look forward to future contributions directed to this problem.

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Filling the Gaps in Ca^{2+} Channel Regulation

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Regulation of the cardiac L-type Ca^{2+} channel has served well as a model for modulation of voltage-gated channels by phosphorylation (Hartzell and Duchatelle-Gourdon, 1992). In heart, norepinephrine released from sympathetic nerves acts on β-adrenergic receptors to increase the Ca^{2+} current (I_{Ca}) via a mechanism that is thought to involve cAMP-dependent phosphorylation of the Ca^{2+} channel. Although phosphorylation clearly controls Ca^{2+} channel function somehow, it has been difficult to prove that the Ca^{2+} channel itself is the ultimate target of phosphorylation and to relate its gating properties to its phosphorylated states. Some steps toward this goal have recently been published, one of which is the paper by Herzig et al. (1993) in this issue.

The problems in understanding how phosphorylation relates to channel gating became evident with the earliest single channel studies. On the basis of macroscopic currents, Reuter & Scholz (1977) proposed in 1977 that the increase in Ca^{2+} current in response to β-adrenergic agonists was due to an "increase in the number of functional conductance channels." When this was first tested at the single channel level in Reuter's (Reuter and Scholz, 1977) and Trautwein's (Brum et al., 1984) labs, however, it appeared that cAMP increased open probability (p_o) by accelerating the rate constants leading to channel opening with no change in the number of channels, N. Shortly thereafter Tsien's lab (Bean et al., 1984) showed that the increase in p_o was not large enough to explain the magnitude of the increase in I_{Ca} and that N increased as well. The apparent contradiction between these studies was resolved by assuming that channels could cycle slowly on the timescale of several seconds between two states, one competent to be opened by voltage and one nonfunctional (Tsien et al., 1986). β-Agonists do not increase the number of channels in the patch, but rather increase the probability that the channel will be in the available state. This conclusion was promoted by the finding (Cavalie et al., 1986; Ochi and Kawashima, 1990) that in a series of depolarizations, sweeps having channel openings ("active sweeps") and sweeps having no openings ("blank sweeps") were clustered in a strikingly nonrandom fashion. Although these studies provided a valuable hypothesis, the goal of elucidating the quantitative relationship between phosphorylation and Ca^{2+} channel function has remained elusive.

One reason for this difficulty is that a quantitative analysis of the clustering of active sweeps has not been accomplished. Traditional continuous-time Markov analysis of open and closed times is useful in analyzing the rapid (millisecond) gating transitions within a short depolarization, but channel inactivation rends traditional Markov analysis inadequate for long depolarizations, because closed time histograms are dominated by transitions from inactivated states. Furthermore, with continuous-time Markov analysis, there is no formal way of dealing with slow transitions that occur in the gaps between the depolarizing steps. Herzig et al. (1993) have filled this gap by applying discrete-time Markov analysis to understanding these slow transitions. This provides the first quantitative framework for dealing with the slow transitions. According to their model, there are multiple gating modes corresponding to permutations of two or more phosphorylation sites. Site 1 must be phosphorylated for the channel to be available to open and yield active sweeps. Phosphorylation of a second and possibly more sites alters the fast gating properties within channels that are available to open. As predicted from this model, isoproterenol increases the forward rate constant for the transition from an unavailable to an available state, and the protein phosphatase inhibitor okadaic acid decreases the backward rate constant for this reaction. In addition, isoproterenol decreases the backward rate constant as expected if cAMP-dependent phosphorylation of protein phosphatase inhibitor-1 were to activate this inhibitor and slow phosphatase activity.

This formal, quantitative analysis of the slow transitions in Ca^{2+} channel gating makes an important biophysical step toward proving Tsien's (Tsien et al., 1986) proposal that "the calcium channel has two phosphorylation sites, one that controls slow transitions between available and unavailable states,

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and a second site that regulates the fast kinetic changes."

Although Eckert and Armstrong (1987) have shown that Ca^{2+} channels in other cell types must be phosphorylated to open, support of this hypothesis for cardiac Ca^{2+} channels has been ambiguous. Recently, though, Katsushige Ono and Harry Fozzard (1992) have provided stronger support by showing that phosphorylation restores Ca^{2+} channel activity after rundown in excised patches. Other data that the cardiac Ca^{2+} channel does not require phosphorylation to function can be reconciled if the probability that the unphosphorylated channel will open is very small, but finite. This gating mode might not have been detected in the experiments of Herzig et al. (1993) and would have appeared as blank sweeps. This idea of dual phosphorylation sites is made even more attractive by a recent paper by Ono and Fozzard (1993) showing that low concentrations of okadaic acid increased channel availability without affecting channel open time, whereas higher concentrations increased both availability and open time. They interpret these data to indicate that one phosphorylation site controls availability and is dephosphorylated by a different phosphatase than the second site that controls open time.

Although the biophysical consequences of phosphorylation are becoming more clearly defined, biochemical correlates have lagged behind (Hartzell and Duchatelle-Gourdon, 1992). Despite convincing evidence that the skeletal muscle Ca^{2+} channel α_1 subunit is phosphorylated by cAMP-dependent protein kinase (PKA), evidence that the cardiac channel is phosphorylated is limited. There have been several reports that the α_1 subunit is not a substrate for PKA (Hartzell and Duchatelle-Gourdon, 1992). Furthermore, only two of the seven consensus sequences for PKA in the skeletal muscle α_1 subunit are conserved in the cardiac channel. Perhaps more problematic is the absence of an effect of cAMP on I_{Ca} generated by the cardiac α_1 subunit expressed in frog oocytes. Steps toward solving these two problems have recently been published. Yoshida et al. (1992) have found that the gene for the cardiac α_1 subunit expressed in CHO cells produces two forms of Ca^{2+} channel: a minor 250-kDa form which is phosphorylated in response to dibutyryl cAMP and a major 200-kDa species (proteolytic fragment?) which is not phosphorylated. Dibutyryl cAMP also produced a one-fold increase in I_{Ba}. In contrast, I_{Ca} produced by the cardiac α_1 subunit expressed in frog oocytes is not increased by cAMP unless the β subunit of the channel is coexpressed (Klöckner et al., 1992). These data raise the question whether one or both of the putative phosphorylation sites that regulates Ca^{2+} channel function is on the α_1 subunit, another subunit, or another regulatory protein. The slow kinetics of the increase in I_{Ca} in response to cAMP may suggest that an intermediate protein is phosphorylated (Frace et al., 1993).

Although the dogma that the cardiac Ca^{2+} channel is regulated by cAMP-dependent phosphorylation of the channel itself is an attractive model, it is clear that there are still many gaps that remain in tying the biophysics of channel gating and availability to channel phosphorylation. If there are indeed multiple phosphorylation sites, possibly on different subunits or affected by the presence of different subunits, site directed mutagenesis of putative phosphorylation sites should be able to distinguish between sites that regulate gating and availability.

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Calcium Oscillations and Waves: Is the IP_3R Ca^{2+} Channel the Culprit?

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In this issue of *Biophysical Journal* Atri et al. (1993) present evidence from modeling studies that the inositol 1,4,5-trisphosphate receptor (IP_3R) is respon-

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