Protein kinases and neuronal excitability

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Like most cells, neurons are regulated by intracellular second messengers formed in response to extracellular signals. Among the components of a neuron that are subject to regulation are its complement of ion channels, which determine the neuron's intrinsic electrical properties. Modulation of these electrical properties influences both the responsiveness of neurons and their capacity to signal to other cells. Also subject to regulation by second messengers are neurotransmitter receptors, proteins that determine the amount of neurotransmitter release, components of the neuronal cytoskeleton, and elements that control gene expression in neurons, including ion channel genes. Regulation by second messengers constitutes a fundamental mechanism that allows neurons to control long-lasting changes in behavior and to change patterns of behaviors in response to sensory input. This is perhaps most apparent in cases where deficits in the brain levels of neurotransmitters, such as dopamine or serotonin, which elicit second messenger production in neurons, result in profound psychopathology, including schizophrenia, anxiety, and depression.

The majority of known second messenger pathways are linked to the activation of protein kinases, enzymes that transfer phosphate groups, from the γ-phosphoryl-phosphoryl group of ATP, to serine, threonine, or tyrosine residues on substrate proteins such as ion channels. The biochemical and conformational changes produced in an ion channel by phosphorylation changes its function and directly impacts neuronal excitability. The two enzymes that have received the most attention for their effects on excitability are cyclic AMP-dependent protein kinase (PKA), which is activated by neurotransmitters that elevate intracellular cyclic AMP (cAMP) levels, and protein kinase C (PKC), which is usually activated by neurotransmitters that stimulate the intracellular formation of 1,2-diacylglycerols. Both PKA and PKC are considered serine/threonine kinases because they phosphorylate these particular amino acids on substrate proteins. Other serine/threonine kinases that have been prominently implicated in ion channel regulation and the control of excitability include Ca^{2+}/calmodulin kinase, cyclic GMP-dependent protein kinase, and casein kinase 2. Kinases that transfer phosphate groups onto tyrosine residues, such as the proto-oncogene *src* or receptor tyrosine kinases like the insulin receptor, can also produce rapid changes in excitability and signalling by altering ion channel function. The *removal* of phosphate from substrate proteins is accomplished by a group of enzymes called protein phosphatases, which often work with kinases for homeostatic control of ion channels.

Over the past twenty years molecular biology has revealed the amino acid sequence of many different types of ion channels. The cloning of ion channel genes, which began with the *Drosophila* K^+channel genes, *Shaker*, *Shab*, *Shaw*, *Shal*, and *Slo*, has allowed the identification of residues in these proteins that are necessary for modulation to take place. Although the function of all of these sites is not yet known, evidence obtained by mutating various residues indicates that phosphorylation at specific sites influences a number of ion channel functions, including responsiveness to membrane potential, the rate and extent of activation or inactivation, as well as the action of other second messengers, such as Ca^{2+}, on channels. Figure 1 shows a schematic of the α-subunit of a typical ion channel, the $K_v3.1$ Shaw family K^+ channel, with the location of key amino acid residues at consensus substrate sites for kinase-dependent protein phosphorylation. A consensus site for phosphorylation is a short stretch of protein which contains the target residue for phosphorylation and is recognized by the kinase according to adjacent amino acids. For example, a general consensus site for PKA phosphorylation is a pair of basic amino acids (arginine or lysine), followed by any amino acid, and ending in a serine or threonine.

One of the first protein kinases implicated in the regulation of ion channel function was in fact PKA. PKA is a complex of two regulatory and two catalytic subunits which separate following the binding of cAMP to the regulatory subunits, thereby freeing the catalytic subunits to phosphorylate substrates. Under physiological conditions, PKA is activated by hormones or transmitters that stimulate cAMP production within a neuron; however, the enzyme may be stimulated directly by the application of membrane permeant cAMP analogues, such as 8-bromo-cAMP and 8-(4-chlorophenylthio)-cAMP, or indirectly by introducing chemicals such as 3-isobutyl-1-methylxanthine (IBMX) or theophylline, which block the enzymatic breakdown of endogenous cAMP by phosphodiesterases.

A well-studied example of ion channel regulation by PKA is the inhibition of a of K^+ channel, known as the S channel, in the sensory neurons of the marine mollusc, Aplysia californica. The "S" refers to serotonin, a neurotransmitter that elevates cAMP levels and activates PKA in the sensory neurons of this snail. Phosphorylation of the S channel by PKA causes the channel to remain closed for long periods of time without effecting its unitary conductance. Inhibition can be observed by directly applying the catalytic subunit of PKA to the cytoplasmic face of an S channel in cell-free patches excised from a sensory neuron. The S channel is weakly voltage-dependent and is essentially a resting conductance that helps to determine sensory neuron responsiveness. When PKA is activated in an intact sensory neuron with cAMP analogues or by serotonin, it inhibits the channel and makes the neuron less leaky to current flow across its membrane. The net effect of S channel closure is enhanced excitability and an increased likelihood of generating action potentials following excitatory synaptic input.

The second general protein kinase that is a potent regulator of voltage-dependent and Ca^{2+}-dependent ion channels in neurons is PKC. This kinase is a single protein molecule consisting of a regulatory domain and a catalytic domain. In the presence of Ca^{2+} and phosphatidyl serine, a normal constituent of plasma membranes, this enzyme is activated by 1,2-diacylglycerols. The Ca^{2+}, phosphatidyl serine, and diacylglycerol all bind to the regulatory domain which causes a conformational change that reveals the catalytic domain for phosphorylation of substrate. Diacylglycerols are formed in cells in response to synaptic or hormonal stimuli that trigger the hydrolysis of membrane phosphoinositides, usually by activation of the enzyme phospholipase C. In reality, there exists a family of protein kinase C enzymes which differ from each other in their sensitivity to intracellular Ca^{2+} levels and fatty acids. There are a number of useful and potent agents that activate PKC pharmacologically, including phorbol esters, such as 12-O-tetradecanoylphorbol-13-acetate (TPA) and phorbol-12,13-dibutyrate (PDBu), as well as synthetic diacylglycerols such as 1-oleoyl-2-acetyl-glycerol (OAG) and 1,2-dioctenyl glycerol (DiC_8).

Activators of PKC commonly increase spontaneous and evoked release of neurotransmitter from neurons. In large part, this effect may result from changes in the secretory machinery of the synapse. Thus, these activators can sometimes increase the amount of release observed in response to a fixed elevation of Ca^{2+} levels in the cytoplasm. Nevertheless, the activators also influence ion channels near release sites. One clear example of this is found in the bag cell neurons of *Aplysia*, in which the activation of PKC may contribute to the rapid remodeling of neuropeptide release sites. These neurons trigger a prolonged sequence of reproductive behaviors by undergoing a transition from a resting state to a state during which the neurons fire repetitively for approximately 30 minutes. During this period of spontaneous firing, the Ca^{2+} component of action potentials in enhanced. Such an enhancement of Ca^{2+} action potentials can be mimicked in isolated bag cell neurons by application of phorbol esters or synthetic diacylglycerols, as well as by microinjection of PKC itself. Voltage-clamp and patch clamp experiments have disclosed that the enhancement of voltage-dependent Ca^{2+} channels occurs through the unmasking of a "covert" species of Ca^{2+} channel that is not detected in cells in which PKC has not been activated. The unmasked Ca^{2+} channel differs from the voltage-dependent Ca^{2+} channel that is observed in unactivated cells in its unitary conductance and its spatial distribution. Of the two Ca^{2+} channels recently cloned from the bag cell neurons, one of the α-subunits, known as the 1a-type, is found in intracellular granules. This may represent a pool of covert Ca^{2+} channels that could be recruited to the membrane through a PKC-dependent mechanism. In support of this, the use of a Ca^{2+} indicator dye in isolated bag cell neurons has shown that activators of PKC produce a rapid expansion of the terminals where neuropeptide release is believed to occur, and that the new Ca^{2+} channels are selectively recruited to these expanded terminal sites. Such restructuring of release sites may contribute to the progressive potentiation of peptide release that occurs over the first 10 minutes of an afterdischarge in the bag cells neurons.

PKC can also regulate the function of ion channels already active at the membrane. For example, PKC-dependent phosphorylation can modulate the type IIA voltage-dependent Na^+channel which is responsible for the up stroke of the action potential in the vertebrate brain. One of the intracellular loops in the α-subunit of this protein, that located between the third and fourth domains, plays an important role in the rate at which the channel inactivates during a depolarization. Phosphorylation of a site in this domain by PKC slows Na^+current inactivation and reduces the peak current. Changes in Na^+channel inactivation can be brought about by applying PKC directly to the inner surface of channels in cell-free patches. Interestingly, the Na^+channel can also be regulated by PKA; specifically, it is inhibited by phosphorylation at PKA phosphorylation consensus sites found on an intracellular loop joining domains one and two of the α-subunit. The effect of PKA, which is only on peak current and not inactivation, can also be achieved by application of PKA catalytic subunit to the inner surface of Na^+channels. During the recording of currents from intact cells expressing the Na^+channel gene, it was found that PKA activation failed to inhibit the Na^+current unless PKC had already been triggered first. Thus, inhibition by PKA requires concomitant phosphorylation by PKC, suggesting there exists a degree of "cross-talk" between the two kinases. Convergence or dependence of one kinase on the action of another provides an integration mechanism and could act as a molecular coincidence detector for the arrival of different inputs. Modulation of Na^+channel properties by PKA and/or PKC would significantly change the threshold for action potential generation and alter the ability of a neuron to integrate synaptic inputs.

A different serine/threonine kinase that has been more recently recognized as capable of modulating ion channel function is casein kinase 2. Casein kinase 2 is widely expressed in the nervous system and appears to be active under physiological conditions. Thus, its targets would most likely be constitutively phosphorylated. For the K_v3.1K+channel, constitutive phosphorylation by casein kinase 2 determines the basal features of the current carried by this channel. Figure 1 has already shown that there are a number of consensus sites for both PKC and casein kinase 2 phosphorylation on K_v3.1. Normally, K_v3.1is constitutively phosphorylated, suggesting that one of these kinases is continuously transferring phosphate to the protein. Treatment of cells expressing K_v3.1with H7 (1-(5-isoquinolinylsulfonyl)-2-methlypiperazine), a general inhibitor of PKC, does not alter whole cell current; however, Figure 2 shows that exposing the same cells to DRB (5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole), a specific inhibitor of casein kinase 2, results in far less current and greater inactivation, which is caused by a shift in the channel's voltage-dependence to more negative potentials. This indicates that the constitutive phosphorylation is due to casein kinase 2, as opposed to PKC, and following inhibition of casein kinase 2 the channel is de-phosphorylated by an as yet unidentified phosphatase. K_v3.1currents show fast activation, fast de-activation, and little use-dependent inactivation, which enables neurons expressing this channel to both rapidly repolarize following an action potential up stroke as well as fire action potentials at high frequency. When such neurons are treated with DRB they fail to generate high frequency output, suggesting that constitutive phosphorylation by casein kinase 2 is crucial in setting these membrane properties.

New and diverse types of protein kinases continue to be discovered and characterized. Much of the impetus for this work has been the unraveling of signalling pathways that transduce the effects of growth factors into changes in gene expression required for cell division and growth in non-neuronal cells. Many of these kinases transfer phosphate onto tyrosine residues on substrate proteins. Such enzymes fall into two groups: receptor tyrosine kinases exemplified by the insulin and epidermal growth factor (EGF) receptors, and non-receptor tyrosine kinases such as the extracellular response kinases (EFKs) and the product of the proto-oncogene src. For receptor tyrosine kinases, which are transmembrane proteins, the binding of a ligand, usually a growth factor, causes the protein to autophosphorylate itself on tyrosine residues in its cytoplasmic domain. Such autophosphorylation allows the receptor to bind to and activate a number of other cytoplasmic enzymes. In contrast, the non-receptor kinases are cytoplasmic enzymes that must be activated indirectly by external stimuli. This is frequently achieved by a cascade of phosphorylation events following the activation of a membrane receptor. In addition, it has been found that signalling may occur by the activation of both receptor and non-receptor phospho-tyrosine protein phosphatases, which catalyze the removal of phosphate from tyrosine residues of substrate proteins.

Several independent lines of evidence indicate that activation of tyrosine kinase signalling pathways may produce rapid changes in the electrical properties of neurons. An example of this is provided by the K_V1.3 and K_V1.5 members of the *Shaker* subfamily of voltage-dependent K^+channels. K_V1.3 is phosphorylated on tyrosine residues when it is co-expressed in cell lines with the EGF receptor. Application of EGF to these cells not only

enhances phosphorylation of tyrosine on the channel but also inhibits $K_V1.3$-mediated K^+currents. Furthermore, similar work showed that $K_V1.5$, is regulated by the non-receptor tyrosine kinase, *src*, which also appears to inhibit this current through direct tyrosine phosphorylation. Notably, it is possible to co-immunoprecipitate $K_V1.5$ with *src* and vice versa, indicating that the kinase and channel are in close physical association. In a similar fashion, the related tyrosine kinase, *fyn*, was found to be closely-associated with $K_V1.5$ in the hippocampus, which has implications for influencing neuronal excitability. Finally, it must be mentioned that a large body of pharmacological and biochemical evidence also indicates that tyrosine kinases, in particular *src*, associate with and influence the activity of the N-methyl-D-aspartate (NMDA) class of glutamate receptors.

The example of $K_V1.5$ being closely-associated with tyrosine kinases points to an emerging theme in the control of ion channels and excitability, namely, the physical grouping of kinases together with channels into signalling complexes. In certain cases, this physical link between ion channel and enzyme is sufficient to maintain kinase-dependent modulation in cell-free patches or following reconstitution into bilayers. Co-localizing kinases with ion channels is achieved by various protein-protein interactions, both directly between kinase and channel (see above) as well as through scaffolding proteins that organize multiple enzyme-channel interactions. For example, the AKAP (cAMP-dependent protein kinase-anchoring protein) family of scaffolding proteins are capable of organizing and targeting several different kinases, including PKA and PKC, as well as phosphatases to the plasma membrane.

The first report of a functional complex between an ion channel and a kinase was that of biochemically co-purified rat-brain large conductance Ca^{2+}-activated K^+channels and PKC. When these channels were reconstituted into bilayers, the addition of ATP, or a non-hydrolyzable analog, to their cytoplasmic face increased the open probability. The effect of ATP appears to be mediated by PKC as it is inhibited by a selective peptide inhibitor of this enzyme. Similar findings have been made for the association of either PKA or PKC with several types of channels, for example, a recent report using rat neurons provided evidence for a large signalling complex between the Cav 1.2 L-type Ca^{2+} channel and the b2 adrenergic receptor, adenylate cyclase, PKA, and PP2A protein phosphatase.

A cation channel whose activity may underlie the prolonged afterdischarge of the bag cell neurons provides an example of how a complex of many enzymes with an ion channel can diversely regulate its behavior. In excised patch clamp recordings, this channel displays two different patterns of gating (Figure 3, A); in the first, termed the burster mode, periods of rapid opening and closing are separated in time by periods of closure lasting for up to several minutes, while in the second, termed the high activity mode, there are no prolonged closures. Activation of a endogenous tyrosine phosphatase that is associated with the cytoplasmic side of the cation channel converts burster mode channels into high activity mode channels. Activation of the channel-associated tyrosine phosphatase is believed to occur on synaptic stimulation of the bag cell neurons. The resultant increase in the opening of the cation channel would be expected to produce a prolonged depolarization, leading to afterdischarge of these neurons. Along with mode-switching, it has been established that the cation channel is regulated by a closely-associated PKC. Similar to the case of Ca^{2+}-activated K^+channels, application of ATP to the cytoplasmic face of the cation channel in excised patches results in an increase in open probability (Figure 3, B), which is prevented by both organic and peptide inhibitors of PKC. The actions of PKC can be reversed by a similarly associated protein phosphatase. PKC activity is upregulated at the onset of that afterdischarge and its effects on the cation channel would be expected to maintain depolarization during this period of repetitive firing.

The cation channel signalling complex is extensive, and includes a serine-threonine kinase, PKC, a serine-threonine phosphatase, and a tyrosine phosphatase; furthermore, there are indications that a Ca^{2+}-sensing mechanism, involved in initiating a period of inexcitability following the afterdischarge, is also in proximity to the cation channel. A recent set of experiments has attempted to dissect the molecular basis of this signalling complex by using different domains and peptide motifs meant to interfere with the association between the cation channel and PKC in excised patches. Modulation of the channel by PKC could be disrupted by applying either the src homology 3 (SH3) protein-protein interaction domain or a src SH3 motif peptide. Presumably, this is due to the disruption of protein-protein interactions between the cation channel and PKC required to position the kinase for successful phosphorylation of the channel. In the bag cell neurons, several distinct protein-protein interactions may be required to bring together the enzymes that regulate the cation channel and influence excitability. Overall, by bringing together all of the relevant protein components, it seems that signalling complexes are a common mechanism for efficiently orchestrating ion channel modulation and controlling excitability.

The acute modulation of ion channels and receptors by protein kinases allows a cell to alter its electrical properties for a short while following synaptic stimulation. At the same time, such stimulation may also alter the phosphorylation state of other cellular constituents such as cytoskeletal proteins and nuclear transcription factors. The challenge now is to determine all of the kinases associated with a particular ion channel, functionally and physically, as well as how those kinases potentially act together in coordinating short-term and long-term changes in neuronal excitability.

1. See also

Signal transduction in neurons by phospholipid breakdown products
Signal transduction, role of heterotrimeric GTP-regulatory proteins [Classic paper]

2. Further reading

Byrne JH, Kandel ER (1996): Presynaptic facilitation revisited: State and time dependence. *J Neurosci* 16:425-435 [MEDLINE]

Chung S, Reinhart PH, Martin BL, Brautigan D, Levitan IB (1991): Protein kinase activity closely associated with a reconstituted calcium-activated potassium channel. *Science* 253:560-562 [MEDLINE]

Davare MA, Avdonin V, Hall DD, Peden EM, et al. (2001): A ß$_2$ adrenergic receptor singalling complex assembled with the Ca^{2+} channel $Ca_v1.2$. *Science* 293:98-101 [MEDLINE]

Holmes TC, Fadool DA, Ren R, Levitan IB (1996): Association of Src tyrosine kinase with a human potassium channel mediated by SH3 domain. *Nature* 274:2089-2091 [MEDLINE]

Knox RJ, Quattrocki EA, Connor JA, Kaczmarek LK (1992): Recruitment of calcium channels during rapid formation of putative neuropeptide release sites in isolated *Aplysia* neurons. *Neuron* 8:883-889 [MEDLINE]

Li M, West JW, Numann R, Murphy BJ, et al. (1993): Convergent regulation of sodium channels by protein kinase C and cAMP-dependent protein kinase. *Science* 261:1439-1442 [MEDLINE]

Macica CM, Kaczmarek LK (2001): Casein kinase 2 determines the voltage dependence of the $K_V3.1$ channel in auditory neurons and transfected cells. *J Neurosci* 21:1160-1168 [MEDLINE]

Wilson GF, Kaczmarek LK (1993): Mode-switching of a voltage-gated cation channel is mediated by a protein kinase A-regulated tyrosine phosphatase. *Nature* 366:433-438 [MEDLINE]

Wilson GF, Magoski NS, Kaczmarek LK (1998): Modulation of a calcium-sensitive nonspecific cation channel by closely associated protein kinase and phosphatase activities.*Proc Natl Acad Sci U S A.* 95:10938-10943 [MEDLINE]

Yu X-M, Askalan R, Keil GJ, Salter MW (1997): NMDA channel regulation by channel-associated protein tyrosine kinases Src. *Science* 275:674-678 [MEDLINE]

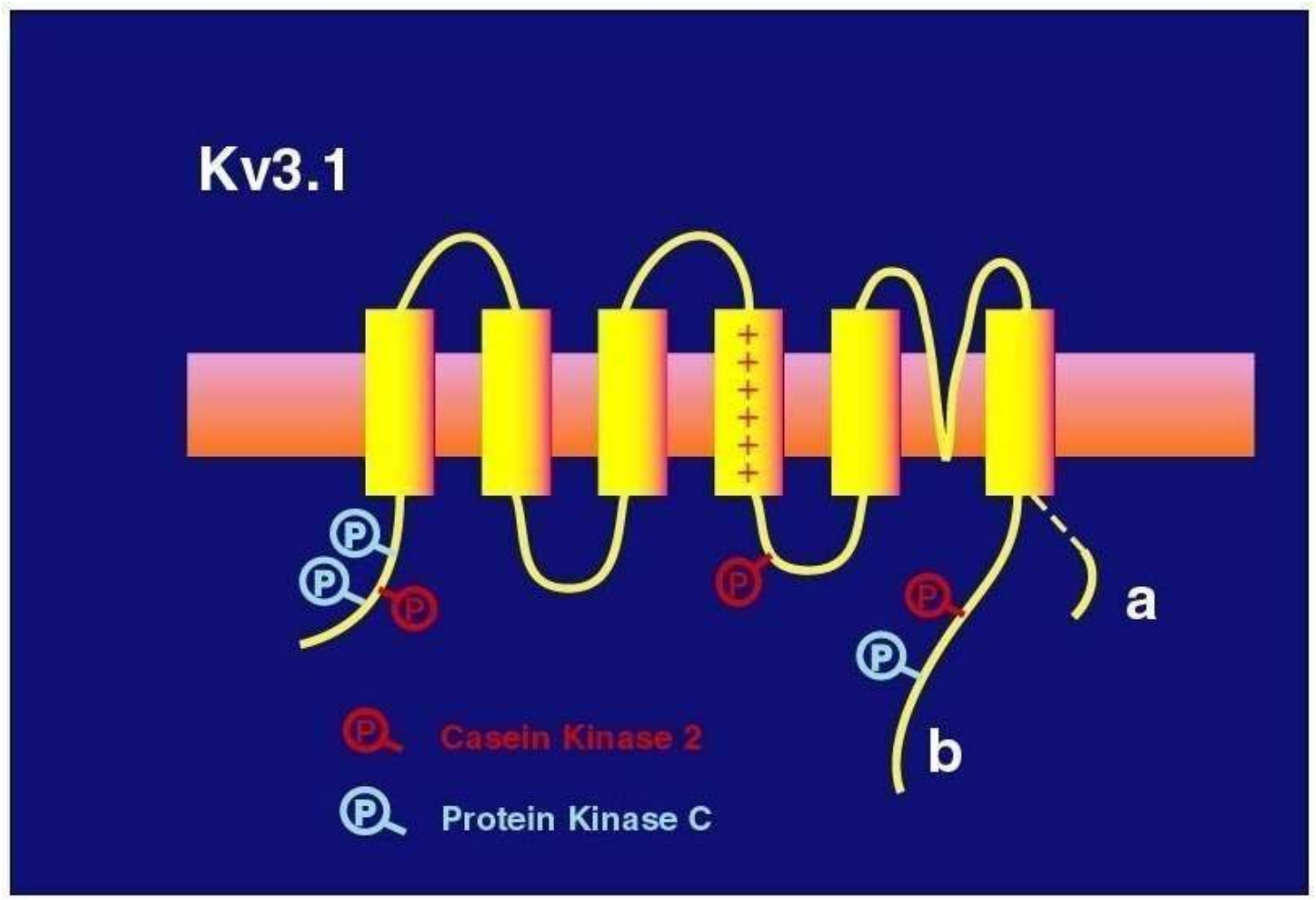

Figure 1. Schematic diagram of the α-subunit for the voltage-dependent, delayed-rectifier Shaw K^+channel, $K_v3.1$. The α-subunit has six transmembrane segments, the fourth of which is charged (indicated by the "+" symbols) to convey sensitivity to membrane potential, while a loop between segments five and six forms the ion conduction pathway. Most cloned K^+channels and non-selective cation channels are tetramers of four such assembled α-subunits, while $Na^+$and Ca^{2+}channels appear to be four K^+channel-like domains linked together into one large α-subunit or monomer protein. For the $K_v3.1$ α-subunit, the location of potential consensus sites for protein phosphorylation by kinases at key serine or threonines amino acid is given.

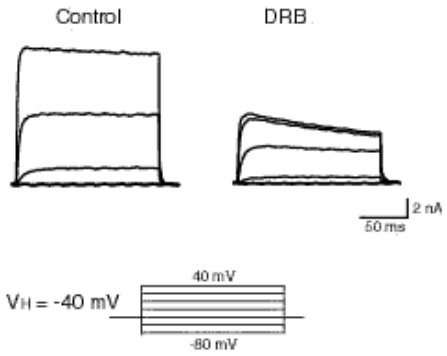

Figure 2. Regulation of K^+channel function by constitutive casein kinase 2 phosphorylation. *Left*, Control macroscopic K^+currents recorded from a cell line expressing the $K_v3.1$ α-subunit. The current sweeps are in response to successively more positive changes to the cell's membrane potential under voltage clamp. *Right*, The same cell following application of the casein kinase 2 inhibitor, DRB. The inhibition of casein kinase 2, and the subsequent de-phosphorylation by endogenous phosphatases, causes both a reduction of the total current and an increase in the rate of inactivation, i.e, an enhanced decline of the current during the depolarization, particularly at more positive potentials. Reproduced from the *Journal of Neuroscience* with permission.

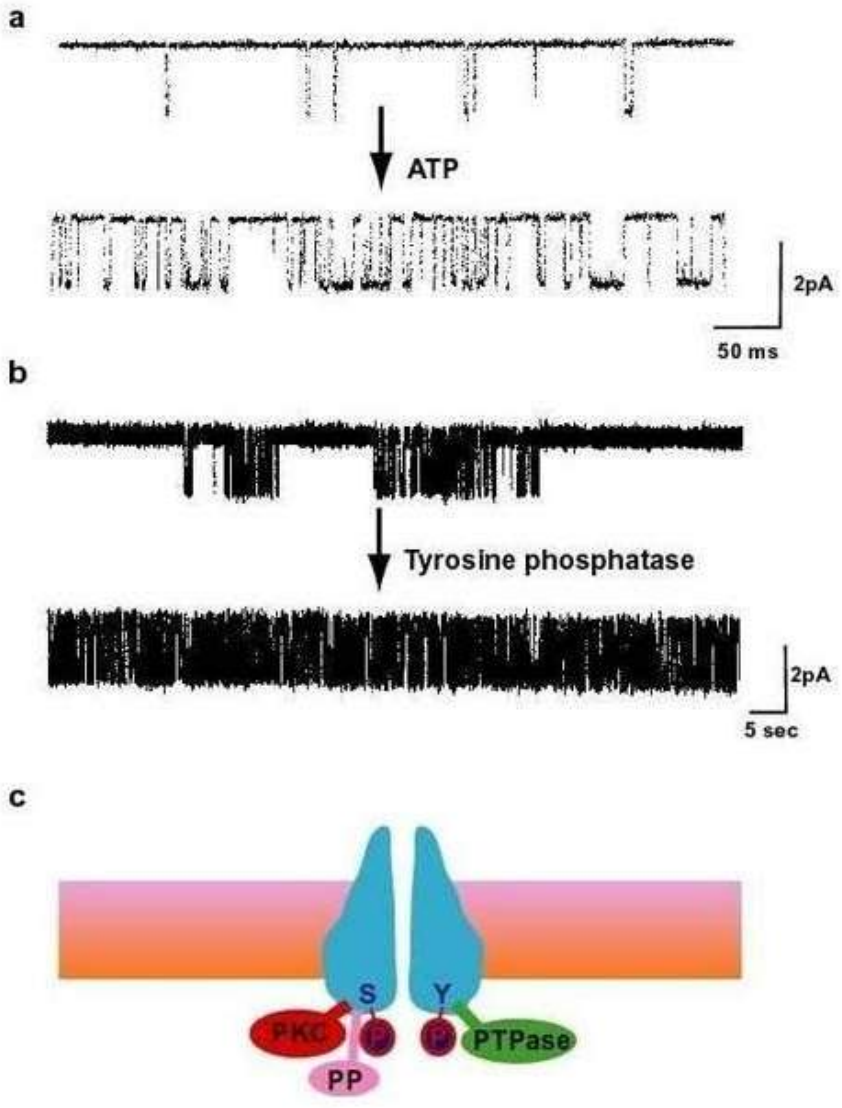

Figure 3. A, The effect of tyrosine phosphorylation on the gating of cation channels from the bag cell neurons of *Aplysia*, recorded in cell-free patches. The activity of channels in the burster mode is characterized by prolonged closures that may each last from several seconds to minutes. Channel opening is seen as downward deflections away from the closed state represented by the baseline at the top. Activation of a tyrosine phosphatase closely associated with the channel creates a high activity channel by eliminating the prolonged closures. Reproduced from *Nature* with permission. *B*, A different high activity mode cation channel in a cell-free patch. When ATP is applied to the inner surface of the channel, the number of openings goes up and the channel spends less time in the closed state. Rather than acting on the channel directly, ATP is actually used as a phosphate source by a closely associated PKC to phosphorylate the channel and enhance activity. *C*, Conceptualized depiction of the cation channel with associated PKC, serine-threonine phosphatase, and tyrosine phosphatase.