

Modulating Modulation: Crosstalk Between Regulatory Pathways of Presynaptic Calcium Channels

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The central nervous system expresses a range of different voltage-gated calcium channels (termed N-, P/Q-, L-, R- and T-types), each with unique subcellular distributions and specialized functions. The N- and P/Q-types are largely found in presynaptic nerve terminals where they are both physically and functionally involved in neurotransmitter release. The modulation of N- and P/Q-type channels by a variety of G protein-coupled receptors and associated intracellular messenger cascades is consequently a key mechanism for regulating synaptic transmission. The activation of presynaptic calcium channels is inhibited by direct interactions with G protein $\beta\gamma$ ($G_{\beta\gamma}$) subunits (1, 2). This phenomenon was first described more than two decades ago (3), but it was not until more recently that we have begun to understand the underlying molecular details, and to appreciate its complex nature (for review, see 4). We now know that $G_{\beta\gamma}$ subunits bind to the cytoplasmic-region linking domains I and II of the N- and P/Q-type calcium channel α_1 subunits (5, 6) (Figure 1), that N- and P/Q-type channels are differentially regulated by distinct types of G_{β} subunits (7–9), and that calcium channel subunit composition regulates G protein efficacy (10, 11).

In contrast to the action of $G_{\beta\gamma}$ subunits, the activation of protein kinase C (PKC) through either the metabotropic glutamate receptor-subtype 1 (mGluR1) or by application of phorbol esters mediates an increase in N-type current activity in *in vitro* heterologous expression systems and in neurons (6, 12, 13). A number of additional protein kinases including cGMP-dependent protein kinase (14) and mitogen-activated protein (MAP) kinases (15) also modulate N-type and P/Q-type channel activity. One unique aspect of the action of PKC is that phosphorylation of a single residue, Thr⁴²², in the linker region between domains I and II of the N-type calcium channel, antagonizes subsequent G protein-dependent inhibition of the channel (6, 12, 16, 17). The crosstalk that occurs between the G protein and PKC pathways is dependent on the nature of the G_{β} subunit isoform (18) and may account for the observation that the channel inhibition mediated by different types of G protein-coupled receptors varies in PKC sensitivity (12, 18).

In addition to second messenger control, presynaptic calcium channel activity is regulated by interaction with synaptic vesicle release proteins. For example, the binding of syntaxin-1A to the domain II-III linker of the N- or P/Q-type calcium channels reduces the availability of the channel for opening (19–23).

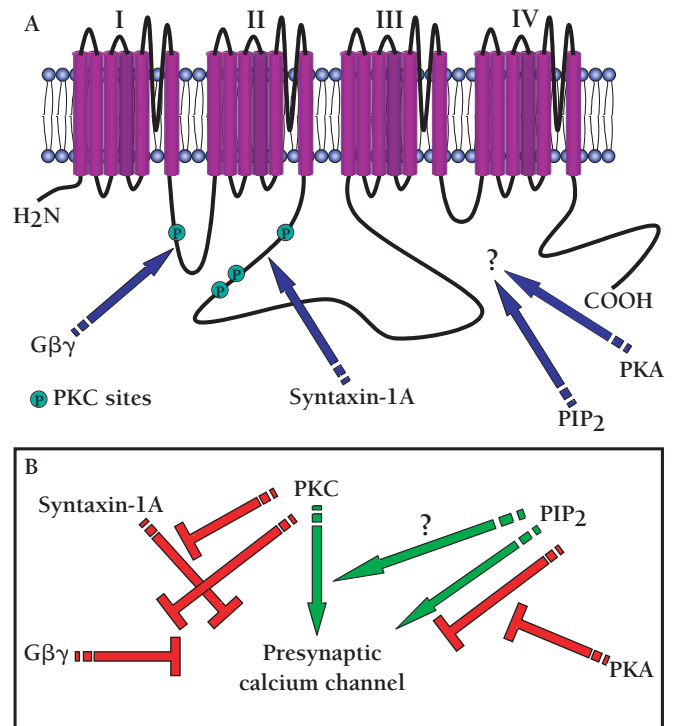


Figure 1. Proposed transmembrane topology of the calcium channel α_1 subunit, illustrating target sites for PKC-dependent phosphorylation, syntaxin binding, and G protein-dependent modulation. A. The sites of action of PIP₂ and PKA remain to be identified, and it remains possible that their effects are mediated by interactions with one of the ancillary calcium channel subunits. B. Crosstalk between regulatory pathways converging on presynaptic calcium channels. Inhibitory (red) and stimulatory (green) interactions are shown.

Interestingly, this regulatory mechanism intersects with second messenger regulation in two ways: First, PKC-dependent phosphorylation of the syntaxin binding site on the N-type calcium channel antagonizes the action of syntaxin (23, 24). Secondly, and perhaps more intriguingly, syntaxin-1A regulates G protein-mediated inhibition of N-type calcium channels (21, 23, 25). The fact that calcium influx through P/Q-type calcium channels triggers syntaxin-1A expression (20) raises the possibility that P/Q-type channels could also indirectly control the activity of N-type channels. Taken together, there appears to be an extensive interaction between individual types of regulatory pathways (Figure 1), most of which occur directly at the level of the calcium channel α_1 subunit.

The level of complexity of calcium channel modulation has increased yet further with a recent article by Wu et al. (26) that demonstrates a novel type of regulation of presynaptic calcium channels by phosphatidylinositol-4,5-bisphosphate (PIP₂). Direct modulation of ion channels by PIP₂ was first reported for ATP-dependent K⁺ (K_{ATP}) channels (27) and was subsequently demonstrated for other types of potassium channels (28, 29) and transient receptor potential (TRP) channels (30). The report by Wu

et al. is, however, the first to reveal that PIP₂ has an effect on any type of calcium channel. The authors show that the time-dependent decrease in current activity (rundown) of P/Q-type calcium channels following membrane patch excision could be slowed and partially reversed by the addition of PIP₂, and accelerated with antibodies against PIP₂. Interestingly, PIP₂ also strongly inhibited the activation of P/Q-type channels, resulting in a ~30 mV depolarizing shift in the voltage dependence of activation. This latter effect is reminiscent of the action of G_β subunits, but it remains unclear as to whether the effects of G_β subunits and PIP₂ are additive or work through a common mechanism. Of particular note, the authors show that activation of adenosine 3',5'-monophosphate- (cAMP)-dependent protein kinase (PKA) antagonized the inhibitory effect of PIP₂. Based on these findings, the authors proposed the presence of regulatory and stabilization domains capable of interacting with PIP₂, with the regulatory domain being a target for PKA-dependent phosphorylation. This model is similar to that proposed for PKC- and G protein-mediated crosstalk discussed above. However, in the absence of site-directed mutagenesis data on the channel complex itself, at this point it remains unclear whether the effects of either PIP₂ or those of PKA occur directly at the level of the channel or through an intermediary. Wu et al. also examined native N-type calcium channels in bullfrog sympathetic neurons. Using luteinizing hormone releasing hormone (LHRH) to effect the hydrolysis of PIP₂, the authors showed that PIP₂ appears essential for stabilizing N-type channel activity. This effect mirrors the protective effect of PIP₂ against P/Q-type channel rundown, but unlike with P/Q-type channels, the authors did not report a second, inhibitory action of PIP₂ on N-type channel function. Nonetheless, the data clearly show that both types of presynaptic calcium channels are regulated by PIP₂, suggesting yet another layer of complexity in the regulation of presynaptic calcium homeostasis.

It thus appears that there are at least two sets of regulatory systems that fine tune presynaptic calcium channel activity. On one hand, there is an intricate interplay between G proteins, PKC, and syntaxin, and on the other hand, there is crosstalk between PKA and PIP₂ modulation of channel activity. It seems likely that there may be additional interplay between the two regulatory systems. PIP₂ is the precursor for IP₃ and diacylglycerol, both of which are involved in the activation of PKC. Regulating PIP₂ concentrations could therefore conceivably affect the activity of some isoforms of PKC, and potentially, its crosstalk with G protein- and syntaxin-dependent regulation of the channels. Why do nerve cells need such a complex web of regulatory mechanisms converging on the calcium channel molecule? The notion that the individual regulatory pathways intersect at the level of the channel protein rather than simply regulating each other's activities per se ensures that crosstalk is specific for the target channel. Thus, the integration of multiple regulation pathways with different time courses ranging from milliseconds, for direct G_{βγ}-dependent

inhibition, to minutes, for kinase activity, allows for the precise temporal control of, and maximum specificity for, calcium influx and consequently neurotransmitter release. 🍷

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Toward a Role for Statins in Immunomodulation

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Statins are a group of drugs defined as inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase (1) and have been described as the principal and the most effective class of drugs to clinically reduce serum cholesterol levels (2). These clinical benefits, directly attributed to the cholesterol lowering effect of statins, have been extensively demonstrated in patients with atherosclerosis and cardiovascular diseases with or without coronary artery disease symptoms (3, 4). At present, five statins are available in the US and most other Western countries: atorvastatin, fluvastatin, lovastatin, pravastatin, and simvastatin. It is estimated

that about twenty-five million people worldwide are currently being treated with a statin. The number of prescriptions for statins issued in developed countries in 2000 was almost thirtyfold greater than the number written in 1991. World Health Organization statistics indicate that there are about 200 million people worldwide with coronary artery disease, stroke, other occlusive vascular diseases, or diabetes mellitus. Consequently, a proposal has been made that tens of millions of people at increased risk of heart attacks and strokes should begin statin treatment before onset of disease (5).

In addition to their recognized positive effects in cardiovascular diseases, statins might also have some additional clinical benefits. Although not yet demonstrated systematically and convincingly in human studies, such additional benefits of statins have been the subject of much speculation. The prevailing view is that such additional effects, if real, could result from indirect consequences of cholesterol lowering by statins. Others have argued in favor of pleiotropic effects of statins, through unknown mechanisms unlinked to cholesterol-lowering properties (6). For instance, the suggestion of beneficial effects of statins in cardiac transplantation was generally thought to result from favorable consequences of stringent and adequate lowering of cholesterol levels (7, 8). Laufs et al. showed that statins regulate nitric oxide (NO) levels in vitro, contributing to the view that statins might have pleiotropic effects (9). But despite this interest in the possibility of additional effects of statins, there was until recently no scientific rationale for statins to be considered as a method of treatment outside the field of atherosclerosis and cardiovascular diseases. In particular, they had not been considered or employed as immunomodulators or as drugs relevant to immune-related diseases.

The first direct scientific and mechanistic evidence that statins might have a role in immunomodulation reported that, unexpectedly, statins inhibited the expression of major histocompatibility complex (MHC) class II genes [also referred to as Immune Response (IR) genes] (10, 11). More specifically, statins inhibited the transcription of CIITA (MHC class II transactivator), a transcription factor essential for the expression of MHC II genes. This specific mechanism, together with the key role of the regulation of MHC class II expression in the control of immune responses in general, provided for the first time a scientific rationale for advocating statins as immunomodulators and immunosuppressive agents, independently of their well-known effects on lowering cholesterol levels. This finding led to the suggestion that statins might become novel therapeutic agents in the area of immunosuppression, anti-inflammation, and immune-related disorders such as auto-immune diseases (12). Indeed, the demonstrated ability of statins to block expression of MHC class II qualified them for use in novel immunomodulation therapeutic strategies. Recently, certain additional effects of statins, also distinct from cholesterol lowering, have been reported, such as inhibition of expression of CD40 (13, 14), binding to LFA-1 (15), inhibition of expression of adhesion molecules (13), cytokines, and chemokines (16).

In addition to these findings, several very recent publications identify a therapeutic use for statins in the treatment of multiple sclerosis (17, 18), and in particular to the mouse model of multiple sclerosis—termed experimental autoimmune encephalomyelitis (EAE)—are particularly interesting and not entirely unexpected. Youssef et al. (17) describe how oral statin treatment prevents or reverses chronic and relapsing paralysis and suppresses clinical and histological EAE. In these experiments, statin treatment reduces CNS infiltration (T_H1 lymphocytes) and MHC class II expression, inhibits the activity of CD40, CD80, and CD86 co-stimulatory molecules, largely inhibits the secretion of numbers of proinflammatory cytokines [for example, tumor necrosis factor- α (TNF α), interferon- γ (IFN- γ), interleukin-12 (IL-12)], and even induces the production of anti-inflammatory cytokines [such as tumor growth factor- β (TGF β) and IL-10]. In addition, statin treatment of either antigen-presenting cells (APC) or T lymphocytes from EAE-susceptible mice suppressed antigen-specific T-cell activation. These statin-mediated effects are specific for the inhibition of the enzyme HMG-CoA reductase. In their conclusion, the authors suggest that statins may be beneficial for multiple sclerosis and other T_H1 lymphocyte-mediated autoimmune diseases. These findings were largely confirmed in another recent report published by Neuhaus et al. (18). It is important to bear in mind that one of the current treatments of choice for multiple sclerosis, IFN- β , also inhibits expression of MHC class II, although the exact mechanism is unknown. Investigations on possible synergy between statins and IFN- β treatment on immunomodulation and proinflammatory molecules are currently being explored and may result in novel therapeutic strategies to influence the clinical issue of multiple sclerosis.

In a similar vein, it is tempting to argue that the clinical benefit observed with statins in cardiac transplantation (7, 8) is not the result of the lowering of cholesterol levels, as was suggested, but rather the result of their demonstrated role as inhibitors of expression of the MHC class II regulator CIITA. Indeed, the disruption of the CIITA gene in mice has a very strong, favorable effect on the outcome of cardiac transplantation (19), confirming that inhibiting CIITA, and thus MHC class II expression, is beneficial in organ transplantation.

The practical implications of new roles for statins as immunomodulators, as deduced from their effect on MHC class II expression (10, 11) and from their effect in preventing an autoimmune disease in mice (17, 18), are interesting. First, statins will, no doubt soon, be tested in several human autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis, Crohn's disease, and psoriasis. Second, the effect of combination therapy with IFN- β and statins in multiple sclerosis should be attempted. And third, direct pharmacological inhibition of expression of MHC class II, acting either on CIITA or on the three other MHC class II-specific transcription factors that form the regulatory factor X (RFX) complex (20), should be explored. In view of the recently recognized effect of statins—not only on the expression of MHC

class II genes, but also on certain key immunoregulators such as CD40, or on the pathway mediated by LFA-1—one can expect to hear about other new examples of clinical benefit from statin treatment in autoimmune or inflammatory diseases. We have recently observed, for instance, remarkable effects of statins in mice models of skin transplantation and of collagen-induced arthritis (21). Although it is to be hoped that statins may prove to be clinically useful in autoimmune diseases, one should also keep in mind that some degree of immunosuppression over a very long time period might have negative consequences. It is thus relevant to mention that in the recent human clinical trial PROSPER (22), it was reported that the use of statin in elderly individuals might increase the risk of cancer. Nonetheless, the future seems bright for the statin family in immune regulation. 🍀

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Multiple Levels of Telomerase Regulation

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Telomeres are the nucleoprotein complexes found at the end of all linear chromosomes. These complexes consist of a six-base pair DNA repeat (TTAGGG)_n and a growing list of associated proteins (1, 2). Proper maintenance of these structures is required for ongoing cellular replication and genomic stability. Indeed, as normal cells divide in culture their telomeres progressively shorten, and upon reaching a critical length, cells cease to divide (3). In contrast to what is observed in normal human cells, immortal tumor cells display stable telomere lengths despite continued cellular division (4, 5). Overexpression of the catalytic component of telomerase (hTERT) in normal human cells leads, in many cases, to immortalization, demonstrating an important role for hTERT in cellular immortality (6, 7). These observations form the basis of the telomere hypothesis that states that maintenance of telomere length is a prerequisite for cellular immortality (8). Recent work by numerous groups has discovered that telomere maintenance is more complex than simply the maintenance of length, and it is clear that the overall structure of the telomere is also important for continued cellular proliferation (9).

Expression of hTERT and the RNA template component hTR is sufficient for in vitro telomerase activity (10, 11). Most human cells constitutively express hTR throughout development, whereas the expression of hTERT is more tightly regulated (5). Early human embryonic cells express hTERT (12); however, hTERT expression and telomerase activity is not detectable in the majority of differentiated somatic cells (5). Notable exceptions to this observation are tissue-specific stem cells and both activated B and T lymphocytes (13–16). Although these cell types do express hTERT, the expression is not sufficient to maintain telomeres. In contrast to somatic cells, greater than 90% of human cancer cells utilize telomerase to maintain stable telomere lengths (5). The remaining cancer cells utilize an alternative mechanism of telomere maintenance (ALT), which is defined operationally as telomerase-independent telomere maintenance and may consist of a recombination mechanism (17). Ectopic expression of hTERT is sufficient to immortalize many normal human cells, indicating that hTERT activity is the rate-limiting component (6, 7). A paradigm has emerged over the last few years whereby cancer cells utilize a number of approaches to activate the telomerase holoenzyme, and the recent report by Wong et al. adds yet another possible level of telomerase regulation (18).

Because tumor cells express hTERT and have robust telomerase activity, the majority of studies to date have focused on the transcriptional regulation of *hTERT* in tumor cells. The *hTERT* gene is located on the distal arm of chromosome 5p (5p15.33) (19,

20). Mutations in this region are rare, although amplifications have been detected in some types of cancers, suggesting that increased copy number may be one mechanism that increases telomerase expression in human tumors (19, 21). The *hTERT* promoter contains a number of regulatory sites including two c-Myc binding sites (22). The transcription factor c-Myc and its antagonist Mad (23, 24) affect the transcription of *hTERT*. Because *MYC* and *MAD* are sometimes perturbed in human cancers, this may provide another level of telomerase deregulation. [Estrogen also modulates *hTERT* transcription (25, 26).] It has also been suggested that the *hTERT* promoter might contain a site that negatively regulates its expression in a p53-dependent manner (27). Because the majority of human cancers are deficient in p53 protein or in functional p53-mediated pathways, this too could contribute to increased *hTERT* expression.

In a recent publication, Wong et al. demonstrated that subnuclear (nucleolar) shuttling of hTERT may also modulate telomerase activity at the telomere (18). In the current study, this group created a green fluorescent protein–telomerase (GFP–hTERT) fusion protein and followed its fate throughout the cell cycle; ectopic expression of this fusion protein was capable of maintaining stable telomere lengths and immortalizing cells. They observed that in normal human fibroblasts this fusion protein was restricted to the nucleoli throughout much of the cell cycle; however, the protein relocalized to the nucleoplasm during late S/G2, the time at which human telomeres are thought to be replicated. Analyses of in vitro telomerase activity (TRAP) indicated that there was no change in enzymatic activity throughout the cell cycle. This suggests that the cellular localization of hTERT does not affect the enzymes' ability to elongate telomeres but rather that the restricted localization of hTERT may sequester it from the 3' termini of the chromosome, thus inhibiting telomere elongation at inappropriate times.

Unlike what was observed in normal cells, telomerase-positive transformed cells displayed a nucleoplasmic distribution throughout the cell cycle. In addition, when hTERT was overexpressed in ALT cell lines, it was also found in the nucleoplasm throughout the cell cycle. Similarly, introduction of the SV40 Large T antigen (LT) into normal human cells abrogated the nucleolar localization of hTERT, suggesting that it is the deregulation of the p53- and/or retinoblastoma- (Rb)-dependent pathways that leads to inappropriate localization of hTERT. This observation is interesting because it suggests that p53 may influence telomerase activity at two distinct levels. At one level, loss of p53 may contribute to increased transcription of *hTERT* (27). This study suggests that p53 expression may also control telomerase activity at a second level by altering the subcellular localization of the holoenzyme. The potential role of the Rb pathway in this regulation remains to be determined. Together, these observations suggest that telomerase activity is sequestered in the nucleoli in cells with intact cell cycle checkpoints. Upon inactivation of these pathways, hTERT is released from the nucleoli, presumably giving telomerase access to the telomeres throughout the cell cycle, resulting in inappropriate

elongation and maintenance of the telomere.

Maturation of the telomerase complex requires nucleolar components (28); therefore, it is not necessarily surprising to find hTERT in the nucleolus. However, the fact that hTERT is restricted to the nucleolus during particular phases of the cell cycle suggests that there is an important consequence to hTERT localization. To demonstrate whether this observed nucleolar localization was of functional importance, Wong and colleagues exposed cells expressing GFP-hTERT to γ -radiation. They found that when normal cells expressing GFP-hTERT were exposed to γ -radiation, nucleolar localization was enhanced. Surprisingly, they also found that in normal cells expressing LT, in transformed cells, and in cells that utilize ALT for telomere maintenance, GFP-hTERT also relocalized to the nucleolus following γ -irradiation. In in vitro assays, the association of telomerase with DNA is promiscuous, and it has been suggested that telomerase can participate in chromosome healing following double-strand breaks (29). Addition of telomeric repeats to internal sites in the chromosome would likely have disastrous effects on the genome. Therefore, one possible explanation for this observation is that sequestration of the telomerase complex in the nucleolus might not only be important for limiting hTERT's access to the telomere at inappropriate times throughout the cell cycle, but also during times of DNA damage. In addition, because this DNA damage-induced relocalization of hTERT occurs in both normal cells and cells expressing LT, it indicates that there is a mechanism that is distinct from the cell cycle control of nucleolar localization of hTERT, which is p53 and/or Rb independent. Understanding this mechanism will require further investigation but highlights the complexity of hTERT regulation.

Telomere homeostasis plays an important role in both normal and malignant cell physiology. Controlling the expression and activity of the telomerase holoenzyme is complex, and the current study (18) unravels another layer of this regulation. A number of other questions arise, including what mechanisms control the subcellular localization of hTERT and how these mechanisms are perturbed in human cancers. Because of its restricted expression, telomerase is an attractive target for future antineoplastic approaches, and the study by Wong et al. (18) provides yet another level at which future therapies can be directed. 🍀

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