied by an increase in $[\text{Ca}^{2+}]_i$ (86). ZT-A fails to cause platelet aggregation or increase $[\text{Ca}^{2+}]_i$ in a Ca^{2+} -free solution. Platelet aggregation and the increase in $[\text{Ca}^{2+}]_i$ induced by ZT-A are inhibited by divalent cations such as Cd^{2+} , Co^{2+} and Mn^{2+} , indomethacin (cyclooxygenase inhibitor), and SQ-29548 (thromboxane A_2 receptor antagonist). Methysergide (5-HT₁ and 5-HT₂ receptor antagonist) inhibits ZT-A-induced platelet aggregation but does not affect the increase in $[\text{Ca}^{2+}]_i$ induced by ZT-A. Genistein and tyrphostin 23 (protein tyrosine kinase inhibitor) inhibits platelet aggregation and tyrosine phosphorylation of a 42-kDa protein induced by ZT-A. Tyrphostin 23 inhibits the phosphorylation of p42 but not p38 mitogen-activated protein kinase (87). These results suggest that ZT-A increases Ca^{2+} influx into platelets, resulting in the secondary release of TXA_2 . It is also suggested that the response to ZT-A is associated with tyrosine phosphorylation of p42 mitogen-activated protein kinase.

III. Elucidation of Excitation-Contraction Coupling of Muscle

III-1. Natural products that affect Ca^{2+} -release-channel function

Molecular properties of the ryanodine receptor

Muscle contracts when the free Ca^{2+} concentration of the myofibrillar space reaches 10^{-6} to 10^{-5} M by the

release of Ca^{2+} from the SR via Ca^{2+} -release channels. Evidence from recent studies has indicated that two classes of intracellular Ca^{2+} -release channels, ryanodine receptors and inositol (1,4,5)-triphosphate (IP_3) receptors, are essential for spatiotemporal Ca^{2+} signalling in cells. Ryanodine (Fig. 6), a plant alkaloid from *Ryaniaspeciosa* Vahl, specifically interacts with Ca^{2+} -release channels of skeletal or cardiac muscle to lock the channel in an open state (88). [^3H]Ryanodine binding to Ca^{2+} -release channels is enhanced by activators, but is decreased by blockers. This indicates that [^3H]ryanodine can be used as a tool for studying the functional state of the channels. Recently, ryanodine receptors from skeletal and cardiac muscle have been purified by monitoring [^3H]ryanodine-binding activity and biochemically characterized (89–91). Purified ryanodine receptors incorporated in lipid bilayers are permeable to Ca^{2+} with a single-channel conductance of about 100 pS (92). The sequence of 5037 amino acids composing the skeletal ryanodine receptor has been deduced by cloning and sequencing the complementary DNA (93). The ryanodine receptor has recently been shown to be expressed not only in skeletal (RYR1) and cardiac (RYR2) muscle, but also in the central nervous system (RYR3), and the cloning of RYR3 gene has revealed that this subtype is widely expressed in several tissues and cells (94).

Characterization of Ca^{2+} -release channels by eudistomin D analogues

Caffeine, a plant alkaloid from *Thea sinensis* L, has been used most extensively as a typical inducer of Ca^{2+} release from SR, but is not used as a chemical probe for molecular characterization of the caffeine binding site because of its low affinity. Recently, we have found that eudistomin D and related substances isolated from a marine tunicate *Eudistoma olivaceum* induce Ca^{2+} release from a heavy fraction of the fragmented SR (HSR) (95). Structure-activity relationship studies to find derivatives that are more potent and can be radiolabeled have led to the discovery of 7-bromoeudistomin D (BED) and 9-methyl-7-bromoeudistomin D (MBED) (Fig. 7, Table 2) (95–98). The rate of $^{45}\text{Ca}^{2+}$ release from SR was increased markedly by BED, MBED or caffeine. The 50% effective concentrations of BED, MBED and caffeine are approximately 2 μM , 1 μM and 1 mM, respectively, indicating that BED and MBED are 500 and 1,000 times more potent than caffeine, respectively (95, 97). Procaine, ruthenium red or Mg^{2+} inhibits BED- or MBED-induced Ca^{2+} release. The bell-shaped profile of Ca^{2+} dependence for each compound is very similar to that of caffeine. These stimulatory effects of BED and MBED on the Ca^{2+} release from skeletal muscle SR are almost indistinguishable from those of caffeine. These results suggest

Puff-adder lectin

Fig. 6. The chemical structures of natural products that affect Ca^{2+} release channels.

noteworthy that there is a close correlation between the increase in Ca^{2+} release and enhancement of [^3H]ryanodine binding to SR in these analogues.

Recently Imaizumi et al. have successfully provided evidence using the whole cell clamp technique that in smooth muscles, MBED as well as caffeine are able to cause Ca^{2+} release from the intracellular Ca^{2+} stores that

Fig. 7. The chemical structures of bromoeudistomin D (BED), 9-methyl-7-bromoeudistomin D (MBED) and the analogues.

are needed for activation of Ca^{2+} -activated K^+ channels (99). However, MBED unlike caffeine failed to induce a contraction of skinned smooth muscle strips even at high concentrations. These data strongly suggest that intracellular Ca^{2+} stores available for Ca^{2+} -induced Ca^{2+} release channels in smooth muscles may consist of two functionally distinct types, only one of which is sensitive to MBED.

It is of great interest that analogues with a carbazole skeleton and bromine at C-6 inhibit both Ca^{2+} and caffeine-induced Ca^{2+} release (Fig. 7, Table 2) (98). 4,6-Dibromo-3-hydroxycarbazole (DBHC) inhibits $^{45}\text{Ca}^{2+}$ release induced by Ca^{2+} from SR (Fig. 7, [A]4) (100). The inhibitory effects of blockers such as procaine, ruthenium red and Mg^{2+} on $^{45}\text{Ca}^{2+}$ release are clearly observed at Ca^{2+} concentrations from pCa 7 to pCa 5.5, whereas that by DBHC is found over a wide range of Ca^{2+} concentrations. [^3H]Ryanodine binding to HSR is suppressed by ruthenium red, Mg^{2+} and procaine, but is not affected by

DBHC. [^3H]MBED binding to HSR is decreased by DBHC and the drug increases the K_d value without affecting the B_{max} value, indicating a competitive mode of inhibition. These studies lead to the conclusion that DBHC binds to the caffeine/MBED binding site to block Ca^{2+} release from SR (100). DBHC is a novel type of inhibitor for Ca^{2+} release channels in SR and may provide a useful tool for clarifying the Ca^{2+} -release mechanisms in SR.

[^3H]-Radiolabeled MBED with a higher specific activity has been successfully synthesized. [^3H]MBED binds to terminal cisternae (TC)-SR membranes in a replaceable and saturable manner, indicating the existence of a specific binding site (101). Caffeine inhibits the [^3H]MBED binding to the TC-SR membranes from skeletal muscle with an IC_{50} value of 0.8 mM, in close agreement with the concentration that causes Ca^{2+} release from SR. Scatchard analysis of [^3H]MBED binding to the TC-SR membranes revealed a competitive mode of inhibition of

Table 2. Effects of bromoeludistomin D analogues on $^{45}\text{Ca}^{2+}$ -releasing activity

Compound ^a	Concentration (μM)	Change in $^{45}\text{Ca}^{2+}$ -releasing activity (%) ^b	
		pCa 7	pCa 6
Bromoeludistomin D	10	+150 $\pm$ 10	+20 $\pm$ 6
A [1]	100	+65 $\pm$ 3	+9 $\pm$ 2
A [2]	100	+60 $\pm$ 4	+9 $\pm$ 3
A [3]	100	+30 $\pm$ 1	+4 $\pm$ 2
A [4]	100	-30 $\pm$ 5	-88 $\pm$ 5
A [5]	100	+138 $\pm$ 6	+9 $\pm$ 3
A [6]	100	+62 $\pm$ 7	+0 $\pm$ 5
A [7]	100	+66 $\pm$ 3	+0 $\pm$ 3
A [8]	100	+30 $\pm$ 3	+4 $\pm$ 7
A [9]	100	+120 $\pm$ 7	+5 $\pm$ 4
A [10]	100	-35 $\pm$ 4	-68 $\pm$ 3
A [11]	100	+27 $\pm$ 5	+40 $\pm$ 2
A [12]	100	-96 $\pm$ 2	+34 $\pm$ 1
A [13]	100	+69 $\pm$ 11	+30 $\pm$ 1
A [14]	100	+5 $\pm$ 9	-40 $\pm$ 2
A [15]	100	+69 $\pm$ 11	+30 $\pm$ 1
A [16]	100	-5 $\pm$ 9	-40 $\pm$ 2
A [17]	100	+27 $\pm$ 3	+21 $\pm$ 2
A [18]	100	+64 $\pm$ 6	+32 $\pm$ 0
A [19]	100	+27 $\pm$ 3	+25 $\pm$ 2

^aThe derivatives designated as A [1] through A [19] are illustrated in Fig. 7. ^bThe activities are presented as values relative to the control, and each value is expressed as the mean $\pm$ S.E.M. (n=3 or 4).

MBED binding by caffeine. [^3H]MBED binding to TC-SR membranes is inhibited by procaine, but is not changed by Mg^{2+} , suggesting that procaine, but not Mg^{2+} , may exert its inhibitory effect on Ca^{2+} -induced Ca^{2+} release by affecting the caffeine-binding sites. A significant point that was raised is that MBED shares the same binding sites as caffeine in Ca^{2+} release channels and thus may provide an useful biochemical tool for elucidating this site at the molecular level.

It has been demonstrated that [^3H]MBED specifically binds to microsomes of bovine aortic smooth muscle (102) or guinea pig brain (103). Caffeine competitively inhibits [^3H]MBED binding to the membranes prepared from smooth muscle and brain, indicating that MBED shares the same binding site as caffeine. Furthermore, tissue and subcellular distributions of the binding site of [^3H]MBED have been investigated in several tissues (104). All binding is completely inhibited by caffeine. This shows that all [^3H]MBED binding sites are modulated by caffeine. In liver microsomes the mode of inhibition by caffeine is allosteric, indicating that the hepatic [^3H]MBED binding site is distinct from that of skeletal SR (105).

Activation of Ca^{2+} -release channels by peptide toxins

Myotoxin α (MYTX) (Fig. 6), a polypeptide toxin purified from the venom of the prairie rattlesnake *Crotalus viridis viridis*, has been found to induce Ca^{2+} release from HSR in the concentration range between 30 nM and 10 mM, being the most potent Ca^{2+} releaser in HSR (106). The Ca^{2+} -dependency of MYTX-induced $^{45}\text{Ca}^{2+}$ release has a bell-shaped profile, but is quite different from that of caffeine. MYTX-induced $^{45}\text{Ca}^{2+}$ release consists of both early and late components. The early component induced by MYTX at low concentrations is completed within 20 sec, while the late component induced at higher concentrations is maintained for at least 1 min. Both components are almost completely inhibited by Mg^{2+} , ruthenium red and spermine. However, procaine abolishes the early component, but not the late one, suggesting that at least the early component is mediated through Ca^{2+} -induced Ca^{2+} release channels. These results suggest that the properties of Ca^{2+} release induced by MYTX is quite different from that in the case of caffeine or AMP-PCP and that MYTX induces Ca^{2+} release having novel characteristics in HSR (107, 108).

MYTX has been [^{125}I]-radiolabeled for use in a ligand-binding assay. [^{125}I]MYTX, which has high Ca^{2+} -releasing ability, specifically binds to a single class of binding sites in HSR. However, [^{125}I]MYTX does not bind to the purified ryanodine receptor. The binding to HSR is markedly decreased by spermine. Scatchard analysis of [^{125}I]MYTX binding indicated that the B_{max} value is decreased by spermine without altering the K_d value, indicating a noncompetitive mode of inhibition. Most significant is the finding that calsequestrin is essential for MYTX-induced Ca^{2+} release from HSR in single channel recording experiments (109). A probable explanation of these findings is that MYTX binds to an important regulatory protein of Ca^{2+} release, which is not the ryanodine receptor. Another possible interpretation of all these observations is that MYTX activates two Ca^{2+} -releasing pathways of the ryanodine receptor and a new class of Ca^{2+} release channels to increase the Ca^{2+} permeability of the SR membrane. MYTX will be a useful pharmacological tool for clarifying the molecular mechanism of Ca^{2+} release from skeletal muscle SR.

Recently, we have found that puff-adder lectin (PAL), a novel lectin venom purified from *Bitis arietans* (Fig. 6), causes Ca^{2+} release ($\text{EC}_{50}=10 \mu\text{M}$) from the heavy fraction but not from the light fraction of skeletal muscle SR (110, 111). The potency of PAL is approximately 200-fold higher than that of caffeine. The bell-shaped profile of Ca^{2+} dependence for PAL is almost the same as that for MYTX, but is different from that for caffeine. Typical blockers of Ca^{2+} release channels including Mg^{2+} , procaine, ruthenium red and ryanodine, markedly in-

hibits PAL-induced Ca^{2+} release from SR. An interesting finding is that PAL inhibits [^{125}I]MYTX binding to HSR with an IC_{50} of 20 μM . Scatchard analysis reveals that the mode of inhibition by PAL is noncompetitive. This suggests that PAL binds to a site different from the MYTX binding site. PAL does not affect [^3H]ryanodine binding to SR. It is concluded that PAL binds to a different site than MYTX does to cause Ca^{2+} release from SR with novel properties.

Two peptide toxins called "imperatoxin inhibitor" and "imperatoxin activator," from the venom of the scorpion *Pandinus imperator*, have been shown to modify the activity of ryanodine receptor Ca^{2+} -release channels (112). Imperatoxin inhibitor has an Mr of $\gg 10,500$, inhibits [^3H]ryanodine binding to skeletal and cardiac SR with an ED_{50} of $\gg 10$ nM, and blocks opening of skeletal and cardiac Ca^{2+} -release channels incorporated into planar bilayers. In whole-cell recordings of cardiac myocytes, imperatoxin inhibitor decreases twitch amplitude and intracellular Ca^{2+} transients, suggesting a selective blockade of Ca^{2+} release from the SR. Imperatoxin activator has an Mr of $\gg 8,700$, stimulates [^3H]ryanodine binding in skeletal but not cardiac SR with an ED_{50} of $\gg 6$ nM, and activates skeletal but not cardiac Ca^{2+} -release channels. These ligands may serve to selectively "turn on" or "turn off" ryanodine receptors in fragmented systems and whole cells.

Activation of Ca^{2+} -release channels by macrolides

It has been reported that FKBP (FK506-binding, 12-kDa protein) belongs to growing class of cytosolic proteins that regulate signal transduction pathways essential to immune function. Recently FKBP has been demonstrated to play a modulatory role in the Ca^{2+} -release function of SR (113–115). FK506, an immunosuppressant from the prokaryote *Streptomyces tsukubaeni*, promotes dissociation of FKBP from SR membrane and decreases the rate of active Ca^{2+} uptake into FKBP-deficient SR vesicles. The reduced Ca^{2+} accumulation in FKBP-deficient SR is restored to control values in the FKBP-reconstituted SR. These results suggest that FKBP stabilizes the closed conformation of Ca^{2+} release channels in skeletal muscle and may thereby play an important role in regulating the kinetics of channel gating during excitation-contraction coupling in skeletal muscle.

It has been reported that macrocyclic natural products derived from bromotyrosine isolated from the sponge *Ianthella basta* selectively modulates the skeletal isoform of the ryanodine-sensitive SR Ca^{2+} channel by a novel mechanism involving the FKBP/RyR-1 complex (113). Bastadin 5 (Fig. 6) has been demonstrated to increase the [^3H]ryanodine binding capacity of SR membranes by stabilizing the high affinity conformation of RyR-1 for

ryanodine. Ca^{2+} uptake into SR vesicles is inhibited by bastadin 5, while Ca^{2+} -induced Ca^{2+} release is increased by it. Bastadin 5 prolongs the single-channel open dwell time without altering unitary conductance for Cs^+ or open probability. An important finding is that the unique actions of bastadin 5 on [^3H]ryanodine binding and Ca^{2+} transport are antagonized by the immunosuppressant FK506. Unlike FK506, the activity of bastadin 5 is not associated with its ability to directly dissociate the FKBP/RyR-1 complex, but rather, it enhances FK506-induced release of FKBP from RyR-1. On the basis of these results, it is suggested that the bastadin 5 effector site is a novel modulatory domain on FKBP. Bastadins represent a new class of compounds to gain insight into the functional interactions between FKBP and RyR-1.

It has been demonstrated that quinolidomycin A_1 , a 60-membered macrolide from an actinomycete *Micromonospora* sp (Fig. 6), markedly induces $^{45}\text{Ca}^{2+}$ release from the heavy fraction of skeletal muscle SR ($\text{EC}_{50}=20$ μM), but induced only slight release from the light fraction of SR, showing a lack of ionophoretic activity even at a high concentration (116). The potency of quinolidomycin A_1 is 100-fold higher than that of caffeine. The bell-shaped profile of Ca^{2+} dependence for quinolidomycin A_1 is different from that for caffeine. Blockers of Ca^{2+} -release channels such as Mg^{2+} , procaine and ruthenium red partially blocked quinolidomycin A_1 -induced $^{45}\text{Ca}^{2+}$ release from SR. Quinolidomycin A_1 potentiates [^3H]ryanodine binding to SR with decreasing K_d but without altering B_{max} . These results suggest that quinolidomycin A_1 -induced Ca^{2+} release from SR consists of two components, one that is sensitive and another that is insensitive to blockers of Ca^{2+} -release channels, and the former component is associated with the ryanodine receptor.

III-2. Natural products that affect Ca^{2+} -pump function

Inhibition of Ca^{2+} -pump activity by thapsigargin

Thapsigargin, a plant-derived sesquiterpenoid lactone (see Fig. 8), has been identified as a non-phorboid tumor promoter whose effect appears to result from the emptying of intracellular Ca^{2+} stores, as a consequence of inhibiting the uptake pathway. For this reason, thapsigargin has recently attracted the attention of numerous investigators as a tool to define intracellular Ca^{2+} pools.

It has been found that thapsigargin discharges intracellular Ca^{2+} stores in rat hepatocytes (117). It induces a rapid, concentration-dependent release of stored Ca^{2+} from liver microsomes. Pretreatment of microsomes with thapsigargin blocks subsequent loading with $^{45}\text{Ca}^{2+}$, suggesting that its target is the Ca^{2+} pump of the endoplasmic reticulum. Thapsigargin causes an inhibition of the

Fig. 8. The chemical structures of natural products that modulate Ca^{2+} -pumping ATPase.

microsomal Ca^{2+} -ATPase activity without changing the activity of Ca^{2+} -ATPase on the hepatocyte or erythrocyte plasma membrane. These observations clearly indicate that thapsigargin is a highly specific inhibitor of the endoplasmic reticulum Ca^{2+} pump (Ca^{2+} -ATPase) (117).

Thapsigargin increases $[\text{Ca}^{2+}]_i$ in many cell types, probably as a result of Ca^{2+} release from an intracellular pool (117–119). In cardiac cells, however, both the contraction and $[\text{Ca}^{2+}]_i$ transient have been shown to be inhibited by thapsigargin (119). Oxalate-stimulated Ca^{2+} uptake into SR is largely decreased by thapsigargin, whereas Ca^{2+} -induced Ca^{2+} release is not affected by it. Furthermore, it has been demonstrated that the sarcolemmal current or transport system is not affected by thapsigargin. Detailed experimental data on the effect of thapsigargin on Ca^{2+} movements suggest that thapsigargin inhibits the contractile response of cardiac muscle by inhibition of SR Ca^{2+} -ATPase. Thapsigargin has become a valuable probe for clarifying the control of intracellular Ca^{2+} pools as well as for structure-function studies of the Ca^{2+} -ATPase molecule itself.

Activation of Ca^{2+} -pump activity by gingerol

The Ca^{2+} -ATPase of SR is intimately involved in regulation of the contractile activity of muscles, and the Ca^{2+} uptake into SR by Ca^{2+} -ATPase causes relaxation of muscle fibers. In research aimed at finding cardiotoxic substances having unique mechanisms of action from crude drugs, [8]-gingerol (gingerol) was isolated as a cardiotoxic principle from the rhizome of ginger, *Zingiber officinale* Roscoe (Fig. 8) (120). Gingerol has been demonstrated to induce a positive inotropic effect on isolated guinea pig left atria (121). The inotropic effect of gingerol is abolished by ryanodine, but is little affected by propranolol, chlorpheniramine, cimetidine, TTX, diltiazem or reserpine. In the atria, gingerol increases the contractile force without changing the action potential. Gingerol increases the Ca^{2+} uptake of fragmented cardiac SR and the Ca^{2+} -ATPase activity of cardiac SR at concen-

trations similar to those that produce cardiotoxic action. These results provide the first evidence that the direct enhancement of the Ca^{2+} -pumping activity of SR plays an important role in the cardiotoxic action of gingerol in atrial muscle.

Gingerol increases the SR Ca^{2+} -pumping rate of not only cardiac SR, but also skeletal SR. The rate of $^{45}\text{Ca}^{2+}$ uptake of the heavy fraction of SR is also stimulated markedly by gingerol without affecting the $^{45}\text{Ca}^{2+}$ efflux from the light fraction of SR. Furthermore, gingerol elevates Ca^{2+} -ATPase activities of skeletal or cardiac SR. Kinetic analysis of the activating effects of gingerol suggests that the activation of SR Ca^{2+} -ATPase is both uncompetitive and competitive with respect to Mg^{2+} -ATPase (122). Kinetic analysis also suggests that the activation by gingerol is of the mixed type with respect to free Ca^{2+} , and this enzyme is activated probably due to the acceleration of enzyme-substrate complex breakdown. Gingerol may provide an essential chemical tool for investigating the regulatory mechanisms of SR Ca^{2+} -pumping systems and the causal relationship between the Ca^{2+} -pumping activity of SR and muscle contractility.

III-3. Natural products that affect myosin function

Myosin is an ATPase whose activity is stimulated by interaction with actin. There is much evidence suggesting that the conformational changes of actin and myosin are tightly linked to cross-bridge cycling. Although the binding sites involved in the interaction between myosin and actin molecules have been determined, the role of these conformational changes and the interactions in muscle contraction remain to be elucidated. Therefore, novel tools that provide information on conformational changes and interactions of contractile proteins will be useful.

Modulation of myosin ATPase activity by purealin

Purealin has been isolated as an ATPase modulator

Fig. 9. The chemical structures of natural products that affect contractile proteins.

from an Okinawan sea sponge, *psammaphysilla purea* (see Fig. 9). It has been reported that purealin enhances the superprecipitation of skeletal muscle actomyosin (123) and the actin-activated ATPase activity of myosin from rabbit skeletal or cardiac muscle (124, 125). Of particular interest is the activation of myosin K^+ (EDTA)-ATPase by purealin, since no other drug is known to activate K^+ (EDTA)-ATPase. Furthermore, similar results concerning the modification of ATPase activities by purealin were obtained with myosin subfragment-I instead of myosin. These results suggest that purealin enhances the super-

precipitation of myosin B by affecting the myosin heads directly. This is the first evidence that there is a correlation between the activities of myosin K^+ (EDTA)-ATPase and actomyosin ATPase of myosin B.

In contrast, in smooth muscle, the Ca^{2+} - and Mg^{2+} -ATPase activities of dephosphorylated myosin are elevated by purealin, whereas that of K^+ (EDTA)-ATPase is decreased by it (126, 127). Purealin lessens the ATP-induced decrease in light scattering of dephosphorylated myosin. Thick filaments of dephosphorylated myosin are preserved by purealin even after administration of ATP.

These findings clearly indicate that purealin modulates the ATPase activity of dephosphorylated smooth muscle myosin by increasing the stability of myosin filaments against the disassembling effect of ATP.

Modification of specific sulfhydryl (SH) groups of myosin by xestoquinone

In the course of our survey of cardiotonic substances having a novel mechanism of action from marine sources, xestoquinone (XQN) has been isolated as a cardiotonic principle from an Okinawan sea sponge, *Xestospongia sapra* (Fig. 9) (128). XQN causes cardiotonic effects on guinea pig left and right atria (129). An important observation is the direct action of XQN on the contractile machinery of cardiac myofilaments in chemically skinned fiber preparations from papillary muscles. The concentration-contractile response curve for Ca^{2+} in the atria is substantially shifted to the left by XQN, and this effect is reversed by verapamil. In whole-cell patch-clamped atrial myocytes, XQN increases the slow inward current. cAMP phosphodiesterase activity is inhibited and tissue cAMP content is increased by XQN, suggesting that the elevated cAMP levels may be responsible for the Ca^{2+} channel activation by XQN. Increases in cAMP content, however, do not occur in parallel with increases in contractile response. These observations suggest that enhancement of intracellular cAMP content and Ca^{2+} influx across the cell membrane contribute to the late phase of XQN-caused cardiotonic responses, whereas the early phase may largely be elicited through direct activation of contractile elements. XQN may provide a novel lead compound for developing cardiotonic agents.

Also important is the finding that XQN enhances Ca^{2+} -induced tension development of chemically skinned fibers from guinea pig cardiac muscle, even at free Ca^{2+} concentrations as low as pCa 9 to 8 (130). XQN markedly enhances the rate and extent of superprecipitation of natural actomyosin and produces a concentration-dependent increase in the myofibrillar ATPase activity of canine cardiac muscle. These findings clearly indicate that XQN directly activates the actomyosin ATPase activity of cardiac and skeletal myofibrils, thus producing an enhanced superprecipitation activity as well as an increase in skinned fiber contractility.

XQN has been reported to decrease the activities of both Ca^{2+} and K^{+} (EDTA)-ATPase of skeletal muscle myosin. This inhibition is abolished by dithiothreitol, suggesting modification of myosin SH groups by XQN. Recent work in our laboratory showed that unlike *N*-ethylmaleimide, a well-known SH reagent, modification of 2 mol of SH groups per myosin by XQN causes a marked increase in the actomyosin ATPase activity (131). It is noteworthy that *N*-ethylmaleimide is still able to

modify both the SH_1 and SH_2 groups after modification of 2 mol of SH groups by XQN. XQN modifies myosin SH groups, which causes changes in the tryptophan fluorescence intensity and circular dichroism. These results suggest that XQN strengthens the interaction between actin and myosin through inducing a conformational change in the myosin molecule. These observations raises the possibility that XQN modifies specific SH groups in myosin distinct from SH_1 and SH_2 , resulting in activation of actomyosin ATPase. Being a novel positive inotropic agent, XQN should be a valuable chemical tool for studies aimed at elucidating the molecular mechanisms of muscle contraction.

III-4. Natural products that affect actin function

Actin is one of the most abundant and common components of the cytoskeleton. It also regulates various cell functions such as muscle contraction, cell motility and cell division. It is well-known that cytochalasins, a group of fungal metabolites, serve as actin filaments and shift the polymerization-depolymerization equilibrium toward net depolymerization of F-actin.

Investigation of the relationship between structure and function of actin by goniodomin A

Goniodomin A, isolated from the dinoflagellate *Goniodoma pseudogoniaulax* (see Fig. 9) (132), has been demonstrated to modulate skeletal actomyosin ATPase activity (133). The effect of goniodomin A is dependent on the concentration of actin, but not that of myosin. The actomyosin ATPase activity is increased by pretreatment of actin (but not myosin) with goniodomin A. This toxin causes a sustained increase in the fluorescence intensity of actin. However, the ATPase activity and fluorescence intensity of myosin are not changed by goniodomin A. It is of great interest that goniodomin A induces a remarkable but transient increase in the fluorescence intensity of actomyosin in a concentration-dependent manner at concentrations similar to those that stimulate the actomyosin ATPase activity. These data suggest that activation of actomyosin ATPase activity by goniodomin A results from the conformational changes of actin molecules. Goniodomin A remarkably decreases the fluorescence intensity of pyrenyl-F-actin to the level of G-actin. Furthermore, electron microscopic observations have clearly demonstrated that actin filaments associate with each other to form a gel in the presence of goniodomin A. These findings suggest that the conformational change of actin molecules, resulting from stoichiometric binding of goniodomin A to actin monomers in filaments, may modify the interaction between actin and myosin. Goniodomin A should become a useful tool for the in-

vestigation of the relationship between the structure and function of actin.

Depolymerization of actin by mycalolide B

Mycalolide B was isolated from the marine sponge *Mycale* sp. as an antifungal or cytotoxic substance and contains trioxazole in a macrolide ring (Fig. 9) (134). The toxin inhibits the ATPase of native actomyosin prepared from chicken gizzard smooth muscle without affecting myosin ATPase activities, suggesting that mycalolide B acts directly on either actin or myosin (135). Mycalolide B does not accelerate actin polymerization, but slowly depolymerizes F-actin (136). Detailed kinetic analysis has indicated that mycalolide B severs F-actin and forms a 1:1 complex with G-actin. The strongest evidence for depolymerization of actin filament by mycalolide B comes from the viscometric and electron microscopic observations. These results suggest that mycalolide B severs F-actin and sequesters G-actin to inhibit actin-activated myosin Mg^{2+} -ATPase activity. Mycalolide B is a "depolymerizing" agent, and it has become an important tool for elucidating actin-mediated cellular functions.

IV. Physiological Role of Protein Phosphorylation and Dephosphorylation

IV-1. Natural products that affect protein kinase activity

The fungally derived indole carbazoles (staurosporine, K252a and modified derivatives), which interact at the ATP-binding site of protein kinases, have been used extensively as inhibitors of serine/threonine (Ser/Thr) kinases (137). The effects of staurosporine (Fig. 10) have been frequently cited as evidence of protein kinase C (PKC) involvement in a cellular response. Aromatic compounds such as erbstatin, herbimycin and lavendustin have been isolated from *Streptomyces* as inhibitors of protein tyrosine kinases. Genistein (Fig. 10), an isoflavonoid purified from *Pseudomonas*, inhibits some tyrosine kinases, including those that regulate T-cell proliferation (138).

It has been reported that certain bioactive, amphipathic polypeptides such as bee venom melittin, antimicrobial antibiotic polymyxin B (Fig. 10), snake venom cardiotoxin, and human neutrophil antibiotic defensin potently inhibit PKC (139). It is worthy of our notice that these

Fig. 10. The chemical structures of natural products that inhibit protein kinases.

surface-active polypeptides inhibit the enzyme competitively with respect to phosphatidylserine, indicating that they could bind to the sites on PKC shared by the phospholipid cofactor or directly interact with the phospholipid, rendering it inactive. Protein kinase inhibitors such as melittin, cardiotoxin and polymyxin B inhibit PKC

stimulation by phosphatidylserine bilayer or arachidonate monomer and blocks binding of [3 H]phorbol 12,13-dibutyrate to PKC in the presence of a phosphatidylserine bilayer, with IC_{50} values of 1–8 μ M. Polymyxin B is much less inhibitory (IC_{50} = 10–20 μ M), whereas melittin and cardiotoxin are similarly inhibitory (IC_{50} = 1–4 μ M),

Fig. 11. The chemical structures of natural products that inhibit protein phosphatases.

when PKC is activated instead by synaptosomal membrane. Kinetic analyses indicate that mastoparan inhibits PKC, assayed using phosphatidylserine or synaptosomal membrane as the phospholipid cofactor, competitively with respect to the phospholipid cofactor, in a mixed manner with respect to CaCl_2 or diacylglycerol, noncompetitively with histone, and uncompetitively with respect to ATP.

IV-2. Natural products that affect phosphatase activity

It is well-known that there are two functional classes of natural inhibitors of phosphatase. First, there are 'endogenous' peptide inhibitors that regulate particular phosphatases within eukaryotic cells; for example, inhibitors 1 and 2, which are specific for protein phosphatase 1. The second group are secondary metabolites produced by bacteria, fungi, plants, dinoflagellates and insects. The type 1 and 2A protein Ser/Thr phosphatases (PP1 and PP2A) are inhibited by okadaic acid (diarrhetic shellfish poisons from the marine sponge *Halichondria okadai* and the dinoflagellate *Dinophysis fortii*), tautomycin (antibiotic polyketide from *Streptomyces spiroverticillatus*), mycrocystin (hepatotoxic cyclic peptide from the blue-green algae *Mycrocystis aeruginosa*) and calyculin A (cytotoxic polyketide from the marine sponge *Halichondria okadai*) (Fig. 11) (140). These inhibitors have been reported to promote tumor formation, suppress cell transformation and induce apoptosis, suggesting that PP1 and/or PP2A are components of both growth-stimulating and growth-suppressing pathways in cells (141). Inhibitors of PP1 and PP2A have been widely used as first-choice reagents for finding out whether a particular cellular process is regulated by reversible protein (Ser/Thr) phosphorylation.

Cyclosporin A is a cyclic undecapeptide of the prokaryote *Tolypocladium inflatum* (Fig. 11). This peptide has been clinically used as an immunosuppressing drug for organ transplant patients. On entering eukaryotic cells, cyclosporin A binds to a protein called cyclophilin. Another immunosuppressing compound, FK506 (see Fig. 6), binds to a distinct protein, FKBP. Both of these drug-protein complexes (cyclosporin-cyclophilin and FK506-FKBP) bind specifically to the Ca^{2+} -activated-protein phosphatase 2B (PP2B, calcineurin) to decrease its enzymatic activity (142). Inhibition of PP2B prevents induction of interleukin 2 (IL-2) expression and hence T-cell activation. These drugs are useful as tools for defining the role of PP2B in intracellular signalling.

Acknowledgments

I wish to thank Dr. K. Matsunaga, this Department, for his helpful advice in the preparation of this review.

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