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COMMENTARY

Blue flash sheds light on the roles of individual phosphoserines in CFTR channel activation

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Light-controlled availability for phosphorylation reveals dominant roles of select R-domain serines in CFTR channel activation.

In a recent issue of the *Journal of General Physiology*, a study by Infield and colleagues (Infield et al., 2023) presented breakthrough novel technology that provides the first true insights into the functional consequences of individual phosphorylation events within the regulatory domain of the CFTR channel.

The CFTR anion channel, essential for salt–water transport across several epithelia, is an ATP-binding cassette (ABC) protein. In addition to ABC-typical sequences that form its transmembrane pore and two cytosolic nucleotide binding domains (NBDs), it contains a unique cytosolic regulatory (R) domain. Structure and function of the canonical domains is highly similar to that of other ABC proteins and is relatively well understood. Pore opening and closure are linked to formation and disruption, respectively, of a tight intramolecular NBD dimer which occludes two molecules of ATP (Vergani et al., 2005; Liu et al., 2017; Zhang et al., 2018). Given the continuous availability of cytosolic ATP, CFTR channel activity is strictly regulated through phosphorylation of its R domain by cAMP-dependent protein kinase (PKA): the vanishingly low open probability (P_o) of unphosphorylated channels increases by ∼100-fold upon phosphorylation (Mihályi et al., 2020).

The R domain (residues ∼640 to ∼840) is an unstructured polypeptide segment with no known homolog in the GenBank database. It emerged together with CFTR in early vertebrates (Cui et al., 2019), and contains a large number of serines in dibasic consensus sites (R/K-R/K-X-S) for PKA, which have all been shown to become phosphorylated in vitro (Picciotto et al., 1992; Townsend et al., 1996) or in vivo (Cheng et al., 1991). The regulatory function of the R domain is predominantly inhibitory, as deletion of a large part of (residues 708–835; Rich et al., 1991) or the entire (residues 634–835; Csanády et al., 2000) R domain yields channels that can gate in ATP without prior exposure to PKA. That feature was explained by recent cryo-EM structures in which the dephospho-R domain is seen to intercalate between the two NBDs preventing their dimerization, whereas the phospho-R domain is entirely disordered and is released from its occluded inhibitory position (Liu et al., 2017; Zhang et al., 2018).

But the molecular details of activation by phosphorylation are little understood, and especially the contributions of individual phosphoserines to channel activation have remained unclear. The conventional way to address these questions has been targeted replacement of consensus serines by either alanine or aspartate/glutamate, with the underlying assumption that the uncharged alanine side chain mimics the unphosphorylated, whereas the negatively charged acidic side chains the phosphorylated form of the native serine side chain. Using that approach, a multitude of early studies have arrived at the general conclusion that the ∼11 consensus R-domain serines are funcionally redundant: open probability (P_o) was reduced by less than twofold by alanine substitutions of any individual position. Even more surprisingly, even combined mutagenesis of 4 or 8–10 serines reduced P_o only by approximately twofold and approximately threefold, respectively (Rich et al., 1993; Chang et al., 1993; Winter and Welsh, 1997), prompting efforts to discover additional potentially relevant phosphorylatable positions (Seibert et al., 1995).

Interestingly, whereas alanine replacement of most tested positions reduced P_o, mutations S737A and S768A facilitated channel activation, both in response to forskolin exposure of intact cells, and in inside-out patches exposed to ATP + PKA (Wilkinson et al., 1997; Vais et al., 2004; Csanády et al., 2005), suggesting that these two positions act as "inhibitory sites." However, such an effect could not be demonstrated in current recordings from purified, pre-phosphorylated CFTR channels reconstituted in lipid bilayers (Hegedus et al., 2009).

Several factors complicate correct interpretation of the above large pool of early data. First, the redundancy concept has been

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recently questioned by the demonstration that simple binding of the PKA catalytic subunit to CFTR provides strong reversible stimulation, additive to the irreversible activation caused by phosphorylation. That activation by PKA binding is at least partly retained even following alanine substitution of all dibasic PKA target sites (Mihályi et al., 2020), which explains the surprisingly large P_o values reported in the early studies for the various multi-alanine mutants, all of which were assessed in the presence of PKA. Indeed, for the all-alanine construct, only ∼10% of the current survives PKA removal, suggesting a vanishingly small P_o (∼0.01) in the absence of PKA binding (Mihályi et al., 2020)—consistent with the behavior of pre-phosphorylated purified multi-alanine mutants studied in lipid bilayers in the absence of PKA (Hegedus et al., 2009). Second, the basic assumption that an alanine side chain functionally resembles an unphosphorylated serine might not be correct, e.g., the network of contacts that stabilizes the unphosphorylated R domain in its wedged-in position might include hydrogen bonds formed by polar serine side chains which are disrupted by alanine substitutions.

Due to these limitations, the true functional consequence of phosphorylation of any individual R-domain serine is currently unclear, and conventional mutagenesis provides little hope for deciphering these details. The study by Infield et al. (2023) presents breakthrough novel technology that provides first true insights into these questions.

Instantaneous release of a biologically active compound by light-induced cleavage of a photolabile group of an inactive precursor molecule ("the caged compound") is an elegant strategy invented decades ago (Kaplan et al., 1978), and has found numerous applications in physiology (Ellis-Davies, 2020). More recently, the conventional mutagenesis toolkit for protein structure–function studies was largely enhanced by the targeted incorporation of unnatural amino acids into proteins, made possible by the nonsense codon suppression method (Pless and Ahern, 2013). Infield et al. (2023) now creatively combine those two methods to express in HEK293 cells several variants of the CFTR channel in which one particular PKA-target serine in the R domain is "caged," i.e., contains a protective photolabile adduct attached to its side chain. Whereas the targeted position cannot be phosphorylated in its caged form, photo uncaging instantaneously regenerates a native serine side chain which is henceforth available for phosphorylation. That innovative approach allows the authors to study for the first time the effects on CFTR whole-cell currents, as well as the in vivo kinetics, of phosphorylation of individual R-domain serines.

To that end, cellular cAMP levels are first maximally stimulated by continued exposure to 10 µM forskolin, keeping endogenous PKA maximally active throughout the experiment. Once the CFTR current has reached a plateau reporting full phosphorylation of all available (native) serines, a brief light pulse is delivered to uncage the target position. The ensuing current response reveals the kinetics and functional consequence of phosphorylation of the target position in the background of a CFTR protein that is already phosphorylated at all other sites. One might be concerned that, although uncaging regenerates the native phosphorylatable serine, *before* uncaging the target position might not conform to its resting state functional role. If that were the case, then an abrupt change in current would be expected upon the applied light flash. However, no such instantaneous effect is observed for any of the tested positions, suggesting that the subsequent slow current relaxations indeed report the functional effects of phosphorylation of the individual sites. From the variable responses to uncaging at different positions two interesting patterns emerge.

Quantitation of current response *amplitudes* reveals a clear spatial pattern. Whereas uncaging of positions near the N-terminal end (700, 737) or in the center (768) of the R domain evokes modest (less than approximately twofold) responses, uncaging of positions near its C-terminal end (795, 813) is followed by robust, approximately eightfold current activation. Assuming that the efficiency of uncaging is near 100% regardless of the target position's location, that pattern suggests a dominant role of the R domain's C-terminal portion in controlling CFTR channel activity. That conclusion is congruent with earlier structural analysis which suggested that the wedged-in R-domain helix resolvable in the cryo-EM structure of unphosphorylated CFTR corresponds to a C-terminal segment (residues 825–843) of the R-domain (Liu et al., 2017). It is also consistent with the functional observation that severing the polypeptide backbone near the C-terminal end of the R domain (between positions 835 and 837) disrupts strict PKA-dependence of channel gating, whereas severing it near the N-terminal end (between positions 633 and 634) produces no phenotype (Csanády et al., 2000).

Focusing on the two high-impact positions, 795 and 813, Infield et al. (2023) also provide first evidence for non-independence between phosphorylation events at two distinct positions. Using a phospho-specific antibody which recognizes the non-phosphorylated S813 motif, they quantitate the level of phosphorylation of position 813 in channels that contain a caged serine at position 795. Surprisingly, they find that upon forskolin stimulation S813 remains unphosphorylated as long as position 795 is not uncaged by a blue-light flash. They conclude that phosphorylation at position 795 is a prerequisite for phosphorylation of S813, and suggest that the relatively fast phosphorylation of serine 795 serves as a "ready" signal, whereas subsequent slow phosphorylation at serine 813 is the actual rate limiting step that drives channel activation.

Comparison of the *signatures* of the current responses to uncaging carries further surprises. Except for position 768, phosphorylation of which causes a small current decline consistent with its proposed inhibitory role, at all other positions uncaging is followed by an increase in current. This includes position 737, another suggested inhibitory site (Wilkinson et al., 1997; Vais et al., 2004 but, cf., Hegedus et al., 2009). That puzzling finding underscores the complexity of CFTR regulation and leaves multiple possible explanations open. First, the reported "inhibitory" role of S737 phosphorylation was suggested to be most prominent at low levels of cellular PKA activity (Wilkinson et al., 1997), whereas the Infield study investigates the effect of S737 phosphorylation in an otherwise fully phosphorylated channel. Given that in a WT channel exposed to PKA S737 is phosphorylated early (Csanády et al., 2005), it is conceivable that the functional consequence of phosphorylation at position 737 is context-dependent, i.e., depends on the pattern of phosphorylation of other sites.

Alternatively, it is conceivable that the serine cage at position 737 interferes with the activating effect caused by PKA binding (Mihályi et al., 2020), and the current response upon uncaging is dominated by the appearance of binding-induced activation. Indeed, SPR experiments by Infield and colleagues reveal that the serine cage, when introduced into a PKA substrate peptide, lowers its binding affinity to PKA by ∼1,000-fold. Finally, it is also possible that the increased activity reported for the S737A mutant (Wilkinson et al., 1997; Vais et al., 2004) is not a consequence of lack of phosphorylation at that site, but a direct consequence of the serine-to-alanine mutation. In that view the unphosphorylated form of the S737 side chain would serve an inhibitory role (e.g., by forming a hydrogen bond which helps to stabilize the unphosphorylated R domain in its wedged-in position) and the mutation itself would cause disinhibition. Such an interpretation would be in line with the increased P_o observed for unphosphorylated S737A compared to unphosphorylated WT CFTR (Hegedus et al., 2009), but would also imply that the serine cage at position 737 does not interfere with that inhibitory interaction.

In addition to the above conundrum, a multitude of further interesting questions remain to be answered. The suggested interplay between nearby positions 795 and 813 assumes that the cage itself at one position does not interfere with PKA binding to the other—if true, then the effect should not be reciprocal. The puzzlingly different fractional suppression of P_o caused by the S813A (twofold; Winter and Welsh, 1997) and S813-cage (eightfold; Infield et al., 2023) mutations also awaits clarification. The possibility of functional interactions between other pairs of positions remains to be addressed. Deconvolving the entire complexity of channel regulation will continue to entertain CFTR physiologists, and the powerful novel caged serine approach will certainly help decipher many of these open questions. Moreover, it should be applicable also to the broader field of phosphoregulation of enzymes in general.

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References

Chang, X.B., J.A. Tabcharani, Y.X. Hou, T.J. Jensen, N. Kartner, N. Alon, J.W. Hanrahan, and J.R. Riordan. 1993. Protein kinase A (PKA) still activates CFTR chloride channel after mutagenesis of all 10 PKA consensus phosphorylation sites. *J. Biol. Chem.* 268:11304–11311. https://doi.org/10.1016/S0021-9258(18)82125-1

Cheng, S.H., D.P. Rich, J. Marshall, R.J. Gregory, M.J. Welsh, and A.E. Smith. 1991. Phosphorylation of the R domain by cAMP-dependent protein kinase regulates the CFTR chloride channel. *Cell*. 66:1027–1036. https://doi.org/10.1016/0092-8674(91)90446-6

Csanády, L., K.W. Chan, D. Seto-Young, D.C. Kopsco, A.C. Nairn, and D.C. Gadsby. 2000. Severed channels probe regulation of gating of cystic fibrosis transmembrane conductance regulator by its cytoplasmic domains. *J. Gen. Physiol.* 116:477–500. https://doi.org/10.1085/jgp.116.3.477

Csanády, L., D. Seto-Young, K.W. Chan, C. Cenciarelli, B.B. Angel, J. Qin, D.T. McLachlin, A.N. Krutchinsky, B.T. Chait, A.C. Nairn, and D.C. Gadsby. 2005. Preferential phosphorylation of R-domain Serine 768 dampens activation of CFTR channels by PKA. *J. Gen. Physiol.* 125:171–186. https://doi.org/10.1085/jgp.200409076

Cui, G., J. Hong, Y.W. Chung-Davidson, D. Infield, X. Xu, J. Li, L. Simhaev, N. Khazanov, B. Stauffer, B. Imhoff, et al. 2019. An ancient CFTR ortholog informs molecular evolution in ABC transporters. *Dev. Cell*. 51: 421–430.e3. https://doi.org/10.1016/j.devcel.2019.09.017

Ellis-Davies, G.C.R. 2020. Useful caged compounds for cell physiology. *Acc. Chem. Res.* 53:1593–1604. https://doi.org/10.1021/acs.accounts.0c00292

Hegedus, T., A. Aleksandrov, A. Mengos, L. Cui, T.J. Jensen, and J.R. Riordan. 2009. Role of individual R domain phosphorylation sites in CFTR regulation by protein kinase A. *Biochim. Biophys. Acta*. 1788:1341–1349. https://doi.org/10.1016/j.bbamem.2009.03.015

Infield, D.T., M.E. Schene, F.S. Fazan, G.D. Galles, J.D. Galpin, and C.A. Ahern. 2023. Real-time observation of functional specialization among phosphorylation sites in CFTR. *J. Gen. Physiol.* 155:e202213216. https://doi.org/10.1085/jgp.202213216

Kaplan, J.H., B. Forbush III, and J.F. Hoffman. 1978. Rapid photolytic release of adenosine 5′-triphosphate from a protected analogue: Utilization by the Na:K pump of human red blood cell ghosts. *Biochemistry*. 17:1929–1935. https://doi.org/10.1021/bi00603a020

Liu, F., Z. Zhang, L. Csanády, D.C. Gadsby, and J. Chen. 2017. Molecular structure of the human CFTR ion channel. *Cell*. 169:85–95.e8. https://doi.org/10.1016/j.cell.2017.02.024

Mihályi, C., I. Iordanov, B. Töröcsik, and L. Csanády. 2020. Simple binding of protein kinase A prior to phosphorylation allows CFTR anion channels to be opened by nucleotides. *Proc. Natl. Acad. Sci. USA*. 117:21740–21746. https://doi.org/10.1073/pnas.2007910117

Picciotto, M.R., J.A. Cohn, G. Bertuzzi, P. Greengard, and A.C. Nairn. 1992. Phosphorylation of the cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem.* 267:12742–12752. https://doi.org/10.1016/S0021-9258(18)42339-3

Pless, S.A., and C.A. Ahern. 2013. Unnatural amino acids as probes of ligand-receptor interactions and their conformational consequences. *Annu. Rev. Pharmacol. Toxicol.* 53:211–229. https://doi.org/10.1146/annurev-pharmtox-011112-140343

Rich, D.P., H.A. Berger, S.H. Cheng, S.M. Travis, M. Saxena, A.E. Smith, and M.J. Welsh. 1993. Regulation of the cystic fibrosis transmembrane conductance regulator Cl^- channel by negative charge in the R domain. *J. Biol. Chem.* 268:20259–20267. https://doi.org/10.1016/S0021-9258(20)80723-6

Rich, D.P., R.J. Gregory, M.P. Anderson, P. Manavalan, A.E. Smith, and M.J. Welsh. 1991. Effect of deleting the R domain on CFTR-generated chloride channels. *Science*. 253:205–207. https://doi.org/10.1126/science.1712985

Seibert, F.S., J.A. Tabcharani, X.B. Chang, A.M. Dulhanty, C. Mathews, J.W. Hanrahan, and J.R. Riordan. 1995. cAMP-dependent protein kinase-mediated phosphorylation of cystic fibrosis transmembrane conductance regulator residue Ser-753 and its role in channel activation. *J. Biol. Chem.* 270:2158–2162. https://doi.org/10.1074/jbc.270.5.2158

Townsend, R.R., P.H. Lipniunas, B.M. Tulk, and A.S. Verkman. 1996. Identification of protein kinase A phosphorylation sites on NBD1 and R domains of CFTR using electrospray mass spectrometry with selective phosphate ion monitoring. *Protein Sci.* 5:1865–1873. https://doi.org/10.1002/pro.5560050912

Vais, H., R. Zhang, and W.W. Reenstra. 2004. Dibasic phosphorylation sites in the R domain of CFTR have stimulatory and inhibitory effects on channel activation. *Am. J. Physiol. Cell Physiol.* 287:C737–C745. https://doi.org/10.1152/ajpcell.00504.2003

Vergani, P., S.W. Lockless, A.C. Nairn, and D.C. Gadsby. 2005. CFTR channel opening by ATP-driven tight dimerization of its nucleotide-binding domains. *Nature*. 433:876–880. https://doi.org/10.1038/nature03313

Wilkinson, D.J., T.V. Strong, M.K. Mansoura, D.L. Wood, S.S. Smith, F.S. Collins, and D.C. Dawson. 1997. CFTR activation: Additive effects of stimulatory and inhibitory phosphorylation sites in the R domain. *Am. J. Physiol.* 273:L127–L133. https://doi.org/10.1152/ajplung.1997.273.1.L127

Winter,M.C., and M.J.Welsh. 1997. Stimulation of CFTR activity by its phosphorylated R domain. *Nature*. 389:294–296. https://doi.org/10.1038/38514

Zhang, Z., F. Liu, and J. Chen. 2018. Molecular structure of the ATP-bound, phosphorylated human CFTR. *Proc. Natl. Acad. Sci. USA*. 115:12757–12762. https://doi.org/10.1073/pnas.1815287115