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A MOLECULAR DESCRIPTION OF NERVE TERMINAL FUNCTION

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PERSPECTIVES AND SUMMARY

The nerve terminal is a specialized region of a neuron, separated from the neuronal soma by an axon that can be exceedingly long, whose function is to release neurotransmitter when stimulated by an electrical signal carried by the axon. In this review, we describe the enzymes, channels, and other proteins presently thought to be important in nerve terminal function. Recent progress in the area has been so dramatic that it is becoming possible to relate simple changes in behavior to modifications of defined proteins in particular nerve terminals.

Neurotransmitters are stored in synaptic vesicles and are released by fusion of these vesicles to the plasma membrane. Vesicle fusion is triggered by Ca^{2+}-influx through specific Ca^{2+} channels that open in response to depolarization of the plasma membrane and is terminated by the disappearance of Ca^{2+} from the vicinities of the active zones. The voltage signal that opens the Ca^{2+} gates is not constant, but also subject to regulation. The key elements are the Na^{+} and K^{+} channels in the nerve terminal. These channels are localized to distinct regions of many neurons and different neurons have different quantities of the different channel types. The voltage sensitive Na^{+} channel is responsible for depolarizing the membrane. This channel has recently been purified and reconstituted in artificial membranes, so many of its properties are known. K^{+} channels are responsible for repolarizing the membrane. Several K^{+} channels have been identified: some activated by voltage, some by intracellular Ca^{2+} and others by neurotransmitters. The properties of several of these have recently been shown to be altered by cAMP-dependent protein kinases, resulting in long-term changes in neuronal activity and the efficiency of synaptic transmission. The voltage-dependent Ca^{2+} channels can also be modified by cAMP-dependent protein kinases. Many of the neurotransmitters and hormones that modulate the efficiency of transmitter release apparently do so by modifying the Ca^{2+} channels. Much of the recent progress in molecular studies on the K^{+} and Ca^{2+} channels has been made possible by a dramatic new technique called “patch clamping” that permits individual ion channels to be examined on the tip of a microelectrode. In many ways this technology circumvents the need for biochemical isolation and reconstitution.

Maintenance of the Na^{+} and K^{+} concentrations in neurons requires the classical $Na,^{+}$ K^{+} -ATPase that is found in all cell types. Recent experiments suggest that a novel form of this enzyme exists in neural tissues. This pump appears to be localized to anatomically and functionally distinct regions of many neurons. To restore the cytoplasmic Ca^{2+} concentrations to original levels, the nerve terminal has an array of Ca^{2+} removal systems including a Na^{+}:Ca^{2+} antiporter in the plasma membrane, a Ca^{2+} porter in the mitochondrial inner membrane and several distinct ATP-dependent Ca^{2+} uptake systems in the plasma membrane, smooth endoplasmic reticulum, and synaptic vesicles.

The mechanism of vesicle fusion is poorly understood despite recent progress in isolating highly purified synaptic vesicles and presynaptic plasma membranes. All the elements required for exocytosis—vesicles, Ca^{2+} channels, and components mediating membrane fusion—appear to be localized in small active zones. The Ca^{2+} that enters the nerve terminal must

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interact with a class of molecules just inside the nerve terminal that regulates vesicle fusion. These Ca^{2+}-binding molecules induce exocytosis within 100–200 μs at Ca^{2+} concentrations of 1–10 μM. Recent research has strongly suggested, but not proven, that calmodulin mediates Ca^{2+} action in exocytosis at the nerve terminal. Identification of the Ca^{2+} target and the components that mediate membrane fusion is a high priority for future research.

Studies on the synthesis and packaging of peptide and classical neurotransmitters have revealed that the two transmitter classes are regulated by fundamentally different mechanisms. Peptide transmitters are synthesized as precursors, packaged in the cell soma, and processed in secretory granules before arrival at neuronal terminals. The components of these granules are brought to the nerve terminal by an ATP-dependent, fast (3–5μm/sec) transport system that is likely to involve microtubules and actin. Once released by exocytosis, there is no convincing evidence that peptides are retrieved or reused. In contrast, classical neurotransmitters, such as catecholamines, are mostly synthesized in the nerve terminal and can be recycled. These transmitters are retrieved from extracellular space by transmitter-specific Na^+ driven coporters. The uptake of classical transmitters into synaptic vesicles requires an ATP-dependent proton translocase, similar to the mitochondrial F_1 ATPase, that is found in all secretory granules and maintains their interiors at acidic pH. The uptake of catecholamines into chromaffin granules utilizes a proton-catecholamine antiporter. Uptake of other transmitters has not been described with comparable precision. Several different mechanisms couple the rate of synthesis of classical transmitters to their rate of depletion from the nerve terminal.

Nerve terminal functions are regulated by changes in cyclic nucleotide and Ca^{2+} levels in response to membrane depolarization or the binding of transmitters to receptors. Nerve terminals contain high levels of calmodulin and adenylate cyclase. They also contain high levels of cAMP-dependent protein kinase, several Ca^{2+}-calmodulin-dependent protein kinases and a Ca^{2+}-phospholipid activated protein kinase, each of which has a distinct set of protein substrates. Changes in phosphorylation of specific proteins are likely to modulate many of the rapid changes in the rate of energy metabolism, activity of ion pumps and channels, and rates of transmitter uptake and synthesis that are known to occur in individual nerve terminals in response to electrical activity. In several cases there is now direct evidence that regulation of nerve terminal function by second messengers does indeed involve phosphorylation of specific proteins. Most dramatically, recent work with *Aplysia* has shown that changes in the phosphorylation of specific ion channels alter the properties of individual synapses and cells, which in turn cause changes in the animal's behavior.

BIOCHEMICAL AND MOLECULAR STUDIES ON ION-SELECTIVE CHANNELS AND PUMPS

For the most part, transmitter release is modulated by Ca^{2+} entry and removal. Ca^{2+} enters the cytosol through a Ca^{2+}-selective channel that is opened by depolarization of the plasmalemma. Although the conductance of the calcium channel can be controlled directly by covalent modification (1), its important physiological regulator is the membrane potential, which in turn reflects the conductance of channels and the activity of pumps that are selective for other ions, most notably Na^+ and K^+ (e.g. 2). The sensitivity of Na^{2+} and K^+ channels to transmembrane voltage has been known for many years to be responsible for electric signalling in the nerve system. To understand the regulation of transmitter release, therefore, it is first important to examine the regulation of ionic conductances in the nerve terminal.

The electrical properties of excitable cells reflect changes in the selective permeability of the plasmalemma to different ions. Hodgkin & Huxley (3) found that the initial depolarization of the plasmalemma during an action potential in the squid giant axon could be attributed to an initial large increase in Na^+ permeability, depolarizing the cell membrane. The subsequent

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repolarization to the starting condition required inactivation of the permeability to Na^+ and also an increase in K^+ permeability. The Na^+ and K^+ permeabilities were distinguished experimentally by replacing one of the ions with an equivalent impermeant ion and measuring the permeability of the other. After a step depolarization the Na^+ current rises to a peak quickly but then because of inactivation decays to a low value. The K^+ current differs from the Na^+ in that it rises more slowly, and does not decay. The opening and closing of both these channels is sensitive to membrane potential.

One major motive for pursuing biochemical studies on the channels has been to obtain molecular insights into the aspects of their structure that determine their ion-selectivity properties and voltage sensitivity. A second motive has been to determine the distribution of individual channel types on individual neurons. Na^+, K^+, and Ca^{2+} channels have precise and often distinct distributions on neurons, and these distributions have important functional consequences. Antibodies to purified channel proteins would be powerful reagents in neuroanatomy. Information on channel properties and distribution is crucial to developing models describing properties of neurons or circuits of neurons in the brain.

Sodium Channels

DESCRIPTION—Voltage-sensitive Na^+ channels play a key role in conducting the action potential to the nerve terminal and are the one channel type that has been significantly purified and studied with traditional biochemical methods. The channels in different tissues have very similar properties, suggesting they are either a single molecular species or family of closely related molecules (e.g. 4). Recent research on the physiology, biophysics, and toxicology of Na^+ channels have been extensively reviewed (e.g. 4–7), so this review focuses on recent developments. Models describing the Na^+ channel have usually postulated two “gates” or subsections of the channel, both of which must be open to permit ion flow through the channel (e.g. 4,5). At typical resting potentials, the *m* gate is closed and *h* gate open. Depolarization results in opening of the *m* gate and ion flow. Depolarization also results in closing of the *h* gate with slower kinetics, stopping ion flow. Channels are not reactivable by depolarization until the *h* gates have reopened. Both opening and closing of these gates are sensitive to transmembrane potential. Recently, patch clamps have shown that the individual Na^+ channels have a conductance of approximately 5–20 pS (e.g. 8). The channels do not have to open before being inactivated (9), so the *m* and *h* gates appear to function independently, confirming the essential feature of the two gate model.

BIOCHEMICAL CHARACTERIZATION—Several toxins have specific high affinity binding sites on the Na^+ channel and have been invaluable reagents for biochemical studies. Two toxins in particular, tetrodotoxin and saxitoxin, inactivate the channel at nanomolar concentrations. These toxins can be radiolabeled and have served as probes to measure channel density (10) or monitor channel purification (e.g. 11). A second set of alkaloid ligands, among which are batrachotoxin and veratridine, bind the channels at a distinct site in its lipophilic domain and induced persistent activation (e.g. 12). A third set of ligands are peptide toxins from certain scorpions and sea anemones (e.g. 13) that show voltage-sensitive binding to the extracellular surface of the Na^+ channel (14). These inhibit channel inactivation and act synergistically with the alkaloids to enhance persistent activation. The peptide toxins can also be radiolabeled, but do not bind the channel in the absence of a substantial transmembrane potential, making them of little use for purification. Beneski & Catterall (15) used arylazido [^{125}I]-scorpion toxin to radiolabel the subunits of the Na^+ channel in intact rat brain synaptosomes. They identified two peptides with apparent molecular weights of 32,000 and 250,000 as putative Na^+-channel subunits. Catterall (16) also used [^{125}I]-scorpion toxin to measure the distribution of Na^+-channels on living neuroblastoma cells and spinal cord neurons (see next section, on Localization of Na^+ Channels in Neurons).

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Using saxitonin as a probe, investigators estimated the size characteristics of the solubilized Na^+ channel from rat brain by gel filtration and sucrose gradient sedimentation in D_2O and H_2O. Channels solubilized by Triton or cholate give complexes of M_r 600,000 and 1,000,000, respectively (17,18). Assuming a reasonable protein density, the protein in these complexes would appear to be about 300 kd in size (17), implying that the channel could not contain more than one 250 kd subunit. Similar analysis of the sodium channel in rat muscle suggests that it also has a mass of about 300 kd (18a).

The Na^+ channel has been purified substantially from *Electrophorus* electroplax (11,19), rat brain (20), and rat muscle (21). The channel from *Electrophorus* appears to contain only one polypeptide of 270 kd that copurified with saxitonin-binding activity (19). Since this channel has recently been shown to contain 30% carbohydrate and to behave anomalously on gels (22), the value of 270 kd is clearly only an approximation of the molecular weight. The Na^+ channel from rat brain, on the other hand, appears to contain three subunits of about 270, 39 and 37 kd (20,23), two of which are labeled by arylazido [^{125}I]-scorpion toxin. The unlabeled M_r 39,000 subunit is linked to the large subunit by disulfide bonds. The Na^+ channel from rat muscle has been purified to approximately the same specific activity and contains a large glycoprotein with anomalous behavior on acrylamide gels and a 38-kd doublet (23a), which are almost certainly closely related to the 270-, 39-, and 37-kd brain channel subunits (23), and an additional protein of 45 kd (23a).

Recently, highly purified preparations of the eel Na^+ channel have been visualized in the electron microscope (24). Negatively stained preparations consist of clusters of 41-Å × 170-Å rods. Assuming the rod represents the protein portion of the glycoprotein, one can calculate the mass of each cylinder to be approximately 180 kd. If the unit contains a single glycoprotein with 30% carbohydrate, the total molecular weight would be about 260,000.

Crude preparations of Na^+ channels from rat brain can be used to reconstitute a voltage-sensitive Na^+ permeability in liposomes. Goldin et al (18) preloaded phospholipid vesicles containing sodium channels with Cs^{2+}. Addition of the channel-specific alkaloid veratridine allowed Cs^+ to escape, reducing the density of the liposomes in a surcrose gradient. These ingenious experiments not only demonstrated functional reconstitution of the channel, but also yielded a 50-fold purification of the vesicles containing Na^+ channels. Tamkun & Catterall (25) reconstituted the Na^+ channels into liposomes that had a Na^+ concentration gradient across their membranes (inside high). Incubation with veratridine generated a transmembrane diffusion potential that was assayed by the voltage-dependent binding of scorpion toxin. Recently, purified preparations of both the rat brain and the rat muscle sodium channel have been successfully reconstituted in phospholipid vesicles (26,27). Both preparations exhibit veratridine-induced Na^+ uptake that is sensitive to tetrodotoxin and saxitoxin. The purified eel protein has not yet been successfully reconstituted. Since open channels have very high conductances, the opening of a single Na^+ channel in a phospholipid vesicle will allow that Na^+ to equilibrate within msec. Incorporation into lipid bilayers will be essential for accurate biophysical analysis of Na flux. Recently, Barchi et al (27a) used quenched-flow kinetic techniques to compare the permeability to different alkaline metal cations of batrachotoxin-activated Na^+ channels. The selectivity of the purified, reconstituted channel is very similar to that of the channel in its original membrane, suggesting no subunits have been lost during purification. Normal responsiveness of the reconstituted channel to transmembrane voltage changes has not yet been demonstrated.

The large subunits identified in the electroplax, rat brain, and muscle preparations seem very likely to be homologous. All are similar in apparent size and contain carbohydrate. The presence of small subunits in purified sodium channels from rat brain and muscle, but not electroplax, is not understood.

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Modulation of Na^+ channel activity by covalent modification has not been observed in vivo, but the 270-kd subunit is a preferred substrate for the cAMP-kinase in vitro (28), suggesting that such modifications may be demonstrated to be of functional importance in the future.

LOCALIZATION OF NA^+ CHANNELS IN NEURONS—The distribution of Na^+ channels in dendrites, axons, and nerve terminals is functionally important in modulating integration of different synaptic inputs (e.g. 29), initiation and conductance of action potentials (e.g. 30), and neurotransmitter release. In at least some dendrites, Na^+ channels are present, probably in excitable patches (e.g. 29). [^{125}I]-scorpion toxin binding has been used to demonstrate that initial segments of cultured spinal cord neurons, the normal initiation sites for action potentials, have high concentrations of Na^+ channels, suggesting a molecular basis for the lower threshold for action-potential generation often observed at this part of the neuron in vivo (16). In myelinated axons, the density of channels, whether assayed by focal, extracellular recording or density of saxitoxin receptors, is several hundred-fold higher in nodal than internodal segments of axonal membrane (31,32). In the nodes, the channels appear to be present at very high densities— 3000–5000 channels perμm^2 (4,31)—and must be a predominant membrane protein. Indeed, the major particles visualized in freeze-fracture replicas of the nodal membrane have dimensions similar to purified channel protomers (24). Channel density is at least 30-fold lower, but more uniformly distributed in unmyelinated axons (30,31). Recently, monoclonal and polyclonal sera, specific for the 270-kd protein in the *Electrophorus* Na^+ channel, and a new class of Na^+ channel-specific scorpion toxins that do not require a transmembrane potential for binding have been characterized (33,33a,34). In myelinated or acutely demyelinated axons, antibody binding is restricted to nodal regions (33a). We anticipate these probes and others specific for the sodium channel polypeptides to be useful for mapping with more precision the position of these channels in neurons, particularly in the nerve terminal.

Potassium Channels

DESCRIPTION—Hodgkin & Huxley (3) showed that a current of K^+ flowing outward from the axon helps restore the membrane potential to its original value after a depolarization induced by Na^+ entry. The K^+ flows through channels that are activated with slower kinetics than the Na^+ channel, do not inactivate, and are resistant to toxins that block Na^+ channels. K^+ channels are found in virtually all excitable cells and probably have even wider distributions than Na^+ channels. While there may be only one type of Na^+ channel, there are clearly many different types of K^+ channels. Typical vertebrate or invertebrate neurons have a mixture of at least three channel types in different ratios: channels that are open at or near the resting membrane potential and are major regulators of the resting potential and excitability (35–37), voltage-sensitive channels, activated by depolarization, that give rise to the fast outward K^+ current seen in the restorative phase of the action potential (3), and Ca^{2+}-dependent K^+ channels, which only open when intracellular Ca^{2+} levels are high (38). Many neurons also have channels whose permeability is regulated by neurotransmitters (e.g. 39–42). Different channels have been shown clearly to regulate different physiological parameters, such as resting potential, shape of the action potential, Ca^{2+}-influx, adaptation to different firing rates and frequency of action potentials (see e.g. 37,43,44). Different neurons have different ratios of different types of K^+ channel, so these channels are particularly important generators of diversity between neurons in the nervous system. The different currents in molluscan neurons have been reviewed recently (45).

Even though no representatives of K^+ channels have yet been purified and subjected to traditional methods of biochemical analysis, K^+ channels are important to discuss in this review because they are clearly crucial in regulating exocytosis and changes in synaptic efficiency (e.g. 44). Many different types are clearly regulated by protein kinases and phosphatases (e.g. 44,46,47). Finally, biophysical studies using “patch clamp” procedures to isolate single channel

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types and to modify the environments at the cytosolic and extracellular surfaces have provided a wealth of information that was unimaginable only a few years ago.

ELEMENTARY CHANNEL TYPES: ***Channels open near rest:*** A variety of K^+ channels active near the resting membrane potential have been described in molluscan neurons (45), muscle (48), and other preparations (49). Single K^+ channel currents open at the resting potential have been studied by patch clamping cultured rat muscle (48). The channels are present at low density, approximately one per μm^2. The conductance (~ 10 pS) and kinetics are not dramatically different from other channel types. In some cells, neurotransmitters, whose activity is mediated through a cAMP-induced kinase, can increase the conductance of this type of channel (46). By increasing K^+ conductance, such neurotransmitters would act to buffer the membrane against changes in transmembrane potential.

Voltage clamp studies on molluscan and vertebrate neurons reveal a distinct class of channels that carries a transient current that is activated at membrane potentials more positive than −60 mV, but is then rapidly inactivated (36,42). The current that flows through these channels is conventionally called the A-current. This class of channel exerts a major influence on neuronal excitability, slowing the rate of depolarization in response to a stimulus and regulating the rate of action-potential generation in response to sustained stimuli (37). Recently, *Drosophila shaker* mutants have been shown to have altered A-current in muscle and probably nerve (50). Some mutations alter the voltage-dependence of this class of K^+ channel (51). *D. shaker* mutants have abnormally prolonged transmitter release, suggesting this class of channel is important in repolarizing the nerve terminal (52). Genetic and molecular analysis of this locus is likely to contribute significantly to our understanding of K^+ channel function.

Delayed, voltage-sensitive channel: The K^+ channel responsible for repolarizing the membrane in the latter phase of the action potential in the squid giant axon (3) is widely distributed in excitable tissues, usually carrying a significant fraction of the K^+ current during later phases of the action potential (e.g. 53). The channel is defined by the speed of its response to a voltage change, its slow inactivation rate and its sensitivity to triethylammonium. The K^+ channel has been analyzed by patch clamping (54). It has a conductance of approximately 10 pS at physiological K^+ concentrations. Its mean open time is 12 msec, although short interruptions of current flow are seen within this open state. Depolarization seems to alter the probability of opening a channel without changing the conductance or mean open time of single channels (54). It is present in muscle at densities about one-twentieth that of the Na^+ channel (55). Recently, a novel scorpion toxin was discovered with submicromolar affinity for this channel in the squid axon (56). One hopes that this presages the isolation of toxins with tighter binding constants or identification of species in which K^+ channels bind this toxin more avidly. At this point, purification attempts have not been made and biochemical studies are a formidable proposition.

Ca^{2+} -activated potassium channel: The Ca^{2+}-activated K^+ channel requires elevated internal Ca^{2+} to open and can remain open for long periods after trains of impulses, reducing neuronal excitability and exocytosis (e.g. 38). The channel is found not only in excitable tissues such as neurons (38,53), chromaffin cells (57), and muscle (58), but also in hepatocytes and red blood cells (59,60). Although these channels can carry currents comparable to those of the voltage-sensitive K^+ channels, they have a unit conductance that is tenfold higher, so their density must be much lower, less than one per μm^2. While there are reported exceptions (61), the typical conductance of single channels, determined by patch clamping, is 100–200 pS at physiological K^+ (53,57,58). Increased internal Ca^{2+} seems to increase the number of open channels, not the conductance of those channels that are open (57,58,61). Although the channel present in red blood cells is reported to be insensitive to calmodulin (59), the Ca^{2+}-induced opening of the channel in dog heart can be blocked by micromolar trifluoperazine, an antagonist of calci-

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calmodulin action (62). It is thus possible that calmodulin is a tightly bound component of the channel complex. While the channel requires Ca^{2+} for activation, it is sensitive to transmembrane potential at suboptimal Ca^{2+} concentrations (53,57,58,63). Depolarization increases the number of open channels, perhaps by changing the affinity of the channel for Ca^{2+}. Recently, the bee venom toxin apamine, which blocks neuronal Ca^{2+}-dependent K^+ channels (64), has been shown to bind a protease-sensitive receptor in rat brain with a dissociation constant of only 10 pM (65). The density of this receptor is only 1/150 to 1/300 that of Na^+ channels, approximately the density expected for Ca^{2+}-dependent K^+ channels. Apamine also blocks the Ca^{2+}-dependent K^+ channels in cultured rat myoblasts and myotubes, where it binds a receptor with a dissociation constant of 36–60 pM (65a). The density of this receptor is also low, approximately sevenfold lower than the density of Na^+ channels. Although the toxin provides a potential ligand for assay and purification of this channel, the low density of the channel in membranes means that purification will be a formidable task.

In spite of its low density, the Ca^{2+}-dependent K^+ channel has been the subject of several biochemical studies because it is a common target of intracellular kinases, providing a means for second messengers, such as cAMP and Ca^{2+}, to modify ion fluxes in the nerve terminal. In internally perfused *Helix* neurons addition of the catalytic subunit of the cAMP-dependent protein kinase results in an increased Ca^{2+}-dependent K^+ conductance (66). The channel still requires Ca^{2+} to open. One possible consequence of phosphorylation would be to reduce the K_m for Ca^{2+}. Recently, evidence that catecholamines induce the opening of a Ca^{2+}-dependent K^+ channel in mammalian pyramidal cells has been presented (67). Cyclic AMP is also likely to mediate this response (68).

Surprisingly, in other invertebrate neurons, cAMP-dependent phosphorylation seems to reduce, not increase, the conductance of the Ca^{2+} -activated potassium channel. Either bath application of cAMP analogs or microinjection of the catalytic subunit of the cAMP-dependent protein kinase reduces a late K^+ current in *Aplysia* bag cells (47). The channel is suppressed by Co^{2+} or Ni^{2+}, arguing that it is a Ca^{2+}-activated channel (69). The physiological effects of elevated cAMP on the cells are dramatic. A train of impulses in the fibers innervating the bag cells induces a prolonged period of spontaneous activity in these cells. Cyclic AMP analogs have the same effect. If the kinase catalytic subunit can enhance or reduce Ca^{2+}-activated K^+ conductances in different cell types, either the channels or proteins that modify them must differ in some way.

Transmitter-sensitive K^+ channels: Several distinct K^+ -selective channels, restricted to particular classes of neurons, have been demonstrated to be opened or closed as a consequence of neurotransmitter action (e.g. 40,42,70).

One type of transmitter-sensitive channel, the M channel, has been found in sympathetic neurons (71) and several populations of central neurons (see 42). In frog sympathetic neurons, for example, the M channel is selective for K,$^+$ opens at voltages below the threshold for initiation of action potentials, and does not inactivate with time. Thus, it exerts a strong stabilizing effect on the membrane of cells that are depolarized by other actions. Acetylcholine and muscarinic agonists suppress this current and thereby increase the excitability of sympathetic neurons (42). Closing of M channels does not appear to be mediated by second messengers, but M channels are closed by Ba^{2+} (72).

Another type of channel, the S channel, has been found in *Aplysia* sensory neurons (73,74). The S channel is open at rest and is not sensitive to voltage or Ca^{2+}. It is closed, however, by serotonin binding to a receptor (40). The action of serotonin is mediated by a cAMP response that activates a cAMP-dependent protein kinase (e.g. 44). Both the M and S channels are distinct from the fast voltage-sensitive (A), delayed voltage-sensitive (K), and Ca^{2+}-activated

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(C) K^+ channels that coexist in these cells. The M and S channels are only two of a large number of receptor-sensitive K^+ channels. Many cells contain more than one channel of this type (70). K^+ channels are often regulated by peptide transmitters (e.g. 75).

DISTRIBUTION OF K^+ CHANNELS—The existence of so many diverse types of K^+ channels and absence until recently of specific ligands has limited studies on distribution of K^+ channels in neurons. Recent physiological experiments have shown that K^+ channels exist in nodal, paranodal, and internodal regions of the frog myelinated nerve fiber (76). In mammalian myelinated fibers, K^+ channels appear to be excluded from the node (77), but are present in the paranodal axolemma (78). These preliminary results suggest that both the concentration and distribution of different K^+ channel classes will be functionally important in describing the properties of neurons and neuronal circuits. One must hope that specific reagents for K^+ channel polypeptides will be useful for mapping their distribution more precisely.

SUMMARY—K^+ channels exist in a tremendous diversity that is only beginning to be appreciated. The different channel types serve as a reservoir for generation of the multitude of different electrophysiological properties seen in the different neurons. These channels seem also to be a frequent substrate for agents that modify synaptic efficiency, animal behavior, and memory (e.g. 44). Diversity in the channels permits very selective responses in different neurons to the same second messenger. More detailed studies on factors regulating the synthesis and distribution of these channels will be very important for future studies on synaptic function. Generating probes specific for these channels, using molecular biology or hybridoma technologies, will be a formidable but rewarding task.

Restoration of Na^+ and K^+ Gradients

DESCRIPTION—To maintain ionic gradients, neurons use a variety of ion pumps and exchange mechanisms, the majority of which are also used by nonneural cells. The major mechanism for removing internal Na^+ and restoring internal K^+ after action potentials is an ATP-driven Na^+ -K^+ exchange pump that is detected in biochemical assays as an $Na,^+$ K^+ -ATPase (79,80) and is the same enzyme as is used by other cells in the body. The pump occupies a central role in neuronal function. The Na^+ gradient that it maintains is essential to control intracellular osmolality and cell volume. The Na^+ gradient also drives several exchange reactions. One of these, an Na^+ -Ca^{2+} exchange, removes Ca^{2+} from the cytoplasm and contributes to the termination of exocytosis and other consequences of Ca^{2+} action (81,82). The Ca^{2+}/Na^+ antiporter from bovine heart has been solubilized, partially purified, and reconstituted (83), but the proteins responsible for mediating Na^+/Ca^{2+} exchange have not yet been identified. Several cotransport reactions, using the energy of the Na^+ gradient, are responsible for accumulation of sugars, amino acids, neurotransmitter precursors, and neurotransmitters. The presence of an avid Na^+-dependent uptake mechanism for neurotransmitter precursors, such as choline, or neurotransmitters such as norepinephrine, correlates closely with the transmitter specificity of neurons (84) and in some neurons is specifically localized to the nerve terminal (85).

In neurons, activity of the Na^+ pump is limited by internal Na^+ (86,87). Increased cytoplasmic Na^+, introduced by action potentials or micropipettes, activates this pump, which can actually hyperpolarize the cell, since three internal Na^+ are exchanged for only two K^+ (79,80). Ouabain-sensitive hyperpolarizations are prominent features in many neurons following trains of action potentials (e.g. 88) and can block action-potential initiation (89).

BIOCHEMICAL CHARACTERIZATION—The structure and function of the $Na,^+$ K^+-ATPase have been extensively studied (reviewed in 90–92). The enzyme has been purified

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from several sources including rat brain (93). In each case, two polypeptide chains of M_r 120,000 and 50,000 are found in highly purified preparations. The larger polypeptide has an internal binding site for ATP and external binding site for ouabain (e.g. 94). The smaller subunit is a glycoprotein that cannot be removed without losing enzymatic activity. Definitive evidence that these two subunits are the only proteins required for activity came from reconstitution experiments (e.g. 95), in which it was shown that no other large proteins were present in high enough concentrations to be present in each vesicle with reconstituted active Na^+ and K^+ transport. A small acidic proteolipid has been labeled with a photoaffinity ouabain derivative and hence is a possible additional component of the ATPase (96). The stoichiometry of this proteolipid in purified and reconstituted preparations has not been determined.

There is evidence for multiple forms of Na,$^+$ K,$^+$-ATPase in neurons, even though the subunit composition, kinetic properties, and sensitivity to cardiac gangliosides of the neuronal enzymes indicate that neurons use the same basic pump as nonneuronal cells. Two forms of the neuronal ATPase have been identified in vertebrates and invertebrates that are closely related, but differ in sensitivity to proteolysis and cardiac ganglioside-mediated inhibition (93). One of these is the standard (α) form of enzyme that was the only form seen in kidney, muscle, adrenal cortex, and astrocytes. The specific neuronal form of the enzyme (α^+) has a lower affinity for strophanthidine and is the only form found in myelinated axons. The α^+ form appears after the standard form in developing brain. It does not appear to be in all neurons, since cultured sympathetic neurons contained only the standard (α) enzyme.

DISTRIBUTION ON NEURONS—Distribution of the Na,$^+$K$^+$- ATPase has been examined with immunocytochemical methods (97). On neurons, it is restricted to the plasmalemma, where it is distributed over the surface of cell somas and dendrites. It appears to be concentrated in nodes of Ranvier in myelinated axons. Two classes of synaptic terminals were observed, one with and one without high concentrations of the ATPase. Observed differences in concentration between different nerve terminals is likely to be important in regulating the response properties of these terminals to different patterns of stimulation. Concentration in the nodes of Ranvier is also of physiological significance. The antibodies used in these experiments almost certainly recognized both forms of the enzymes.

REGULATION BY HORMONES AND TRANSMITTERS—Hormones and transmitters, such as vasopressin and the catecholamines, regulate activity of the Na,$^+$ K$^+$-ATPase in neuronal and nonneural tissues. Many of these effects seem likely to be mediated by cAMP and cAMP-dependent protein kinase (e.g. 98–102). Direct effects of catecholamines on the ATPase have been seen in brain synaptosomes (99). Thyroid hormone increases the activity of the ATPase by increasing the number of pumps in the plasmalemma (103). These studies suggest that hormones and transmitters will prove to regulate synaptic function by controlling the density and activity of the Na,$^+$ K$^+$-ATPase. Endogenous compounds with ouabain-likc binding have also been identified (104,105). Further studies will be needed to determine whether activity of the Na,$^+$ K$^+$-ATPase is also regulated by such compounds at the ouabain binding site.

Voltage-Sensitive Calcium Channels

DESCRIPTION—Voltage-gated calcium channels have been described in a wide range of neural and nonneural tissues [for recent review see (106)]. Since Ca^{2+} channels in different tissues vary markedly in their kinetic properties and in their sensitivity to inhibitors, it had been assumed that there was not one but many types of calcium channel (106). With the advent of patch clamping this view has been challenged (107) on the grounds that snail, chick and rat channels are remarkably similar to each other and to those found in heart muscle (108) and chromaffin cells (109).

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Measurement of single Ca^{2+} channel conductance by patch clamping has not yet been applied to nerve terminal membranes. Voltage-dependent Ca^{2+} channels in heart cells, chromaffin cells, and snail neurons have, however, been recognized by this procedure. These are open for one or two msec with currents of 0.5–2 pA. At high depolarization, channel openings appear in clusters. Reuter et al (108) suggest that the Ca^{2+} channel enters an activated state from which it rapidly flickers open and closed. The heart cell Ca^{2+} channel also inactivates with prolonged depolarization. The Ca^{2+} channel in heart has a conductance of about 25 pS (108). A detailed study of calcium currents in the nerve terminals of the squid stellate ganglion demonstrated some parallels between the Na^+ and Ca^{2+} channels, but also some striking differences (2). The Ca^{2+} channel opens with applied voltage but only after a significant delay. Consequently the Ca^{2+} channel opens during the falling phase of the presynaptic action potential (110). The total Ca^{2+} current is about 1/20 that of the sodium current. When the voltage is removed the channels close with simple exponential kinetics ($\tau = 630\ \mu$sec). Unlike Na^+ channels, however, the Ca^{2+} channels in this synapse stay open during a prolonged depolarization.

During repetitive stimulation, the amount of Ca^{2+} entering the squid nerve terminal per impulse remains constant. This was shown by injection of the Ca^{2+} sensitive dye Arsenazo III into nerve terminals. A single impulse causes detectable increase in light absorption owing to the formation of Ca^{2+}-Arsenazo III complexes. The increase reaches its maximum at the peak of transmitter release (111). During repetitive stimulation the increase in light absorption increased linearly with the number of stimuli (111,112), showing that the channel is not altered by activity.

The use of Arsenazo III as a calcium-sensitive dye allows the sites of Ca^{2+} entry to be identified. In the squid giant synapse, changes in light absorption were only seen in the nerve terminals and not in preterminal axon regions, suggesting that nerve terminal membranes are enriched in Ca^{2+}channels (111).

Comparison of the change in Arsenazo III absorption induced by injecting a known amount of Ca^{2+} to that induced by a single action potential has made it possible to estimate that approximately 2×10^8 Ca^{2+} ions enter per impulse. The number of ions that flows through a single channel per impulse could be calculated if the density of channels were known. Large intramembranous particles are seen clustered in squid nerve terminals in putative active-zone regions (113). If it is assumed that all large particles in the clusters are Ca^{2+} channels, then each channel passes about 150 Ca^{2+}/impulse and has a conductance of about 0.14–0.21 pS (114). This conductance is in reasonable agreement with that determined from noise measurements on *Helix* neurons (115), but is two orders of magnitude lower than that measured by patch clamping (107). Unless squid nerve terminal Ca^{2+}-selective channels have a smaller conductance, comparatively few of the large intramembranous particles can be functional Ca^{2+} channels.

BIOCHEMICAL NATURE OF THE Ca^{2+} CHANNEL—Progress in Ca^{2+} channel purification has been prevented by lack of specific ligands with nanomolar binding constants and the absence of tissues, equivalent to electric organ, that are rich sources of channels. The Ca^{2+} channel activity also decays rapidly in perfused cells (e.g. 116), so purification may require identification of the factors important for maintaining Ca^{2+} channel activity in the intact cell. One potentially useful approach is the separation of reconstituted phospholipid vesicles containing Ca^{2+} transporters on the basis of Ca^{2+} flux-induced density fluxes (117). ATP-driven Ca^{2+} pumps have already been purified by this procedure. Another is the report that batrachotoxin, long known to interact with voltage-sensitive Na^+ channels, can also inhibit Ca^{2+} channels in a neuroblastoma cell line (118). While 40 nM batrachotoxin gave 50% inhibition, the lipophilic nature of this toxin will limit its utility in providing an assay for the Ca^{2+} channel. Another candidate is maitotoxin, which increases Ca^{2+}-influx into

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pheochromocytoma cells (119). Ca^{2+}-influx induced by this toxin is blocked by classical Ca^{2+} channel inhibitors, such as verapamil, $Mn,^{2+}$ and tetracaine. Drugs of the dihydropyridine type are thought to block slow-acting calcium channels. ^{3}H-Nitrendipine appears to bind to the target of these drugs. Its binding requires calcium or strontium and is blocked by lanthanum or cobalt, whereas verapamil and D-600, classic Ca^{2+} channel blockers, have no effect (120). Identification of a soluble Ca^{2+} channel-specific ligand, equivalent to tetrodotoxin, would greatly facilitate attempts at purification.

REGULATION OF THE CALCIUM CHANNEL—All types of short-term synaptic plasticity that have been investigated in detail involve modulation of Ca^{2+} levels in the terminals, though the mechanisms differ (121,122). The amount of Ca^{2+} -influx during an action potential is primarily regulated by the opening and closing of Na^{+} and K^{+} channels (e.g. 121). In addition internal Ca^{2+} levels depend on steady state Ca^{2+} currents (123), Ca^{2+} accumulation from previous action potentials (122), and direct modification of Ca^{2+} channel activity (124,125).

Synaptic facilitation is the increase in transmitter release sometimes observed on repetitive stimulation. Facilitation does not appear to be due to changes in the Ca^{2+} channel but to accumulation of Ca^{2+} in the nerve terminal. Recent experiments on the squid stellate ganglion synapse (112) show that an alteration of Ca^{2+} current of sufficient amplitude to cause facilitation would have been readily detected. Experiments on *Aplysia* neuron L10 show a close correspondence between facilitation and internal calcium as measured by the activity of the Ca^{2+}-dependent K^{+} channel (122). The kinetics of disappearance of facilitation are thought to be regulated by the time course of Ca^{2+} removal (126).

The steady-state membrane potential also has an important influence on internal Ca^{2+} levels and exocytosis. Studies on *Aplysia* neuron L10 have shown that depolarization of the nerve terminal enhances exocytosis induced by an action potential in two ways: a steady state Ca^{2+} current is increased, resulting in a high concentration of internal Ca^{2+}; and voltage-sensitive K^{+} channels, are inactivated, prolonging the duration of the action potential (123). Hormones and transmitters may thus modify synaptic efficiency by inducing small changes in the resting membrane potential near the terminal.

It was noted earlier that the Ca^{2+} channels in the squid stellate ganglion synapse do not inactivate rapidly as a result of prolonged depolarization (2,127). Under physiological conditions, repetitive stimulation of the synapse gives a linear increase with time in intraterminal Ca^{2+} (111,112). In many cells, however, a slow inactivation of Ca^{2+} channels is seen (128,129). Half times of inactivation are typically about 100 msec, but range from 5 to 1000 msec (106). Inactivation has often been associated with the accumulation of high levels of internal Ca^{2+} (reviewed in 106). Indeed, inactivation of Ca^{2+} channels can be induced by injection of Ca^{2+} into the cytoplasm. If internal Ca^{2+} does control inactivation, differences in surface to volume ratio may account for the differences in observed inactivation rates.

In one case, repetitive stimulation has been shown to reduce intraterminal Ca^{2+} influx and exocytosis. Repetitive stimulation of a sensory neuron in *Aplysia* gives rise to a synaptic potential in a postsynaptic motor neuron that gradually decrements in amplitude. This decrease is thought to explain the habituation observed in the gill-withdrawal reflex in *Aplysia.* By blocking all the known Na^{+} and K^{+} channels Klein et al (130) could show that the decrease in postsynaptic size correlated with a decrease in Ca^{2+} current in the presynaptic cell. The decrease, however, is not likely to be due to internal Ca^{2+}-induced Ca^{2+} channel inactivation, since it occurred when Ba^{2+} was substituted for Ca^{2+} and the time course of recovery was much slower than that observed after Ca^{2+} injections.

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Ca^{2+} channels are also regulated by neurotransmitters and hormones. Ca^{2+}-dependent potentials in cell bodies of the chick dorsal root ganglia (131,132) and in the rat superior cervical ganglion (133) decrease in duration and amplitude in the presence of noradrenaline, γ-aminobutyric acid, enkephalin, and somatostatin. Voltage clamping of sensory neurons in the absence of Na^+ and K^+ currents has provided evidence that reduced current is carried through Ca^{2+} channels (124). While a second messenger hypothesis is attractive to explain the synergistic actions of so many different transmitters, bath application of dibutyryl cAMP did not reduce Ca^{2+} conductance and no evidence was found for involvement of internal Ca^{2+}. Similar results have been obtained using an *Aplysia* neuron. Stimulation of presynaptic inhibitory neurons reduces both transmitter release and Ca^{2+} currents in voltage-clamped L10 neurons (125). Reduced current through the Ca^{2+} channel appeared to reflect direct transmitter action on the Ca^{2+} channel that was not mediated by changes in membrane potential or internal Ca^{2+}. The mechanism of action of the inhibitory transmitter is not known. Ca^{2+} channels are not regulated exclusively by neurotransmitters. Nanomolar to micromolar concentrations of extracellular ATP are also reported to increase Ca^{2+} currents by 25%–30% (134). The stimulation does not require ATP hydrolysis. Since ATP is released with many neurotransmitters, this effect could be physiologically significant.

The modifications of neuronal Ca^{2+} channels that result in altered conductance are not known. In heart muscle, unlike sensory and sympathetic neurons, epinephrine acts to increase Ca^{2+} current. Patch clamp analysis of single Ca^{2+} channels suggests that epinephrine increases the mean time that channels remain open (108). Injection of the catalytic subunit of protein kinase prolongs and enhances the Ca^{2+} action potential, and epinephrine has no further effect (1). The inward Ca^{2+} current increased about threefold. Injection of the regulatory subunit on the other hand decreased the duration and amplitude of the Ca^{2+} current. It would appear therefore that the positive inotropic effect exerted on heart muscle by epinephrine is due to stimulation of cAMP followed by phosphorylation of a protein that is part of, or affects, the Ca^{2+} channel. Isolated cardiac sarcolemmal membranes retain the capacity to show depolarization-sensitive Ca^{2+} uptake (134a). When such membranes contain both radioactive ATP and the catalytic subunit of protein kinase, only one protein is phosphorylated during Ca^{2+} uptake (M_r 23,000). This membrane protein has been termed calciductin. Proteins of such small size are not likely to be channels but may be regulatory elements. A possible model for these molecules is provided by phospholamban, a small, heart-specific protein that binds the ATP-dependent Ca^{2+} transporter in the sarcoplasmic reticulum. Phosphorylation of phospholamban results in increased ATI-dependent Ca^{2+} sequestration in the sarcoplasmic reticulum (e.g. 135,135a).

Ca^{2+}-Removal from the Cytoplasm

DESCRIPTION—The Ca^{2+} that enters during an action potential is removed from the cytoplasm by several mechanisms acting in concert. The plasmalemmas of the squid giant axon and synaptosomes contain both a Na^+/Ca^{2+} exchange activity and a Ca^{2+}-dependent ATPase, which extrude Ca^{2+} (81,82,136,137). The proton electrochemical driving force generated by the mitochondrial ATPase provides the energy required to drive a high capacity Ca^{2+}-uptake into mitochondria (reviewed in 138), which has been seen in both squid (139) and synaptosomes (140,141). In both systems, the cytoplasmic Ca^{2+} level is normally below that which can be effectively removed by mitochondria (e.g. 142), so the mitochondrial system is likely to be only functionally important under conditions of high Ca^{2+} influx (141). Nonmitochondrial sites of internal Ca^{2+} sequestration include smooth endoplasmic reticulum sacs (143) and synaptic vesicles (144).

An ATP-dependent-Ca^{2+} uptake system has also been seen in synaptic vesicles (e.g. 145, 146), but convincing Ca^{2+}-dependent ATP hydrolysis has not been observed (e.g. 147). Vesicles generate a proton electrochemical gradient that is probably responsible for

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neurotransmitter uptake (148,148a) and may well supply the energy required for Ca^{2+} uptake. In cholinergic vesicles from Torpedo electroplax nerve terminals, the ATP requirement for ATP-promoted Ca^{2+} uptake has been shown in part to reflect activation of Ca^{2+} transport by a calci-calmodulin-activated endogenous protein kinase (147).

Ca^{2+} must eventually be released by intracellular organelles and transported out of the cell. Ca^{2+}-efflux from brain mitochondria occurs primarily by Na^{+}/Ca^{2+} exchange (149). The efflux mechanisms of Ca^{2+} from other neuronal organelles, such as synaptic vesicles, have not yet been investigated.

PURIFICATION AND CHARACTERIZATION OF Ca^{2+} PUMPS—The major Ca^{2+} -transpprting ATPase from brain synaptosomes have been investigated by several groups and shown to be distinct in kinetic properties from the muscle sarcoplasmic reticulum Ca^{2+} transporter (e.g. 150). The major Ca^{2+}-dependent ATPase activity detected in membrane vesicles from synaptosomes is found in the same vesicles as Na^{+}-Ca^{2+} exchange activity, which has been detected in intact synaptosomes (82), so it is almost certainly in the plasmalemma.

Neuronal ATP-dependent Ca^{2+} translocators have been purified (117,151). The synaptosomal ATP-dependent uptake system was solubilized in cholate and reconstituted in phospholipid vesicles at high ratios of phospholipid to protein. The vesicles were formed in the presence of oxalate, which forms an insoluble complex with Ca^{2+}. Addition of ATP and Ca^{2+} to the phospholipid vesicles results in Ca^{2+} transport and a shift in density of vesicles incorporating the Ca^{2+}-dependent ATPase caused by the formation of an insoluble Ca^{2+}-oxalate complex. The transporter is purified 100-fold by this procedure. Analysis on gels reveals two bands of M_r 94,000 and 140,000. Both bands are labeled by ATP to form an acyl phosphate (151,152).

The M_r 140,000 protein from synaptosomes is the same molecular weight as the (Ca^{2+} + Mg^{2+}) ATPase in the plasmalemma of red blood cell ghosts and many other tissues (e.g. 153). Recently, the neuronal protein has been purified by calmodulin affinity chromatography (151–152a), the same procedure used to purify the red blood cell enzyme (154). This enzyme is likely to be the same or very similar to the Ca^{2+} -dependent ATPases in the plasmalemma of nonneuronal cells that are strongly stimulated by calmodulin (e.g. 153,155).

The M_r 94,000 Ca^{2+} transporter is not purified by calmodulin-affinity chromatography (151, 152). Immunological evidence (152) indicates that it may be nervous system specific. Antibodies do not cross react with sarcoplasmic reticulum or red blood cell Ca^{2+} transporters. These antibodies should make it possible to identify the location of this transporter.

In summary, nerve terminals contain at least two Ca^{2+} transporters directly dependent on ATP and others that are dependent on ion gradients. Ca^{2+} that enters the cytoplasm during the action potential is sequestered by virtually every intracellular organelle before eventually being secreted from the cell.

MOLECULAR BASIS OF EXOCYTOSIS

Description

Ca^{2+} entering the nerve terminal by voltage-gated channels triggers fusion of synaptic vesicles to the plasmalemma and release of neurotransmitter. Exocytosis also requires a transport system to carry vesicles to the nerve terminal and a docking apparatus to localize them within a short distance of sites of release. The molecules that mediate exocytosis in the nerve terminal remain unknown. Constraints on the mechanisms, however, come from studies on the morphology and physiology of the synapse.

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Kinetics of the Release Process

Electrophysiological recording gives the time course of neurotransmitter release from nerve terminals with a precision unparalleled in any other exocytotic process. Again, the best described synapse is the squid stellate ganglion (156). There is a lag of about one msec between the arrival of the action potential at the nerve terminal and the opening of channels on the postsynaptic membrane. The majority of this time (800 μsec) is due to the lag in opening the Ca^{2+} channels, as described above. Only a few microseconds are required for transmitter to diffuse from the nerve terminals to the very close postsynaptic receptors. This leaves only about 200 μsec for Ca^{2+} to interact with its target and initiate the fusion of synaptic vesicle with presynaptic plasma membrane. Since these experiments were performed at 18°C, it is very likely that in mammalian synapses, the Ca^{2+}-triggered transmitter release takes even less time. The duration of the release process is also very short. The current through the postsynaptic membrane falls with a half-time of approximately one msec. The rapid kinetics of the release process put several constrains on models of neurotransmitter release. First, the brief time involved would allow only those vesicles within a vesicle diameter of the presynaptic membrane to participate in rapid exocytosis. Electron microscopy of the nerve terminal provides evidence for attachment or docking of a small proportion of synaptic vesicles at morphologically specialized attachment sites in the plasmalemma in both central and peripheral synapses (e.g. 157). Even chromaffin granules may make connections with the plasmalemma before exocytosis (158). Affinity chromatography of detergent-solublized chromaffin cell plasma membrane proteins on a column of glutaraldehyde-fixed chromaffin granules results in purification of a 51-kd protein, distinct from actin or tubulin, that is a possible docking protein (159). Proteins of unknown function that are localized specifically to the presynaptic membrane or synaptic vesicle have been identified in neuronal cells (e.g. 160–162) and are thus also candidates for regulation of synaptic vesicle binding. Only those vesicles attached to the presynaptic plasma membranes appear able to participate in normal exocytosis (163,157), although prolonged exocytosis can result in depletion of almost the entire pool of vesicles (164).

The brief time involved also requires that Ca^{2+} channels be located within 100 nm of the site of exocytosis. The 200 μsec would allow Ca^{2+} to diffuse only 40–100 nm through the cytoplasm (156). In fact, the attachment sites for vesicles are usually associated with arrays of intramembranous particles (165). Vesicle openings ruptured by fast freezing during exocytosis are found almost entirely within 80 nm of this zone (157). Indeed, release takes place within a short distance of these particles even in terminals where the arrays are dispersed by soaking in Ca^{2+}-free solutions (163). Ca^{2+} entry, measured by dye binding, is localized to nerve terminal regions (111). It has been argued that the large particles in the active zones are present in appropriate numbers to be Ca^{2+} channels (114). Unfortunately direct evidence is lacking.

The kinetics of transmitter release seem to follow the kinetics of Ca^{2+} entry quite closely, suggesting that the Ca^{2+} that enters is quickly removed from the exocytotic apparatus (111, 112). Disappearance of intracellular Ca^{2+} however, as monitored by Arsenazo III adsorption, occurs much more slowly (sees) that the cessation of transmitter release (msecs). Also, intracellular Ca^{2+} concentration increases linearly during repeated stimulations, but exocytosis correlates not with the total apparent Ca^{2+} concentration, but with the entry of additional Ca^{2+} during each impulse. To resolve this apparent paradox, it has been proposed that Ca^{2+} entry occurs near release zones, where it promotes exocytosis but then diffuses away from these zones, terminating exocytosis. Binding to Arsenazo III monitors average cytoplasmic Ca^{2+} concentrations. In this model, Ca^{2+} diffusion is primarily responsible for the cessation of exocytosis.

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Concentration-Dependence of Ca^{2+}-Dependent Exocytosis

Even though the Ca^{2+} current is linear with external Ca^{2+} below saturation (2), the rate of neurotransmitter release increases with a higher power dependence in the squid giant synapse (110,112) and frog or crayfish neuromuscular junction (126,166). The slope of Ca^{2+} vs release varies from between 1 and 2 (squid) to 4 (neuromuscular junction), so activation of the Ca^{2+} target is likely to have a nonlinear dependence on Ca^{2+}.

Estimates of the local Ca^{2+} concentration during release have been made from measurements of Ca^{2+} entry per unit area, based on current or dye binding (112,156) and suggest a concentration of 10 μM. Alternative calculations have been based on the ratio of spontaneous to induced release. Assuming a fourth power dependence on Ca^{2+} level, internal Ca^{2+} must increase 16-fold from 0.1 to 1.6 μM to account for the 60,000-fold increase in release rate (126).

Direct measurements of the dependence of exocytosis on Ca^{2+} concentration have been made in chromaffin cells and sea urchin eggs that have been permeabilized to molecules of less than 4-nm diameter by brief high voltage electric shocks. The concentration for half-maximal release is about 1 μM in each system and appears to be cooperative with release proportional to the second power of Ca^{2+} concentration (167,168). The Ca^{2+} requirement can not be replaced by Mg^{2+}. A similar dependence on Ca^{2+} is seen by generating from sea urchin eggs a preparation of cortical granule surfaces attached to polylysine on coverslips (169). The calculations of Ca^{2+} dependence in each system assume that Ca^{2+}-EGTA complexes are biologically inert, an assumption that does not appear to be true for ATP-dependent Ca^{2+} transporters (e.g. 170). The evidence that these quasi-in vitro and in vitro systems measure exocytosis is convincing. In each system, vesicles disappear from the cytoplasm and are fused to the plasma membrane. In permeabilized chromaffin cells, release of catecholamines is accompanied by release of the vesicle protein, dopamine-β-hydroxylase; but not cytoplasmic enzymes.

Possible Ca^{2+} Targets

A molecule of the calmodulin type is an obvious candidate for the Ca^{2+} binding site. Calmodulin is present in all eukaryotic cells, including nervous tissue, at concentrations of approximately 10 μM (171,172). It has a 0.1–10 μM binding affinity for Ca^{2+} depending on conditions (reviewed in 172), which is in the same range as the intracellular Ca^{2+} concentrations during exocytosis. It has four Ca^{2+} binding sites and the activation of some of its targets such as cyclic nucleotide phosphodiesterase, adenylate cyclase, and ATP-dependent Ca^{2+} transporters, require 3 or 4 Ca^{2+} bound (173–175). Thus, a requirement for more than one bound Ca^{2+} could explain nonlinearities in the dependence of release on Ca^{2+}. Finally, since calmodulin and calmodulin-like proteins bind to a subset of their target enzymes, such as phosphorylase kinase and protein phosphatase 2B, even in the absence of Ca^{2+} (176,177), calci-calmodulin could initiate vesicle fusion within the 200 μsec required for exocytosis.

Inhibitors of calmodulin action, in particular the phenothiazines, have been shown to inhibit exocytosis in virtually every Ca^{2+}-dependent system examined, including permeabilized adrenal medullary cells (168) and cholinergic synaptosomes (178). Since these drugs shows some interaction with other proteins, for example the phospholipid-sensitive Ca^{2+} -dependent protein kinase (179), and have general membrane perturbant effects, sensitivity to them is not convincing proof of calmodulin involvement. Inhibition of exocytosis in mast cells, though, has been shown to occur at drug concentrations much lower than that required to produce general cell damage (180). Other inhibitory effects, consistent with interference with Ca^{2+} entry, were also observed. In hamster insulinoma cells, concentrations of phenothiazine that block glucose-stimulated, Ca^{2+} -dependent release do not reduce the glucagon-stimulated,

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cAMP-dependent exocytosis that is independent of extracellular Ca^{2+} (181). In this case, the predominant effect of phenothiazines could be on Ca^{2+} entry rather than on exocytosis, suggesting caution in the interpretation of phenothiazine experiments (182).

Compelling evidence for the involvement of calmodulin in exocytosis has been obtained using sea urchin eggs. Using a preparation of cortical coverslips, Steinhardt & Alderton (183) demonstrated that anticalmodulin antibodies prevented Ca^{2+}-dependent fusion of cortical granules to the plasma membrane. The inhibition could be reversed with excess calmodulin. Extensive endogenous calmodulin was detected on the plasma membrane with immunofluorescence. These experiments strongly suggest that calmodulin will be a general mediator of Ca^{2+}-dependent exocytosis.

Direct evidence, however, for the involvement of calmodulin in exocytosis at the nerve terminal is not nearly as compelling. Calmodulin makes up 0.7% of the protein in isolated nerve terminal preparations (184), certainly a sufficient concentration to play a role in exocytosis. A preparation of brain synaptic vesicles also contains calmodulin that can be removed by washing in chelating agents (184,185). Since the synaptic vesicle preparation contained coated vesicles (186), which also bind calmodulin (e.g. 187) it will be important to verify that the calmodulin is truly bound to the synaptic vesicles. There are preliminary reports that calmodulin binds to purified electric organ synaptic vesicles (188), chromaffin granules (189,190) and platelet alpha granules (191). Both Ca^{2+}-independent and Ca^{2+}-dependent binding sites with ~30nM binding constants have been described on the cytosolic surface of chromaffin granule membranes (191a). The former sites appear to represent binding to membrane proteins of M_r 25,000 and 23,000. Binding to additional membrane proteins of M_r 69,000 and 50,000 is seen in the presence of 1 μM Ca^{2+}. Cytosolic proteins of M_r 70,000, 36,000, 34,000, and 32,000 are recruited to the membrane by Ca^{2+} -calmodulin. The latter two proteins appear to be closely related to the clathrin-associated light chains in coated vesicles. Thus, there is no shortage of calmodulin target proteins that could potentially mediate exoctytosis. Evidence that the nerve terminal or vesicle-associated calmodulin is involved in exocytosis is scarce, however. While brain synaptic vesicle preparations have been reported to aggregate and lose half their norepinephrine content in the presence of Ca^{2+} and calmodulin (184,185), this is not compelling evidence that calmodulin is involved in exocytosis at the nerve terminal. Calmodulin clearly regulates additional synaptic vesicle functions not related to exocytosis. In vesicles from electric organ, for example, it regulates activity of the Ca^{2+}-transporter by activating an endogenous protein kinase (147). Thus, it is not clear which, if any, of the calmodulin binding proteins in the chromaffin granule membrane are required for exocytosis.

Another candidate for the calcium-binding site is synexin, a 47-kd soluble protein found in secretory tissues but also liver cells (192,193). In the presence of Ca^{2+} but not Sr^{2+} or Ba^{2+}, synexin aggregates into rod-like structures (192). Synexin also induces aggregation of chromaffin granules in the presence of Ca^{2+}. This aggregation is blocked by trifluoperazine (193). Synexin may not bind a protein receptor since it enhances the Ca^{2+} -dependent fusion of pure phospholipid vesicles (194). Since synexin is not unique to secretory tissue, works on phospholipid bilayers, causes vesicle-vesicle interaction instead of vesicle-plasma membrane interactions and does not function in the presence of Sr^{2+}, synexin should not be attributed a role in Ca^{2+} -triggered exocytosis until more definitive evidence is available. An alternative hypothesis is that any protein, including calmodulin, that exposes hydrophobic domains on binding Ca^{2+} might either self-polymerize or cross link membranes. These might represent nonspecific analogs of the correct in vivo process.

Mechanism of Vesicle Fusion

In the only case of membrane fusion that is understood, the fusion of a membrane virus attached to the plasma membrane at low pH, one of the viral proteins undergoes a pH-induced change

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in conformation that exposes a new hydrophobic domain (195), resulting in fusion of the viral and host plasma membranes. A similar Ca^{2+} or calci-calmodulin induced hydrophobic domain in a plasma membrane or synaptic vesicle protein could result in fusion of synaptic vesicles and exocytosis. Different groups have noticed correlations between secretion and phospholipase activation (e.g. 196), phosphorylation (e.g. 197), or methylation (198). On this basis, it has been proposed that one or more enzymatic mechanisms, activated by Ca^{2+}, might be required for exocytosis. The kinetics of release make it impossible for extensive catalysis to be required. For example, phospholipases with conventional turnover numbers could catalyze at most one hydrolytic event per enzyme in the time available (199). Therefore, phosphorylation or phospholipid hydrolysis during transmitter release seem more likely a priori to be involved in mobilization of vesicles to their release sites or in regulatory controls than to be primary mechanisms of exocytosis.

The requirements for exocytosis can be investigated in more detail in preparations of permeabilized cells or cortical granule surfaces that are readily accessible to small molecules. A requirement for Mg^{2+} -ATP has consistently been found (167,168,183). The requirement for ATP could not be replaced by S-adenosyl methionine, so there is no evidence for a requirement for methylation (168). The requirement for ATP is consistent with a requirement for phosphorylation, but there are many other explanations including mobilization of secretory granules to the active zone. Detailed analysis of the mechanism of fusion will require identification and characterization of the Ca^{2+}-dependent fusogen.

REGULATION OF NEUROTRANSMITTER LEVELS IN THE NERVE TERMINAL

Metabolism of Peptide and Classical Transmitters

Neurons have evolved several specific mechanisms to maintain neuro-transmitter levels in the nerve terminal. The mechanisms employed to regulate the levels of peptide neurotransmitters, which are derived from proteins, are fundamentally different than those for regulating synthesis of classical neurotransmitters, such as catecholamines or acetylcholine, which are derived from metabolites transported into the cell.

To consider the regulation of neuropeptide levels first, the unusual geometry of the neuron restricts protein synthesis to the cell body, so all proteins and peptides have to be transported from there to the nerve terminal (e.g. 200). Recent work has resulted in the cloning of the genes coding for several peptide precursors and identification of the important steps in peptide processing (e.g. 201–206). The enkephalins, corticotropin, and angiotensin are all synthesized as segments of large proteins that appear to be synthesized on membrane-bound ribosomes, glycosylated in the Golgi apparatus, and transported to the nerve terminal in vesicles or other membrane-enclosed compartments. In each case, the precursor protein contains more than one peptide. Neuropeptides are always separated by a pair of dibasic amino acids (lys-lys or lys-arg). Processing of the angiotensin and vasopressin precursors occurs in membrane vesicles during axonal transport (207). Processing occurs by sequential action of a trypsin-like enzyme followed by a carboxypeptidase B-like activity (208). Enzymes in secretory granules that appear to be responsible for each of these steps have been identified and partially purified (209–211). There is evidence suggesting that the enzymes responsible for processing different precursors may differ slightly from each other (reviewed in 200). The corticotropin precursor is processed into different peptides in different cell types (212). This may prove to be true for other precursors also, such as the enkephalin precursor (e.g. 213). Once released by exocytosis, peptides appear to be degraded, not recycled. Characterization of the proteases responsible for peptide degradation is an active, but nascent field (reviewed in 214). Future studies on regulation of peptide stores in response to different rates of exocytosis will almost certainly focus on gene expression and precursor processing.

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In contrast, much of the regulation of metabolite-derived neurotransmitter levels occurs in the nerve terminal (e.g. 215). As the transporters and enzymes required for synthesis of norepinephrine and acetylcholine, for example, are found in the nerve terminal, very tight coupling between the rates of transmitter release and replacement are possible. Acetylcholine synthesis is regulated by changes in activity of the choline transporter, which limits precursor availability (215a,215b). Catecholamine biosynthesis is regulated by changes in tyrosine hydroxylase, the rate-limiting enzyme. The activity of tyrosine hydroxylase is increased within minutes in active nerve terminals by reduced end product catecholamine-mediated inhibition (215) and increased protein kinase activity (e.g. 215c). In contrast to peptides, classical transmitters in granules are in equilibrium with a cytoplasmic pool (e.g. 216). Increased release, therefore, can rapidly affect the cytoplasmic transmitter pool. Ca^{2+} and cAMP increases in response to stimulation also activate tyrosine hydroxylase by protein kinase action (216a, 217,218). Over hours, high rates of stimulation can result in increased enzyme synthesis in the cell body (219). In terminals containing classic neurotransmitters, vesicle membrane is retrieved after exocytosis and used to form new vesicles without leaving the nerve terminal (220,221). The terminal plasma membrane contains a Na^{+}-dependent cotransporter that functions to recover a significant fraction of the released neurotransmitter or its catabolites (222). Proton-driven antiporters are localized in the vesicle membrane where they function to concentrate neurotransmitter stores. Interference with either uptake or new synthesis of transmitter prevents the maintenance of adequate transmitter stores (223,224).

Storage of Classical Transmitters

The most thoroughly characterized vesicle is the chromaffin granule, which is packed with catecholamines (0.6 M), ATP (0.13 M), protein (0.2 g/ml) and proteoglycans (reviewed in 225). An ideal solution of these components would be hypersomotic and unstable. Direct osmometric measurements, however, have recently shown that a mixture of ATP and catecholamines at these concentrations form a nonideal solution with a reduced osmolality (226). There is no evidence for formation of crystals, however, since NMR measurements have shown that ATP and catecholamines tumble as rapidly in chromaffin granules as in free solution (227).

Catecholamine and ATP uptake into isolated chromaffin granules is driven by pH and membrane potential gradients that are established by an ATP-dependent proton translocase. Isolated chromaffin granules are acidic (pH 5.2), whether measured by methylamine redistribution or protonation of the gamma phosphate of ATP (228,229). Isolated granules in the absence of ATP have a negative internal charge that reflects a diffusion potential induced by the proton gradient (228). With addition of external ATP, though, an ATP-dependent proton translocase transports positive charge into the granule, which becomes positively charged compared to the outside. The voltage buildup is blocked by proton uncouplers. The translocase can be solubilized by bile salts and reconstituted in phospholipid vesicles (230).

Most experiments suggests that the mitochondrial and granule ATP-dependent proton translocases are closely related, but not identical. Both are sensitive to a similar array of inhibitors (231). Both translocases contain a similar proteolipid, which is the target of DCCD, a proton channel blocker. The proteolipid target of DCCD in chromaffin granules, however, is slightly smaller than in mitochondria (232,233). Chromaffin granule preparations contain an ATPase that appears to be almost identical to the F_1-ATPase of mitochondria in size, peptide fragments, and antigenicity of its subunits (234) and an F_1-ATPase-like component with a characteristic lollipop morphology has been visualized in negatively stained chromaffin granules (235). Antibodies to the mitochondrial ATPase block the activity of the granule ATPase and the uptake of monoamines into these granules (234). Although the possibility of contamination by mitochondria or by adsorbed F_1-ATPase has not been completely eliminated,

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the weight of the evidence favors a close similarity between mitochondrial and secretory granule ATPases.

Chromaffin granule ghosts, depleted of internal triphosphates and proteoglycans, have been prepared and used to characterize the catecholamine and ATP transporters. In the presence of ATP, these ghosts generate transmembrane voltage and pH gradients and accumulate catecholamines by a reaction that is sensitive to reserpine, the well-characterized inhibitor of normal catecholamine accumulation in storage granules (e.g. 148). If a pH gradient is produced artificially by suspending the ghosts in a buffer at a higher pH, catecholamine accumulation is also seen. When a transmembrane, interior positive, potential gradient is generated by suspending ghosts filled with NaCl in an KCl solution and adding an ionophore specific for K^+, catecholamine accumulation also is stimulated. Accumulation of catecholamines in response to either gradient is sensitive to reserpine. These results show that the uptake of catecholamines is promoted by both gradients, but does not depend directly on ATP hydrolysis. Similar conclusions have been reached in studies in which the ATP-induced pH gradient or voltage gradient was selectively discharged with NH_4^+ or thiocyanate, respectively (237–242). The responsiveness of catecholamine transport to membrane potential means that the transporter moves charged, not electroneutral species of catecholamines. Its responsiveness to a pH gradient means that it is a proton-driven antiporter. Measurement of the pH-generated driving force suggests that two protons are expelled for each catecholamine. The antiporter binds reserpine and has been solubilized and reconstituted into liposomes (243). ATP uptake, sensitive to atractylosides, has been seen in chromaffin granules. It has been reported to depend only on the transmembrane potential (244).

Accumulation of hyperosmotic concentrations of catecholamines must require other compounds such as ATP that reduce the osmolarity of the granule interior. At physiological cytoplasmic concentrations (20 μM) of catecholamines, the maximum concentration inside ghosts is only 20 mM (245), less than 10% of the concentration in normal granules. Additional accumulation may well require other vesicle constituents, such as ATP, proteins or proteoglycans (246).

Other vesicles appear to use the proton electrochemical gradient to accumulate small molecules. This has been clearly demonstrated in platelet and mast cell granules. Catecholamine uptake into brain synaptic vesicle fractions is stimulated by ATP and inhibited by most of the same drugs that prevent uptake into chromaffin granules (247). Cholinergic vesicles, purified from electric organ, have an internal pH of ~5.5 and contain ~0.15 M ATP in free solution (248–251). The purified vesicles contain an ATPase that shows the same drug sensitivity as the ATPase in chromaffin granule ghosts, sensitivity to DCCD and resistance to oligomycin or efrapeptin (252,253). In the presence of Mg^{2+}-ATP, acetylcholine can be concentrated up to tenfold in these vesicles (254). Bicarbonate converts the ATPase to a high affinity, high velocity form and is required to see significant storage in vitro (253). A similar requirement is seen in cholinergic neurons in vivo (224). Uptake in vitro is blocked by uncouplers of the proton transporter (254), but comparatively little evidence has been obtained for uptake in the absence of ATP (see 255). This may reflect the small size of these vesicles. Alternatively, studies on intact electric organ have suggested that acetylcholine and ATP uptake is restricted to a subpopulation of vesicles with a size and density that differ from those of most vesicles (256–258). If only a small fraction of cholinergic vesicles have a functional uptake system, that could account for many of the difficulties encountered.

Peptide-containing granules can also generate a proton electrochemical gradient. The contents of several granule preparations containing different peptides are also acidic on isolation. Addition of ATP results in generation of an inside-positive membrane potential, which is inhibited by DCCD, but not oligomycin (259–261). An anion-stimulated ATPase with

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properties similar to the cholinergic ATPase has been identified in these granules (261a). Proton translocases in these granules have several probable functions. An acidic interior may be required for segregation of lysosomal and hormone proteins from other proteins of the Golgi lumen (262,263) or activity of the proteases that process the peptide precursors (e.g. 210) may require a low pH. A low pH may also promote the formation of peptide aggregates and, of course, permits vesicles with appropriate transporters to accumulate ATP and classical neurotransmitters. A substantial proportion of peptidergic granules contain classic transmitters. The most prominent example is actually the chromaffin granule, which stores both adrenalin and the enkephalins (264).

MOVEMENTS OF MEMBRANES AND PROTEINS IN THE NEURON

Membrane traffic in the nerve terminal has several components. Components of synaptic vesicles are moved to active zones, fused to the plasma membrane by exocytosis, retrieved by endocytosis, and recycled within the nerve terminal. The terminal is the primary site of insertion of plasma membrane components. It is also the site at which receptor-hormone complexes and other material begin transport back to the cell soma.

Mobilization of secretory vesicles can be divided into transport down the axon and further targeting to the active zone. While the two processes are believed to be similar, most experimental evidence derives from studies of movement in the axon. Transport is measured by pulse labeling of newly synthesized proteins and lipids, accumulation of material at ligatures or cold blocks, and direct visualization of moving particles in the light microscope. Membrane components are transported at exceedingly rapid speeds by mechanisms dependent on local energy. The fastest component, traveling at speeds greater than 250 mm/day includes identified plasma membrane components, such as the $Na,^{+}$ K^{+}-ATPase (265). Mitochondria and at least some components of synaptic vesicles move at somewhat slower rates (265–267). Separate pathways for export of plasma membrane and secretory granule components are seen in nonneuronal peptidergic cells (268), and may be related to some of the different transport components seen in axons. All proteins transported by fast transport appear to be associated with membranous organelles, derived in most cases from the Golgi (268a), and the majority appear to be targeted for the nerve terminal, where many have very fast turnover times (269, 270). A subclass of these proteins does, however, appear to be preferentially deposited along the axon en route (271). Axons also contain membranous elements that are returning to the cell body by retrograde transport, a process typically occurring at 40 mm/day and dependent on local energy sources. About 50% of the membrane protein that reaches the nerve terminal is eventually returned to the cell body (272). Receptors and synaptic vesicle proteins have been identified in this fraction.

Cytosolic proteins not associated with membranous elements are moved to the nerve terminal by a much slower and undirectional process that does not depend on energy in the axon, but does require metabolism in the neuronal cell body. Slow component *b* moves at 3–6 mm/day and includes actin (273), the spectrin-like protein fodrin (273a), clathrin (274), and several metabolic enzymes (275). It has been pointed out that actin and fodrin move together at a similar speed during lymphocyte capping (275a). An even slower system, slow component *a*, moves at 0.7–1.1 mm/day. Unlike the other systems, the majority of the proteins in this class have been identified. They are the α-and β-tubulins, the neurofilament proteins (276), and the tau-like microtubule associated proteins (277). The nerve terminal must contain enzymes to degrade proteins transported by slow transport, since movement is unidirectional and the vast majority of these proteins actually reach the terminal region (278).

Spherical vesicles and small membranous tubules appear to contain the material that is moved by fast transport. Neurotransmitters in spherical vesicles and small tubules accumulate at

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ligatures or cold blocks (279–283). A third membrane system does not participate in fast transport, but is prominent in the axon. An extensively anastomosing network of smooth endoplasmic reticulum is seen in the subaxollemmal regions of neurons, but shows no sign of accumulation on the proximal side or depletion on the distal side of a cold block (283). These experiments make it unlikely that the reticular network participates in fast transport or gives rise to the tubules and vesicles that do accumulate.

The axon contains three major cytoskeletal structures—microtubules, actin filaments and neurofilaments—which could a priori mediate fast and retrograde transport. Morphological evidence favors the involvement of microtubules. Membranous organelles are associated with fascicles of microtubules in axons (284,285,285a) and accumulate at ligatures or cold blocks in association with them (281,282). Movement of membrane particles in vitro, examined by new high resolution microscopy, also follows filaments believed to be microtubule bundles (286). Drugs, such as colchicine, which depolymerize microtubules block fast transport (287), and taxol, which prevents microtubule depolymerization, protect fast transport from colchicine (288).

Some experiments suggest that microtubule-associated dynein may provide the force for fast transport. Low concentrations of erythro-9[3-(2-hydroxy-nonyl)]-adenine, an inhibitor of dynein-dependent motility, block fast transport (288–290). This drug, however, also interferes with other ATPases and thus does not provide unambiguous evidence for the involvement of dynein.

Pharmacological evidence also suggests that actin is involved in fast transport. Injection of DNase I and other actin-depolymerization agents has been observed to block fast transport (290–292). Two agents that should interfere with the function of actin filaments without causing extensive depolymerization, dihydrocytocholasin B and the *N*-ethylmaleimide-modified S1 fragment of myosin, do not block fast transport (290). These results suggest that actin may play a structural role in the cytoplasm rather than a role in transport force generation (290). To summarize, though, it is not yet clear which proteins are responsible for generating the force used to move organelles by fast transport.

Possible clues to the mechanism of polarized movement might be found in the tail-like appendages on membranous organelles reported to be associated with the filamentous network (285), or the higher concentration of cross-bridges found at the leading edge of membranous organelles (283). There is ample support biochemically for direct interactions between secretory vesicles and actin (293–295) and microtubules (296). The latter interactions may involve microtubules associated proteins (297).

One proposed explanation for the range of transport rates is that the transport vectors might transiently be associated with the linear axonal motor. The rate of the motor could be constant and the transport rate regulated by the fraction of time the transport vector associated with the motor (298,299). An appealing feature of this model is its ability to explain the saltatory movement of axonal particles seen in the light microscope. Recent improvements in visualizing axonal particles have made it clear that while large membranous organelles may be moving irregularly, small organelles, presumably elements of the tubulo-vesicular system move at fast speeds (3–5 μm/sec) in a continuous fashion along linear elements (286,300). In addition the slower more intermittent movements of large membranous organelles seem to be not as much saltatory as an “elastic recoil” (286). Thus when particles stop they do not resume at the same speed but at a faster one until they catch up with where they would have been had they not stopped. Such movement suggests stops might come about by transient blocking of movement.

Using organelle movement as an assay of transport, one can remove the permeability barrier of the plasmalemma and examine directly the effects of membrane-impermeable inhibitors on

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axoplasmic transport. Permeabilization can be brought about by electric shock (289), detergent (288), or extrusion of the axoplasm from a giant squid axon (301). Not surprisingly, ATP is absolutely required for transport and GTP will not substitute (289). Unexpected however is the observation that the movement of large particles, presumably mitochondria, is blocked by 2,4-dinitro-phenol even in the presence of ATP (301). Vandate ions at concentrations that inhibit mitosis and ciliary beating also block transport (288,289). An observation difficult to reconcile with earlier observations (302) is that transport occurs in EGTA buffers in which the free Ca^{2+} ion concentration is varied between 10^{-8} M and 5×10^{-4} (303). The discrepancy might arise because different measures of axonal transport were used, or because Ca^{2+} leaks from intracellular organelles in permeabilized tissues. Further experiments should clarify this point and also indicate which proteins are responsible for generating the force used in fast transport.

We look forward to the extension of these studies to vesicle transport in the nerve terminal. At present, we know that transmitter release is blocked ten min after injection of DNase I into the cell body of *Aplysia* neuron L10 (304). We also know that release in permeabilized adrenal medullary cells is unaffected by vanadate, or by agents that disrupt microtubules or microfilaments (168). Much more detailed information using permeabilized systems, immunoelectron microscopy, and well-characterized inhibitors is clearly needed.

MODIFICATIONS IN NERVE TERMINAL METABOLISM INDUCED BY ACTION POTENTIALS AND NEUROTRANSMITTERS

The activity of the nerve terminal is regulated by two exogenous influences—changes in membrane potential communicated via the axon and direct interactions of neurotransmitters with receptors in the terminal plasma membrane. As discussed earlier, depolarization of the nerve terminal increases Ca^{2+} flow into the terminal. Cytoplasmic Ca^{2+} binds calmodulin and other Ca^{2+}-binding proteins, which directly activate several enzymes and indirectly activate many more through the action of Ca^{2+}-dependent protein kinases.

The binding of agonists to receptors on the nerve terminal membrane can result in changes of membrane potential or activation of second messenger systems (305). Large numbers of receptors, specific for a variety of transmitters, have been discovered in preparations of nerve terminals or neuronal cell lines. Activation of different receptors results in specific changes in cAMP and cGMP levels (306). cAMP and cAMP-dependent protein kinases are found throughout the nervous system (307), and hence are believed to be important modulators of neuronal function. The effects of cAMP appear to be mediated largely and perhaps exclusively through cAMP-dependent protein kinases, which alter the activity of many different enzymes and transporters within the nerve terminal (308).

Current evidence suggests that cGMP is important in regulating the metabolism of a small percentage of the cells in the nervous system. cGMP is more specifically localized than cAMP. cGMP is 10–50-fold more concentrated in the cerebellum than other brain regions and the high levels of cGMP within the cerebellum are found in one cell type, the Purkinje cells (307). cGMP-dependent protein kinase is also found primarily in cerebellar Purkinje cells (309, 310).

Changes in Ca^{2+} or cyclic nucleotide levels have several consequences on nerve terminal metabolism. Cytoplasmic Ca^{2+} stimulates not only exocytosis, but also glycogenolysis (311, 312), mitochondrial respiration (311,312), endocytosis (313) and neurotransmitter synthesis (314,315). These changes are basically homeostatic in nature, restoring depleted levels of ATP, neurotransmitters and synaptic vesicles. The actions of elevated cAMP are as pleiotropic as those of Ca^{2+}, but not so simple to summarise. cAMP also regulates energy metabolism (316) and can potentiate neurotransmitter synthesis (315) and release (317). In addition, cAMP

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exerts actions that lead to long-term changes in synaptic efficiency, the basis of phenomena such as habituation and sensitization (44). Mutations in *Drosophila* that alter the enzymes regulating cAMP synthesis or degradation result in pleiotropic defects in learning, habituation, and sensitization (317a–320).

Regulation by cAMP

BRAIN ADENYLATE CYCLASE—Cellular cAMP levels are determined by the activity of adenylate cyclase, the enzyme responsible for its synthesis, and phosphodiesterase, the enzyme that hydrolyzes it. Adenylate cyclase (321) is associated with the cell membrane in neurons and other cells. Stimulation occurs via receptor-mediated formation of an enzyme complex that requires a GTP/GDP-sensitive coupling protein. Synthesis of cAMP requires GTP binding and stops when GTP is hydrolyzed to GDP at the regulatory site. A plethora of neuronal transmitters, most notably dopamine, activates adenylate cyclase probably by promoting an interaction between their receptors and the GTP/GDP-sensitive regulator protein (322). Other transmitters inhibit adenylate cyclase. Some may act by receptor-mediated binding to a distinct GTP/GDP-sensitive coupling protein that inhibits adenylate cyclase. There is suggestive, but not conclusive, evidence for inhibitory GTP-sensitive regulatory proteins (322a). Opiates act by receptor-mediated stimulation of GTP hydrolysis (322b,322c). It has been suggested that they reduce cAMP synthesis by reducing GTP occupancy at the regulatory site in the stimulatory coupling protein (322c).

Neural tissues contain an additional form of adenylate cyclase. It is regulated by both the classical GTP/GDP-sensitive regulatory subunit and by Ca^{2+}-calmodulin (323). Recently, Ca^{2+}-calmodulin has been shown to stabilize and activate fourfold the isolated catalytic subunit of this form of the enzyme (324,325). Addition of the GTP/GDP-sensitive regulatory subunit results in further, additive activation of cAMP synthesis. No evidence for Ca^{2+}-calmodulin binding to this second subunit was found. The cell types that contain this Ca^{2+}-calmodulin form of adenylate cyclase are not known. The results suggest, though, that Ca^{2+} could potentiate the effect of neurotransmitters on cAMP synthesis in those cells that contain this form of the cyclase.

Studies on the time course of sensitization of the gill-withdrawal reflex in *Aplysia*, a process mediated by serotonin-induced cAMP synthesis and cAMP-dependent phosphorylation of a K^+ channel in sensory neurons (44), have shown that the memory for sensitization resides in a persistent elevation of cAMP (326) and not in the slow reversal of a later step in the cAMP-induced sequence of events that results in phosphorylation and closure of the K^+ channels (327). These studies suggest that some neurons may contain forms of adenylate cyclase that are inactivated only slowly after the removal of neurotransmitter. Clearly biochemical studies on the novel forms of adenylate cyclase in nervous tissues will be crucial to understanding the molecular basis of synaptic plasticity.

CAMP-DEPENDENT PROTEIN KINASES—The brain contains the highest levels of cAMP-dependent protein kinases found anywhere in the body, suggesting their importance in neuronal and synaptic function (328). In all systems examined biochemically, the cAMP-dependent kinase consists of a single catalytic (C) subunit that is complexed to one of two classes of regulatory subunit, R_I and R_{II}. The R_I and R_{II} subunits can be distinguished from each other on the basis of size and binding affinities for cAMP and cIMP. In the absence of cAMP, the kinase is a tetramer, consisting of two R and two C subunits. Binding of cAMP to the regulatory unit results in dissociation and activation of the catalytic unit, which phosphorylates protein substrates.

If there is only one protein kinase catalytic unit in brain it is clear that regulation of which protein gets phosphorylated must lie elsewhere. As we shall describe in this section, there are

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many forms of regulatory subunit, varying in their composition and location in the cell, yet all bind the catalytic subunit. It is therefore a reasonable conjecture that specificity arises because regulatory subunits associate with the correct substrate and thus concentrate the kinase in the vicinity of its target.

Brain cAMP-dependent protein kinases appear to be similar to those in other tissues of the body, but there do appear to be some differences in the regulatory subunits. Antibodies to the R_I and R_{II} subunits, purified from bovine lung and heart, respectively, show weak cross-reactivity with the R_I and R_{II} subunits from bovine brain (329). As assayed by binding to photoactivable 8-N_3-cAMP, vertebrate brain has significant quantities of both R_I and R_{II} subunits and no significant amounts of other cAMP-binding proteins (330), supporting the proposal that actions of cAMP in brain are mediated exclusively through activation of protein kinases (308). In contrast to other tissues, though, a fraction of the brain R_{II} class cAMP-dependent protein kinase interacts with calmodulin in the presence, but not absence of Ca^{2+} (331,325). Formation of complexes is promoted by calcineurin (which appears to be protein phosphatase 2B). The Ca^{2+}-calmodulin complex can bind to brain, but not to heart R_{II} subunits. The effect of Ca^{2+}-calmodulin is to inhibit the basal activity of the kinase and lower the enzyme's affinity for cAMP (325). The brain and heart kinases with type II regulatory subunits also have antigenic differences (332). Brain enzyme is poorly bound by antibodies to the heart enzyme.

Labeling cAMP-binding proteins with the photoactivable compound, 8-N_3-cAMP, and subcellular fractionations of these binding proteins also provide evidence for unusual heterogeneity in cAMP-dependent protein kinase regulatory subunits in neuronal tissues. Brain R_{II} consists of a family of molecules with slightly different pI's and, in one case, a slightly different M_r (333). The R_I subunit also is a family with members at more than one pI. Several cAMP-binding proteins, most of which appear to be related to R_I and R_{II} are also found in *Aplysia* sensory neurons (334). Vertebrate brain and Aplysia sensory neurons also contain both membrane and soluble forms of these subunits (334,338). Membrane and soluble forms of the R_I and R_{II} subunits have been detected in nonneural tissues, but the brain appears to be the only tissue with both forms of the same subunit. Nonneuronal tissues in vertebrates and *Aplysia* do not have the same heterogeneity in pI or in membrane association. The multiplicity of forms in vertebrate brain could be attributed to the multiplicity of neuronal and supporting cell types in the central nervous system. The results in *Aplysia*, though, demonstrate clearly that single neurons have multiple forms of cAMP-binding proteins distinguished by subcellular distribution and pI.

Recent studies on vertebrate brain tissue have suggested that the cAMP kinase is concentrated in specific positions in the cell by proteins that bind the regulatory subunit. First, analysis of cytosolic and membrane-bound forms of R_{II} subunit indicate that they have the same M_r, pI, and other properties (332). Secondly, experiments in vitro and in vivo have shown that one protein, MAP 2, has a specific binding site for the R_{II} subunit (335,336). Microtubule-associated protein 2 (MAP 2) consists of two domains, one of which binds the microtubule surface and the other of which appears on a large projection and may mediate interactions with other filaments or oganelles. In neurons, MAP 2 is found on dendritic, but not axonal microtubules (337). The projection portion of MAP 2 binds an R_{II} form of cAMP-dependent protein kinase (335). Exogenous bovine heart R_{II} binds specifically to dendrites in frozen sections of rat brain and the binding is prevented by exogenous MAP 2 (336). It is not clear how many forms of brain R_{II} subunit are bound to MAP 2, but MAP 2 clearly binds the heart form of the enzyme. MAP 2 contains substrates in both of its domains for cAMP-dependent protein kinases. Localized binding may assure preferential phosphorylation in response to cAMP. Phosphorylation would be expected a priori to modify association of MAP 2 with microtubules and other organelles. These experiments suggest that cAMP-dependent protein

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kinases are localized to specific positions by proteins that bind the regulatory subunit, and raise the possibility that the heterogeneity in regulatory subunits controls the site of kinase binding, thus conferring substrate specificity on a nonspecific catalytic subunit.

Despite the possible heterogeneity in regulatory subunits, suggested by binding to calmodulin and MAP 2, a single catalytic subunit appears to function as the actual kinase in all cell types. No heterogeneity in catalytic subunit has been seen in neural or non-neural tissues (328). Injected vertebrate and endogenous invertebrate catalytic subunits phosphorylate the same spectrum of proteins (338), exert the same influence on synaptic transmission (47,339), and are sensitive to the same protein inhibitor, the Walsh inhibitor (46,326,327).

Regulation by Calcium

SUMMARY OF Ca^{2+} ACTIONS—Changes in cytoplasmic Ca^{2+} have multiple consequences on synaptic function. As discussed earlier, cytoplasmic Ca^{2+} stimulates both exocytosis and endocytosis (156,164). The sensitivity of each process to calmodulin inhibitors suggests that calmodulin may be the mediator of Ca^{2+} action in each case (168,340), Ca^{2+}-calmodulin also stimulates activity of several neuronal Ca^{2+} pumps, which would help reduce cytoplasmic Ca^{2+} levels. Ca^{2+}-calmodulin binds directly to the 140-kd ATP-dependent Ca^{2+} transporter (151), which is almost certainly the plasma membrane Ca^{2+}-dependent ATPase (137). Calmodulin also activates a protein kinase that in turn increases activity of a Ca^{2+} transporter in synaptic vesicles (147). Nerve terminal glycogenolysis and respiration are increased in response to activity by a Ca^{2+}-dependent action (311,312). Increased glycogenolysis is probably mediated in part by Ca^{2+}-calmodulin activation of glycogen phosphorylase b kinase, which activates glycogen phosphorylase (316). The kinase has been detected in brain, but not yet shown to be localized in nerve terminals (341,342). Cytoplasmic Ca^{2+} also stimulates the synthesis of several neurotransmitters by increasing the activity of rate-limiting transporters or enzymes. Thus, choline transport in cholinergic neurons, tyrosine hydroxylase in catecholaminergic neurons and tryptophan hydroxylase in serotonergic neurons are activated by cytoplasmic Ca^{2+}, the latter two as a result of phosphorylation by Ca^{2+}-calmodulin-sensitive protein kinases. These effects will be discussed in more detail in the next section. These actions are basically homeostatic in nature, restoring cytoplasmic Ca^{2+}, ATP, and neurotransmitters to original levels. Ca^{2+} also has effects on neurons that are less easy to understand. Ca^{2+}-calmodulin interacts with the cyclic AMP pathway at several steps, activating one form of adenylate cyclase, inhibiting the basal activity of a type II kinase, and activating a form of phosphodiesterase with a comparatively high K_m for cyclic nucleotides. These reactions are mediated by Ca^{2+}-dependent binding of calmodulin to the catalytic subunit of cyclase, R_{II} subunit of kinase, and catalytic subunit of phosphodiesterase, respectively (323, 331,343). Cytoplasmic Ca^{2+} also reduces the activity of certain Ca^{2+} channels and activates Ca^{2+}-dependent K^+ channels, resulting in reductions or increases in synaptic efficiency. Long-term potentiation of synaptic transmission in the hippocampus requires Ca^{2+} and is blocked by trifluoperazine (344), evidence that Ca^{2+} is required for some types of synaptic plastic changes.

Ca^{2+}-calmodulin binds to fodrin, a neuronal cytoskeletal protein that is very similar to spectrin (345), regulates the assembly and disassembly of brain microtubules (346), and controls actin myosin interactions in nonneural cells by activation of a myosin light-chain kinase (347). The role of these activities in short-term synaptic function is not clear. They may be important in generating the changes in synapse number and size that result from long-term facilitation or depression in *Aplysia* and in the mammalian hippocampus (44).

CALMODULIN CALCINEURIN INTERACTIONS—Calmodulin, the virtually ubiquitous Ca^{2+}-binding protein of eukaryotes, is present in high concentrations in nerve terminals

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(184) and appears to be the major, but not exclusive Ca^{2+}-binding protein and mediator of Ca^{2+} transduction in the synapse. Since it is the subject of frequent and thorough reviews (171,172,348,349), we make no attempt to review its properties here.

The existence in brain of other Ca^{2+} -binding proteins, such as parvalbumin and calcineurin, has suggested the possibility that these proteins may modulate the kinetics of calmodulin activation. Bovine brain contains high levels of calcineurin, originally described as an inhibitor of Ca^{2+} -calmodulin activation of brain cyclic nucleotide phosphodiesterase (350). Calcineurin is predominantly found in the nervous system where it is present at approximately 1 μmole per kg. It contains two subunits of 61 kd and 15 kd, which are tightly linked to each other (351). The small subunit binds 4 moles of Ca^{2+} with submicromolar affinity, i.e. more tightly than calmodulin. The large subunit mediates Ca^{2+}-dependent formation of a calmodulin calcineurin complex. Calcineurin could potentially act as an effective calmodulin buffer—both by serving as a sink for Ca^{2+} and for Ca^{2+} -calmodulin complexes. Computer simulations of Ca^{2+} binding indicate that cytoplasmic Ca^{2+} will be bound to calmodulin for the first few msec after entry into the nerve terminal, but will be largely sequestered by calcineurin over the next 100 msec ($t_{1/2}$ = 30 msec) (352). Calcineurin also binds to calmodulin more tightly than to phosphodiesterase, so it would tend to displace calmodulin from other targets. Recent work has shown that the subunit structure and enzymatic properties of calcineurin are identical to those of protein phosphatase 2B, a phosphatase with restricted substrate specificity that dephosphorylates the α-subunit of phosphorylase kinase (353). The enzyme requires Ca^{2+} or Mn^{2+} for activity and is further activated tenfold by Ca^{2+} calmodulin. Immunocytochemical studies in the light and electron microscopes show that it is primarily localized within neurons and is most prominent in dendrites and postsynaptic densities (354). It has been argued that the high concentration of calcineurin in neurons will allow an extremely fast on-off switch for Ca^{2+}-dependent biological processes (352). It now appears that calcineurin can interact with Ca^{2+}-calmodulin at several steps, reducing cytoplasmic Ca^{2+}, sequestering Ca^{2+}-calmodulin, and dephosphorylating proteins activated by Ca^{2+}-sensitive kinases. Further studies will be needed to elucidate the importance of this complex regulatory system.

Ca^{2+} -CALMODULIN-SENSITIVE PROTEIN KINASES—A number of the actions mediated by Ca^{2+} and calmodulin in the nervous system appear to reflect activation of Ca^{2+}-calmodulin-sensitive protein kinases with restricted substrate specificities (355). In contrast to cAMP-dependent protein kinases, which contain only a single catalytic subunit, Ca^{2+}-calmodulin kinases exist as several distinct species with specific targets whose diversity is only now beginning to be explored. These kinases phosphorylate a diverse spectrum of proteins, including some that are also substrates of cAMP-dependent kinases (356,357).

Separation of a soluble extract of rat brain on Sepharose revealed three distinct peaks of Ca^{2+} -calmodulin-sensitive protein kinase activity with different substrate preferences (358). The kinases that phosphorylated glycogen phosphorylase, tryptophan hydroxylase, and myosin light chain most efficiently were detected at positions corresponding to molecular weights of 1,000,000, 500,000, and 100,000, respectively. Kennedy & Greengard (342) separated four distinct Ca^{2+}-sensitive kinases on DEAE. Two of these appeared to be myosin light-chain kinase and phosphorylase b kinase. The other two kinases were detected by their ability to phosphorylate protein I.

Phosphorylase kinase has been detected in brain, but not actually shown to reside in nerve terminals (341). Since nerve terminal activity increases glycogen breakdown, provided Ca^{2+} is present (311,312), it seems likely that cytoplasmic Ca^{2+} accelerates glycogenolysis in the same manner in nerve as has been demonstrated in muscle by activating phosphorylase b kinase, which increases the phosphorylation of both glycogen phosphorylase and glycogen synthase (316). As a result, glycogen synthesis is reduced and glycogen breakdown is

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accelerated. The muscle enzyme contains four subunits of total M_r 1,300,000. One of the subunits is calmodulin, which remains bound without Ca^{2+} (359).

Brain myosin light-chain kinase has been purified and shows Ca^{2+}-dependent binding to calmodulin (331). The purified enzyme is a monomer of 130 kd. A recent study suggests that the brain enzyme may have a broader substrate specificity that myosin light-chain kinases from other sources (359a), but this needs to be confirmed. Myosin light-chain kinase activity has been detected in cultured astrocytes (360), so it is not certain that it is actually present in nerve terminals. It does not appear to phosphorylate efficiently nerve-cytosol proteins in vitro (360a).

One of the two protein I kinases is located in the cytosol and phosphorylates the same region of protein I as cAMP-dependent protein kinase. It is sensitive to low concentrations of trifluoperazine, but not to exogenous calmodulin. To explain these results, Kennedy & Greengard (342) have proposed that it contains calmodulin as a subunit, whether or not Ca^{2+} is present.

The second protein kinase phosphorylates two different sites in protein I and requires exogenous calmodulin to respond to Ca^{2+} (342,361). This enzyme is found in both membranous and soluble fractions from brain homogenates. The particle enzyme can be solubilized in low ionic strength buffers, though, and once solubilized, does not appear to differ from the soluble enzyme by several criteria (361). Both the soluble and particulate enzymes have been purified about 200-fold using calmodulin affinity chromatography (361). The partially purified preparation is completely dependent on Ca^{2+} and calmodulin. Recently, purification of the enzyme has been completed (M. Kennedy, personal communication). The purified enzyme has a M_r of ~ 600,000 and contains both 50-kd and 60-kd subunits in approximately a 3:1 ratio. Both of these subunits are autophosphorylated by the kinase activity. Further studies are needed to determine whether such autophosphorylation is functionally significant. Elevation of cytoplasmic Ca^{2+} results in phosphorylation of protein I at the sites defined by both kinases (362), so there is strong evidence that both protein I kinases are in nerve terminals.

A Ca^{2+}- and calmodulin-dependent protein kinase that seems likely to be related to the calci-calmodulin-dependent protein I kinase has recently been purified 800-fold to homogeneity from rat brain cytosol, using myosin light chain as a substrate (360a). The purified enzyme contains multiple copies of a 49-kd subunit, has a total M_r of 500,000–600,000, and phosphorylates many synaptosomal proteins, including an 80-kd doublet that is probably protein I. This enzyme seems to be slightly smaller than the calci-calmodulin-dependent protein I kinase (M. Kennedy, personal communication) and may well be that enzyme stripped of its 60-kd subunits.

Further research is needed to determine how many additional kinases are required to phosphorylate the diversity of substrates that are detected in brain homogenates (356,357). At this time, it is not clear which kinases mediate phosphorylation of other known substrates such as tyrosine hydroxylase (358) or the Ca^{2+} transporter in Torpedo synaptic vesicles (147). In the future antibodies to each of the purified kinases should demonstrate the cellular and subcellular localization of these kinases in the nervous system.

Ca^{2+}, PHOSPHOLIPID, AND DIACYLCLYCEROL-ACTIVATED PROTEIN KINASE —During the past few years, a new class of kinase has been characterized in many tissues, which has an absolute requirement for Ca^{2+} and phospholipid, but is not activated by calmodulin (363–365). The enzyme is found in higher concentrations in brain than other tissues, but appears to be a virtually ubiquitous enzyme (363,366). It has been detected in virtually every tissue and species, vertebrate or invertebrate, in which it has been assayed

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(366). The spectra of proteins phosphorylated by the endogenous kinase in heart, brain, and other tissues are distinct from those phosphorylated by cAMP, cGMP, and Ca^{2+}-calmodulin-dependent kinases (356,357,364,365,367,368,371).

The Ca^{2+}, phospholipid-dependent protein kinase is found mostly in the cytosol (367,372) and requires unphysiological concentrations of Ca^{2+} for activity. Addition of diacylglycerol, however, reduces the binding constants for both phospholipid and Ca^{2+}, the latter to one μM (373). These experiments suggest that the enzyme is activated by phosphatidylinositol turnover. In the presence of diacylglycerol, the enzyme becomes associated with membranes and phosphorylates endogenous substrates (374). The binding of many different hormones and neurotransmitters to specific classes of receptors results in increased phosphatidylinositol turnover (375), so the enzyme may be an important mediator of hormone and transmitter action.

Studies on platelets have demonstrated that physiological mediators of platelet activation modulate the activity of this kinase in vivo. Thrombin activates both phosphatidylinositol turnover and phosphorylation of a 40-kd protein that is a specific substrate of this kinase in vitro (376,377). Parallel stimulation of diacylglycerol synthesis and phosphorylation of the 40-kd protein are seen in response to thrombin and phospholipase C. Parallel reductions in each occur in response to PGE_1, and cGMP, respectively. Tumor-promoting phorbol esters replace diacylglycerol in vitro and induce phosphorylation of the 40-kd protein in vivo without increasing diacylglycerol synthesis (378). The kinase is thus likely to mediate many of the effects on cell metabolism of this important class of regulators.

Recently, Ca^{2+}-influx into synaptosomes, induced by depolarization, has been shown to promote phosphorylation of an 87-kd neuronal substrate of this kinase (379). Pursuit of these studies on endogenous substrates should reveal the physiological conditions required for activation of this enzyme in different nerve terminals.

The Ca^{2+}, phospholipid-dependent protein kinase has been purified 15,000 times from bovine heart to near homogeneity and consists of one subunit with an M_r of 83,000–100,000, depending on the procedure used for determination of molecular weight (364). The purified enzyme transfers phosphate from ATP to serine residues and has an absolute requirement for Ca^{2+} and phospholipid. Addition of diacylglycerol reduces the K_m for Ca^{2+} sevenfold and the K_m for phosphatidylserine fourfold. The purified enzyme's spectrum of phosphorylation sites is distinct from those of Ca^{2+}-calmodulin and cAMP-dependent kinases. The size and properties of partially purified enzyme preparations from brain appear to be very similar (366,380).

The Ca^{2+}, phospholipid-dependent protein kinase contains two distinct functional domains, one with the kinase activity and the second with sites for Ca^{2+}, phospholipid and diacylglycerol binding. Separation of the two domains by proteolysis produces an active kinase (380) that is fully active without Ca^{2+}, phospholipid, or diacylglycerol (381). Calmodulin antagonists, such as trifluoperazine, inactivate the native enzyme but not the proteolytic fragment by competing with phospholipid, but not diacylglycerol (381). Tumor promoting phorbol esters activate the native enzyme by replacing diacylglycerol (378).

The structure of the enzyme has some similarities to enzymes that are regulated by calmodulin. Many of these, including brain phosphodiesterase and the ATP-dependent Ca^{2+} transporter, contain regulatory and catalytic domains that are separable by proteolysis (366,382). Both classes of enzyme are also directly inhibited by phenothiazines (383,384). It will be very interesting to sequence the Ca^{2+}-binding site on the Ca^{2+}, phospholipid-dependent kinase and determine whether it represents a domain that could have been derived from a Ca^{2+} binding site on calmodulin.

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Regulation by Protein Kinases

The role of protein phosphorylation in the control of cellular metabolism has recently been reviewed with special emphasis on glycogen metabolism (316). In this section, we discuss the regulation by protein phosphorylation of three sets of substrates that are found primarily in nerve terminals: enzymes that regulate transmitter biosynthesis; a protein of unknown function, protein I, that is specifically localized to synaptic vesicles; and ion channels that modify neuronal excitability and synaptic transmission.

REGULATION OF TRANSMITTER SYNTHESIS—Stimulation or depolarization of appropriate nerve terminals results in increased synthesis of acetylcholine, serotonin, and the catecholamines that is not dependent on depletion of release of transmitter stores (215b,385). Stimulation of transmitter synthesis can require Ca^{2+} entry or cAMP synthesis (215b,218, 315,386–388). Depolarization increases the activity of the rate-limiting enzyme in the pathway of biosynthesis for each of these transmitters, either the Na^{+}-dependent high affinity choline transporter (215b), tyrosine hydroxylase (215,387), or tryptophan hydroxylase (389). Activation of these enzymes provides one means of maintaining neurotransmitter levels. Since newly synthesized transmitters are in a pool that is preferentially released (390), stimulation of transmitter synthesis may be important for replacing readily releasable transmitter, even in situations where there is not significant depletion of bulk transmitter stores (391). Tyrosine hydroxylase in nerve terminals is activated by stimulation, depolarization, cAMP, and adenosine whether assayed in vivo or in extracts (215,216a,315,385–388,393,395).

Tyrosine hydroxylase in brain extracts can be activated by either a cAMP-dependent or calci-calmodulin-dependent protein kinase (218,315,358). Tyrosine hydroxylase in adrenal extracts appears to be activated only by the cAMP-dependent kinase (396). While both mechanisms activate the enzyme, the kinetic properties of the two activated enzyme preparations differ, notably in pH optima, suggesting that the different kinases phosphorylate different sites on the enzyme (315).

Purified tyrosine hydroxylase can be phosphorylated by cAMP-dependent kinase in vitro (217,397–399,401) and phosphorylation correlates with increased activity (217). There has been some disagreement over the changes in kinetic properties that result in increased activity (217,297), but more recent studies indicated that differences in assay conditions explain at least some of these observations (400). In appropriate assay conditions, phosphorylation reduces the K_m for pteridine cofactor, increases the V_{max}, and increases the K_i for catecholamines. All these changes are very sensitive to reaction pH (400). In physiological conditions, Lazar & Barchas (391) have argued that these changes will increase tyrosine - hydroxylase activity 8–33-fold.

Purified tyrosine hydroxlase can also be activated by a calci-calmodulin-dependent kinase (218,401). Activation requires a new activator protein in addition to the kinase. This activator protein has been purified and appears to consist of two 33-kd subunits (218). The activator protein has a wide distribution in the nervous system and also is necessary for calci-calmodulin-dependent kinase activation of tryptophan hydroxylase (218). Activation of tyrosine hydroxylase appears to proceed in two steps: phosphorylation of the enzyme precedes activation by the activator protein (401).

While these studies demonstrate that the activity of tyrosine hydroxylase is regulated by protein kinases, the phosphorylation of the enzyme in vivo has not been correlated with enzyme activation. In fact, the only published report of in vivo phosphorylation of tyrosine hydroxylase is in response to treatment of PC12 cells with NGF (402). In view of recent evidence suggesting that insulin and EGF receptors are protein kinases (403,404), NGF binding may result in

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phosphorylation and regulation of tyrosine hydroxylase by a third pathway, independent of the cAMP or Ca^{2+} -dependent kinases.

PHOSPHORYLATION OF PROTEIN I—One of the best-characterized synaptic proteins, protein I (synapsin I) has an unknown function but is phosphorylated during nerve terminal stimulation by depolarization or binding of certain neurotransmitters. Protein I consists of a globular head and a proline-rich tail that is sensitive to collagenase (405). Digestion with collagenase releases a protein I fragment from the membrane (406), so the major site of electrostatic interaction with vesicles appears to be in the tail. Protein I is phosphorylated at one serine residue in the collagenase-resistant head by a type II cAMP-dependent kinase (407,408). A Ca^{2+} dependent kinase in the cytosol also phosphorylates this site (342).

The collagenous tail of protein I is phosphorylated by at least two separate serine residues by a distinct Ca^{2+} -calmodulin-dependent kinase that is found in both particulate and soluble fractions of brain (342,362). Phosphorylation facilitates release of the membrane-bound protein I by salt extraction (409).

Phosphorylation of protein I responds rapidly to the initiation or cessation of orthograde nerve stimulation in nerve terminals in the posterior pituitary and sympathetic ganglion (410,411). Increased phosphorylation during stimulation requires external Ca^{2+} and results in phosphorylation of both the head and tail of the protein, implying activation of both Ca^{2+}-dependent kinases. The protein I in nerve terminals is also phosphorylated in response to cAMP or transmitters, such as dopamine and serotonin, that increase cAMP synthesis (410–412). Only the cAMP kinase-sensitive serine in the globular head is phosphorylated in response to these agents.

Protein I appears to be a synaptic vesicle-associated phosphoprotein (413,414). Much of the protein is clearly bound to vesicles, since it copurifies with them in vitro (409) and is found associated with vesicles in immunocytochemical examination of nerve terminals (414,415). In vivo transport of protein I occurs more slowly than that of the fastest moving elements (26), which in other systems include synaptic vesicles. Slower transport would be expected if protein I in vivo is in equilibrium between vesicle-bound and soluble pools. Since phosphorylation facilitates release of vesicle-bound protein I in vitro (409), it may also do so in vivo. Alternatively, phosphorylation may change the affinity of protein I for other proteins or organelles without reducing the binding of protein I to vesicles. The affinity of vesicles for other organelles, such as the plasma-lemma and cytoskeleton, might then be altered to change the efficiency of vesicle mobilization or fusion.

REGULATION OF NEURONAL EXCITABILITY OF PHOSPHORYLATION OF POTASSIUM CHANNELS—In an earlier section, we discussed the modification of K^+ channels by phosphorylation, and how this phosphorylation affected the probability of channel opening. We end this chapter with a summary of some cases in which such phosphorylation of nerve terminal K^+ channels appears to control behavior. (See also 355.)

Aplysia neuron R_{15} is a large neurosecretory cell, which is believed to be a peptidergic neuron (416,417). The neuron has a slow oscillation in its transmembrane potential that induces bursts of action potentials during the depolarizing phase of the cycle. The oscillations are clearly due to fluctuations in the comparative activities of Ca^{2+} and K^+ channels. Slow inward Ca^{2+} current depolarizes the cell, raising the internal Ca^{2+} concentrations. There is disagreement over whether this internal Ca^{2+} modifies the current flow by inactivating the Ca^{2+} channel or activating the Ca^{2+}-dependent K^+ channel (W. Adams, personal communication). In either case, K^+ currents become a large fraction of the total current and repolarize the cell. When internal Ca^{2+} is reduced, Ca^{2+} entry again exceeds K^+-efflux and the cycle repeats. Action

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potentials are controlled by the classical Na^+, Ca^{2+}, and delayed K^+ voltage-sensitive channels. The period between bursts can be shortened or lengthened by excitatory or inhibitory hormones and neurotransmitters. Application of serotonin, an inhibitor, increases the interburst period and, in sufficient quantities, suppresses bursting altogether by increasing the conductance of a K^+ channel (418). R_{15} membranes contain a serotonin receptor that activates an adenylate cyclase (419). The binding of serotonin results in increased levels of intracellular cAMP (420), protein phosphorylation (421), and K^+ conductance. cAMP analogs also increase the K^+ conductance, and inhibitors of cyclic nucleotide phosphodiesterase potentiate the effect of suboptimal concentrations of serotonin (418). So the effect of serotonin on K^+ conductance appears to reflect activation of a serotonin-dependent adenylate cyclase. Indeed, direct injection into R_{15} of cAMP analogs or an activator of adenylate cyclase, guanylphosphoiminodiphosphate, mimics the effect of serotonin (422,423). Intracellular injection of a specific inhibitor of cAMP-dependent protein kinases, the Walsh inhibitor, prevents the response of R_{15} cells to serotonin but not to transmitters acting by other mechanisms (46), providing evidence that phosphorylation by the cAMP-dependent kinase is required to activate the K^+ channel. The affected channel appears to be a channel that opens near rest (46).

Uncertain at this time is the target of kinase action. Lemos et al (421) have injected [^{32}P]ATP into R_{15} and shown that it is retained within the cell. Application of serotonin results in increased phosphorylation of at least four proteins (M_rs of 230,000, 205,000, 135,000, and 26,000) and decreased phosphorylation of at least one (M_r 43,000). The minute amounts of protein available from single cells make it a formidable problem to relate the phosphorylation of individual proteins to particular functional changes.

Modulation of transmitter release by sensory neurons at synapses on motoneurons regulates a monosynaptic gill-withdrawal reflex in *Aplysia* (44). Behavioral changes in gill withdrawal by *Aplysia* have been traced to such modulation. Habituation of the withdrawal reflex to repeated mild sensory stimuli is caused by reduced Ca^{2+}-influx into the sensory neuron terminals (123). The habituated response can be reversed, or "sensitized," by a painful sensory stimulus. The reflex can also be potentiated by a paired stimulus in a classical conditioning training session (424,425). Sensitization involves an increased Ca^{2+}-influx caused by a reduced K^+ conductance, prolonging the duration of the action potential (130). Sensitization occurs because the painful stimulus causes the release of serotonin by a third neuron onto the sensory neuron terminals (426). Serotonin acts by increasing cAMP synthesis, cAMP-dependent protein kinase activity, and phosphorylation of a protein that results in closing a novel K^+ channel. This K^+ channel is a major channel in *Aplysia* sensory neurons and has been studied by patch clamping (40). These channels are distinguished from the rapid (A), delayed (K), and Ca^{2+}-dependent (C) K^+ channels that are also present in these neurons (427). The serotonin-sensitive channels are not regulated by voltage or internal Ca^{2+}. Both cAMP and serotonin reduce almost to zero the probability of channel opening (40). Intracellular injection of the catalytic subunit of cAMP-dependent protein kinase mimics the effect of serotonin and closes the channels (339). Injection of a specific inhibitor of the cAMP-dependent kinase, the Walsh inhibitor, blocks the effects of cAMP, serotonin, and stimulation of the modulator neurons (428).

Both the strength and duration of the Sensitization correlate with increased levels of cAMP in dissected sensory neuron cell somas (429). Brief stimulation of the modulator neurons or application of serotonin result in elevated cAMP levels that persist for 20 min and correlate with closure of the K^+ channel. Injection of the Walsh inhibitor during this interval reverses the effect of elevated cAMP (428), so phosphate is rapidly turned over on the regulatory site of the protein that closes the K^+ channel. The persistance of sensitization thus requires elevated cAMP, which persists long after the stimulus. [Short-term sensitization can be converted to a

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more permanent increase in efficiency of the same synapse, lasting weeks, by repeated painful sensory stimuli, but the mechanism is not known (44).]

Another dramatic example of changes in neuronal behavior induced by phosphorylation-dependent channel modification is provided by studies on *Aplysia* bag cells (430). Brief stimulation of the innervating nerve generates a burst of action potentials that cause release of an egg-laying hormone. Cyclic AMP plays an important role in generating this response. Cyclic AMP accumulates in the bag cells during the first few minutes of stimulation (431). Addition of cAMP analogs can actually generate bursts of action potentials in the bag cells without electrical stimulation (431). During the burst of action potentials generated by stimulating the nerve, a brief period (< 1 min) of sodium action potentials is followed by 30–40 min of calcium action potentials (432). Cyclic AMP accumulates during the first few minutes of the Ca^{2+} phase of action potentials, but then returns to control levels (431). During the period of elevated cAMP levels, the width of each spike increases (430), because cAMP causes the closure of a K^+-channel (41,69). The K^+ channel that is closed by cAMP action appears likely to be a Ca^{2+}-dependent K^+ channel (69). Microinjection of the catalytic subunit of cAMP-dependent protein kinase also decreases the K^+ conductance (47), suggesting that closure is mediated by phosphorylation.

Activity in bag cells correlates with the level of phosphorylation of specific proteins, measured either by preloading cells with $^{32}P_i$ or labeling extracted proteins with [^{32}P]ATP and the catalytic subunit of the cAMP-dependent protein kinase (338). In the latter case, increased phosphorylation in vivo is revealed as reduced incorporation of ^{32}P in vitro. The phosphorylation of one protein (M_r 33,000) is increased at both early and late times during the discharge. The phosphorylation of a second protein (M_r 21,000) remains low at 2 min, but is dramatically increased after 20 min of discharge. The latter is a bag cell–specific, membrane-associated protein and a prominent substrate of cAMP-dependent phosphorylation in vitro. Since cAMP levels are low at 20 min, phosphorylation in vivo seems more likely to be regulated by a different, perhaps Ca^{2+}-dependent kinase.

The examples of R_{15}, sensory neurons, and bag cells are a few examples of how cells containing different ratios of the basic channel types can have dramatically different resting behavior. These examples also indicate that the cAMP-dependent kinase modifies different channels in different cells. Attention naturally has turned to the possibility that the small phosphorylated proteins, apparently different in each neuron, may act as cell-specific channel modifiers. A possible paradigm is provided by the phosphorylation-dependent activation of Ca^{2+} transport by the heart-specific protein phospholamban (135,135a). Whatever the target, these examples provide a powerful demonstration of the functional importance of ion channels and protein kinases in controlling the behavior of cells, synapses, and animals.

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