The Journal of General Physiology

Commentary

Why Biophysicists Make Models: Quantifying Modulation of the M Current

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For those ion channel biophysicists who are also in the signal transduction business, it can sometimes seem like a marriage of strange bedfellows. In the world of voltage gating and permeation studies the analysis is usually meticulously precise and the results often presented using dense mathematics. Gating charges are calculated to the last e^-, molecular movements debated to the last Å, and state occupancies may be many decimal places in length. However, when we turn to regulation of the channels, we usually become less demanding. When we say that a drug or an analogue blocks a certain signal, we usually do not mean that the signal actually was eliminated, but rather that it was reduced. It can be a messy business, but this messiness is brought on, not by the languor of the investigator, but by the imperfect nature of many of the reagents that are used to test hypotheses and to draw conclusions. So it is possible to apply quantitative rigor to the study of such complex signaling pathways? In this issue of the *Journal of General Physiology*, we are treated to a sterling article that answers strongly in the affirmative (Suh et al., 2004).

The article deals with muscarinic acetylcholine receptor (mAchR) modulation of the "M current", a neuronal K^+ conductance that was so named for its depression by mAchR agonists (Constanti and Brown, 1981). Probably little did the discoverers imagine that this receptor action would be so intensively studied, and that its mechanism would resist elucidation for so long. Although the topic remains controversial, what has been well accepted is that muscarinic stimulation activates the $G_{q/11}$ family of G proteins (Haley et al., 1998), and that some additional intracellular 2nd-messenger is involved (Selyanko et al., 1992); i.e., the action is not due to a direct G protein–channel interaction, as is the case for activation of GIRK K^+ channels or inhibition of neuronal Ca^{2+} channels. Adding to the provocative nature of this field is that although the textbook description of $G_{q/11}$-mediated signaling pathways says that PKC and intracellular Ca^{2+} ($Ca^{2+}{}_i$) are the primary second messengers generated by $G_{q/11}$-coupled receptor stimulation, neither is the primary signal for muscarinic inhibition of the M current (Brown et al., 1997) and, in fact, muscarinic agonists do not alter $[Ca^{2+}]_i$ at all in the sympathetic neurons that have been the model neuron in many of these studies (Beech et al., 1991). The field grew further in stature with the identification of the KCNQ family of K^+ channel genes as the molecular correlates of the M current (Wang et al., 1998) and the use of powerful molecular, biochemical and optical techniques to probe the mechanisms of their regulation. Their identification also allowed immunochemical studies, which revealed M channels to be expressed in neurons throughout the nervous system (Cooper et al., 2001; Roche et al., 2002) and has reinforced the dominant role that KCNQ channels play in the control of neuronal excitability (Jones et al., 1995; Castaldo et al., 2002). Finally, several investigators were impressed with the potent regulatory role, for a variety of membrane transport proteins, ascribed to the phosphoinositide that is the target of PLC, phosphotidylinositol 4,5-bisphosphate (PIP_2), and wondered if its depletion might be the mystery second messenger signal for M currents (Suh and Hille, 2002; Zhang et al., 2003). Although a veritable mosaic of channels have been shown to need PIP_2 around to be functional (Hilgemann et al., 2001), the jump to actual hormonally induced depletion on a global scale seemed quite a leap; after all, there is a lot of PIP_2 in a typical mammalian cell, and PLC would seem to have to be quite a busy guy to get the job done.

Thus, the stage seemed set to ask if this all makes sense. Suh et al. (2004, in this issue) had a number of observations to work with: the dependence of muscarinic suppression and recovery on intracellular ATP and Mg^{2+}, the blockade or slowing of the signal with various GTP analogs or G-protein mutants, and the recent development of some very handy optical probes of PIP_2 hydrolysis developed by the Tobias Meyer lab (Nahorski et al., 2003). Most investigators would have been happy leaving the biophysicist hat off, and to observe that omitting Mg^{2+} from whole-cell pipettes blocks the modulation, for example, and to report that this ion has something to do with the story. However, these authors obviously decided to leave the hat firmly on, and to crunch some very hard numbers. They were helped

J. Gen. Physiol. © The Rockefeller University Press • 0022-1295/2004/06/657/6 $8.00
Volume 123 June 2004 657–662
http://www.jgp.org/cgi/doi/10.1085/jgp.200409095

in this regard by the development of an impressive virtual cell modeling program at the University of Connecticut (Xu et al., 2003), designed initially to model phosphoinositide signaling and the predicted meaning of optical measurements using the PIP_2/IP_3 probe also used in the Suh et al. (2004) study, a fusion construct of the PH domain of PLCδ and EGFP (Stauffer et al., 1998). The basic questions were: given the best available data on the rates of G protein turn-on and turn-off, on the density of PIP_2 molecules in mammalian plasma membrane, and on the speed of PLC, (a) can perturbations of the system with ions, analogs, and mutants be predicted by a model of the system? And (b) would occupancy of a reasonable fraction of mAchRs, and the activation of a reasonable fraction of G proteins hydrolyze enough PIP_2 molecules fast enough to coincide with the canonical observations of M-current suppression? The results of the model say yes to both questions, but in doing the heavy lifting, Suh and colleagues also make sense of some observations that seem utterly counter-intuitive. For instance, most people studying G-protein signaling have used the analogue of GTP, GDP-β-S, in the basic experiment to show that G proteins are involved in a signal; it traps the G protein in a GDP-bound inactive state, such that attenuation of the action by GDP-β-S implicates G-protein involvement. This writer has waited impatiently for seemingly massive amounts of this compound to dialyze into cells, only to be rewarded by a 50% diminution of the action at best. The model of Suh and colleagues quantitatively predicts these effects; but, remarkably, also correctly predicts that for brief applications of receptor agonist, the modulation not only continues, but reaches its peak, after the agonist is removed. Thus, we see why biophysicists make models in the first place. Whereas someone thinking observationally would throw out the cell with continued modulation after removal of agonist, calling it "run-down", a careful analysis predicts that this is exactly the behavior that one expects from the system.

The model is divided into two linked parts. The first half involves the various perturbations of the $G_{q/11}$ G-protein cycle that the authors performed and, as such, are common to any $G_{q/11}$-mediated signal, unrelated to modulation of channels. This part highlights the under-appreciated role of Mg^{2+} in G-protein actions, and correctly predicts the effects of altered $[Mg^{2+}]_i$. The second half contains the steps downstream of PLC activation, and involves the business end of the action, including PIP_2 hydrolysis and resynthesis, and association with KCNQ channels. The initial question is whether sufficient changes in $[PIP_2]$ are predicted to be consistent with the observed rates of KCNQ-channel suppression and recovery in cells. The modeled affinity of the channel for PIP_2 is an important parameter here, but it is constrained by several important considerations. For the channels to be highly sensitive to changes in $[PIP_2]$, the equilibrium constant describing this affinity cannot be much different from resting $[PIP_2]$. Yet, because increasing tonic $[PIP_2]$ by PI(4)P5-kinase overexpression does not appreciably increase tonic M-current amplitudes in neurons (Winks et al., 2003), most channels should have PIP_2 bound to them at rest. The authors choose this value to be 72% of channels, which means than an increase of tonic $[PIP_2]$ might not be noticed. Is muscarinic stimulation predicted to be able to substantially lower PIP_2 levels? Amazingly, the model predicts that if all the $G_{q/11}$ molecules were activated at once, the entire pool of PIP_2 would be consumed with a time constant of 210 ms! Thus, in the model, only 13% of G proteins would have to be activated to achieve a maximal inhibition of the KCNQ current with the observed 8-s time constant, which translates into 26 active $G_{q/11}$ molecules in a typical 1 μm^2 of patch membrane. The perturbations performed in the whole-cell clamp experiments are simulated in the model, and the predicted and experimental results are mostly congruent. It would be interesting to make some biochemical measurements of $[PIP_2]$ in cells in response to such hormonal stimulation, and to compare these with what is suggested here. The authors wisely do not attempt to use translocation of the EGFP-PLCδ-PH probe to quantify $[PIP_2]$ changes. Because EGFP-PLCδ-PH has a 10-fold higher affinity for IP_3 over PIP_2 (Hirose et al., 1999), its translocation from membrane to cytoplasm can reflect IP_3 production or PIP_2 depletion, with the dominant molecule being reported probably dependent upon the concentrations of the probe and of PIP_2, and on the activities of PI kinases. This question has already been well-treated in recent work by others (van der Wal et al., 2001; Xu et al., 2003).

Left out of the equation by Suh and colleagues are any effects of receptor stimulation on the activity of PI kinases, which would increase the synthesis of PIP_2 concurrently with its hydrolysis. In fact, such kinase stimulation has been reported to be dramatic sometimes, and probably dependent on whether the receptor raises $[Ca^{2+}]_i$. A recent paper that measured and modeled PIP_2 signaling found, for stimulation of $G_{q/11}$-coupled bradykinin receptors, which raise $[Ca^{2+}]_i$ in neuroblastoma cells, the initial response to be an increase in $[PIP_2]$, followed by only a modest decline (Xu et al., 2003). This is almost certainly due to potent stimulation of PI4-kinase by bradykinin stimulation, perhaps mediated by neuronal calcium sensor-1 (Koizumi et al., 2002; Winks et al., 2002). The values used by Suh et al. (2004) reveal the potential for a dramatic increase in PIP_2 synthesis: their static rate for PI4-kinase is >100-fold less then that of PI(4)P5-kinase, and the density of PI(4)P molecules some fivefold less than that of PIP_2

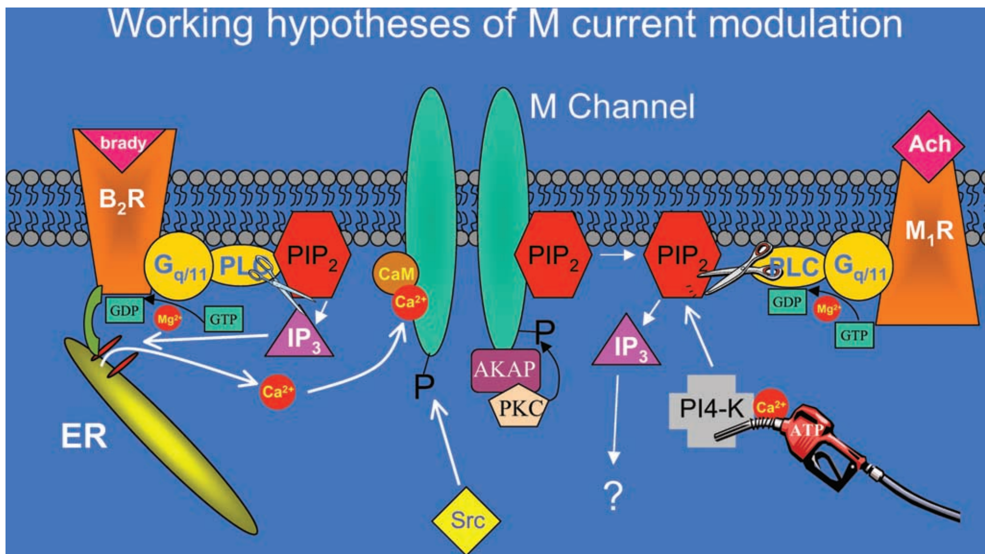

Figure 1. Schematic representation of the several signaling pathways discussed in the text that act on M-type (KCNQ) K^+ channels. The figure depicts the M_1 muscarinic receptor (M_1R) and the B_2 bradykinin receptor (B_2R) both using $G_{q/11}$ G proteins, with the primary mechanism of action being via depletion of PIP_2 in the former case, and Ca^{2+}–calmodulin (CaM) in the latter case. PLC, GTP/GDP exchange, and the Mg^{2+} ion are needed for both signals. PI4-kinase needs ATP to synthesize PI(4)P from PI (not depicted is PI(4)P5-kinase that produces PIP_2 from PI(4)P). Besides the role of the Ca^{2+} ion in the left pathway, is its requirement at sufficient levels for PLC activity, and its putative role in stimulation of PI4-kinase. Association between the plasma membrane B_2R, but not the M_1R, and the endoplasmic reticulum (ER) membrane IP_3R specifies bradykinin, but not muscarinic, stimulation to raise intracellular Ca^{2+}. IP_3 produced by M_1R-induced hydrolysis of PIP_2 has not been shown to have any consequence. A-kinase anchoring protein 79/150 (AKAP) is shown recruiting PKC to the channel, where it may sensitize it to depletion of PIP_2. Finally, Src tyrosine kinase (Src) modulates the channel, seemingly unrelated to G-protein actions. References are given in the text.

and 200-fold less than that of PI. Thus, additional PI(4)P molecules made would be quickly converted into PIP_2, and a sudden ramping up of the PI4-kinase rate might translate into a lot more PIP_2 produced. This brings up the issue of specificity in signaling. If so many different channels and transporters are tightly regulated by membrane PIP_2 levels, and if any $G_{q/11}$-coupled receptor agonist dramatically depletes PIP_2, how could any specific meaningful signal be transmitted in accord with the agenda of the cell? One possibility could be regulator of G-protein *s*ignaling (RGS) proteins, which might be recruited to the vicinity around certain receptors, but not others. Suh et al. (2004) show that overexpression of RGS2, which regulates $G_{q/11}$, severely blunts G-protein modulation. In sympathetic neurons, a major receptor-specific difference is that certain receptors cause rises of $[Ca^{2+}]_i$ (bradykinin B_2, purinergic P2Y), but others do not (mAch M_1, angiotensin AT_1). It will be interesting to examine whether this basic difference underlies distinct mechanisms of action with regard to lipid signals. In the case of M-channel modulation, the actions of two of these $G_{q/11}$-coupled receptors have been the most examined. Fig. 1 summarizes this work from several different labs. M_1-receptor agonists do not raise $[Ca^{2+}]_i$, and inhibit the channels perfectly well with $[Ca^{2+}]_i$ clamped, IP_3 receptors blocked, or internal Ca^{2+} stores depleted (Cruzblanca et al., 1998; Shapiro et al., 2000). Considerable evidence is accumulating that depletion of PIP_2 is central to this action (Suh and Hille, 2002; Ford et al., 2003; Zhang et al., 2003; Suh et al., 2004). Bradykinin, however, does raise $[Ca^{2+}]_i$, and also inhibits M-channels, but the action is severely blunted under any of the above treatments (Cruzblanca et al., 1998). Bradykinin inhibition may be mediated by Ca^{2+} ions, released from stores, binding to calmodulin (CaM), which acts as the channel Ca^{2+} sensor. Thus, overexpression in neurons of a dominant-negative CaM that cannot bind Ca^{2+} blocked most of bradykinin modulation, but has no effect on the muscarinic action; and, in a reconstituted system, CaM was required for KCNQ channels to be Ca^{2+}-sensitive (Gamper and Shapiro, 2003). In addition, regulation of certain KCNQ subunits by Src tyrosine kinase represents a third mode of modulation, unrelated to G-protein signaling (Gamper et al., 2003).

Perhaps in few spheres of thinking in ion channel physiology has modeling proven more useful than that of kinetic states of individual channels. As soon as it was hypothesized that channel proteins could be "open", "closed", or "inactivated," biophysical thinking led to assignment of these conformations to discrete states with precise rate constants governing transitions between them. We observe that receptor stimulation depresses the macroscopic current, and perform tests that implicate alterations of PIP_2 binding as the culprit. Depression of the whole-cell current must translate into lowering of the open probability of the single channel and in the lipid-signaling model, changes in PIP_2 binding must lead to different occupancies of key kinetic

states. The challenge will be to correlate, at the single-channel level, the receptor actions using more physiological experiments with the direct lipid applications using more reduced systems, such as inside-out patches. Sophisticated modeling of M-channel gating has been done, suggesting channels with a very complex gating behavior (Marrion, 1993; Selyanko and Brown, 1999). A simpler scheme, in which a channel must be linearly activated by voltage and by PIP_2 binding, predicts that changes in PIP_2 binding (either by receptor stimulation or directly) should lead to changes in the voltage dependence of gating. In kinetic language, if increased PIP_2 binding stabilizes the open state, then a less depolarized voltage should be necessary to open the channel gate. Interestingly, for KCNQ2/3 channels, muscarinic modulation (which seems to be via depletion of PIP_2) does not alter the voltage dependence of activation (Shapiro et al., 2000), but application of PIP_2 to patches containing highly homologous KCNQ1 channels does (Loussouarn et al., 2003). Could the gating mechanisms be very different between very similar KCNQ channel subtypes? Do M-type channels have only one gate, or are there more? These are the structural questions that biophysicists will love to answer.

When G proteins were first classified into four main groups, and the very idea of different "signaling cascades" as tools of signal amplification and specificity was developed, it was believed that different receptors do different things by triggering different cascades. However, as many receptors were found to use the same pathway, and each second messenger was found to act on many different targets, some new concept of specificity had to be found. For the case of the first discovered second messenger, cAMP, the concept emerged of scaffolding proteins (AKAPs) bringing cAMP-dependent protein kinases to a specific target, thus achieving specificity by clustering the molecules together in microdomains in the cell (Bauman et al., 2004). Fig. 1 also incorporates recent work enticingly suggesting that similar clustering into microdomains underlies specificity in Ca^{2+} signaling and in M-channel modulation (Delmas et al., 2004). Added to the mix is the result that PKC, formerly ruled out as a second messenger involved in M-current modulation, nevertheless is brought to the channels by an AKAP protein, and "sensitizes" the channels to PLC-induced depletion of membrane PIP_2 (Hoshi et al., 2003). Such a coincidence-detection mechanism, long accepted in the synaptic plasticity field, might make sense as a way of ensuring fidelity in the signal, such that fluctuations in [PIP_2] caused by fluctuating PI kinase or phosphatase activity does not "inadvertently" cause modulation. If this is true, then the affinity of the channels to PIP_2 in unstimulated cells could be quite far from resting [PIP_2], with sensitized channels having their affinity for PIP_2 temporarily lowered, and primed for a receptor-induced PIP_2 depletion that otherwise might not have a strong effect. The model by Suh et al. (2004) presented in this issue convincingly makes the case that PIP_2 can handily be depleted almost to zero, which would not require any such coincidence-detector mechanism to be invoked. However, in the nervous system, entire neurons are rarely bathed in supramaximal concentrations of transmitters; rather, signals are usually transmitted one synapse at a time. In addition to control of postsynaptic excitability, M channels have been shown to control neurotransmitter release from presynaptic hippocampal nerve terminals (Martire et al., 2004), and to be expressed as clusters in nodes of Ranvier, where they regulate action-potential propagation (Devaux et al., 2004). It is also unclear how close metabotropic mAchRs are to active zones, and on what scale PIP_2 depletion could be in a region contiguous with unstimulated membrane, from which PIP_2 could quickly diffuse to keep any local [PIP_2] from falling far.

Could this complex system in real neurons, including the clustering of molecules in microdomains, and the spatiotemporal dynamics of localized synaptic actions, be quantitatively modeled as Suh and colleagues do here? I think so. Such computational neurobiology is a hot topic these days, and this feat is increasingly attempted with powerful computers. The number of parameters and variables might be large. The availability of internet-based modeling environments, such as that used here, will encourage this approach. In the face of such a daunting challenge, a good approach is to make a start, and to use a workable system where relatively few variables need to be guessed. This is what Suh and colleagues do in this volume. Explicitly acknowledging the imperfect nature of their model, they successfully predict a host of observations that we patch-clampers like to make. Their paper especially stands out in the field of channel regulation by PIP_2, in which attention to the physiology of real cells has not been a strong suit. Could anyone have guessed a decade ago that the explicit structure of a voltage-gated ion channel would be proposed? I doubt it. As for quantitative models in the channel structure/function field, the quantification of lipid signaling presented in this issue will likely stimulate a lively debate. Such quantitative biophysical approaches usually do. For sure, a model is only as good as the assumptions and parameters that it is based upon. The initial computer simulations by NASA scientists in the days after the space-shuttle accident indicated that a piece of foam could not possibly do serious damage to a wing, but an actual chunk fired at a mock wing here in San Antonio blasted a gaping hole. Nonetheless, biophysicists have used models powerfully to know what questions to be asked experimentally, and to

formulate what conclusions are realistic, given the data. Discrete state kinetic models that predicted the multi-ion occupancy of a channel pore (Hille and Schwarz, 1978) presaged the pore's crystal structure that showed multiple ion binding sites (Doyle et al., 1998). Early models of how inactivation might work (Armstrong and Bezanilla, 1977) proved remarkably predictive when the molecular era allowed its structural mechanism to be shown (Zagotta et al., 1990). We look forward to the increasingly quantitative approach to the study of signaling cascades, and to further modeling of interactions between signaling proteins and their effectors.

I would like to thank Drs. N. Gamper, Y. Li, and B. Rothberg for helpful comments on this commentary.

The work in the author's lab is supported by grants from the American Heart Association-Texas Affiliate, the Epilepsy Foundation, and by NIH/NINDS grant RO1 NS43394.

Olaf S. Andersen served as editor.

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