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Phosphorylation and the Control of Calcium Fluxes

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Phosphorylation and the control of calcium fluxes

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Cell activation, e.g. stimulus–contraction or stimulus–secretion coupling, is brought about by a 100-fold increase in cytosolic free Ca^{2+} concentration from 0.1 to 10 μM, upon release of Ca^{2+} from intrareticular or extracellular stores along the concentration gradient. A return to steady state is achieved by either Na^{+}–Ca^{2+} exchange or ATP-dependent Ca^{2+} transport against the concentration gradient.

Both processes, Ca^{2+} influx and Ca^{2+} efflux, are regulated by sophisticated covalent mechanisms. The positive inotropic effect of adrenalin is mediated by the cyclic-AMP-dependent phosphorylation of cardiac sarcolemmal proteins, among which calciductin is the major phosphate acceptor. Upon cyclic-AMP-dependent phosphorylation, the slow Ca^{2+} channel is activated 3.5 times above its basal low- conductance state, and retains its characteristics, competition by divalent metals, inhibition by La^{3+} and Ca^{2+} entry blockers. The adrenalin-induced abbreviation of systole is also explained in terms of the dual phosphorylation of the cardiac sarcoplasmic reticulum calcium pump activator, phospholamban, by cyclic-AMP-dependent protein kinase on the one hand and Ca^{2+}–calmodulin-dependent phospholamban kinase on the other. Calciductin and phospholamban are closely similar acidic proteolipids. A phospholamban-like protein is also found in platelet Ca^{2+}-accumulating vesicles, where its cyclic-AMP-dependent phosphorylation doubles the rate of Ca^{2+} efflux. These observations raise the possibility that calcium fluxes are regulated by phosphorylation of membrane-bound proteolipids. More generally, phosphorylation modulates K^{+}, Na^{+} and Ca^{2+} fluxes through membranes, i.e. the general excitability properties of the cell.

Introduction

Ca^{2+} ions play a major role in excitation–contraction coupling in all systems where movement is generated by the actin activation of myosin Mg^{2+}-ATPase activity (Ebashi, 1980), in muscle as well as in non-muscle cells. Even though Ca^{2+} triggers contraction in different systems through different mechanisms, some common features account for its general use as a fast signal.

In the steady state of a resting cell, Ca^{2+} ions are segregated in three major compartments, if the role of mitochondria is considered as negligible over a short timescale. The first compartment, the cytosol, is characterized by a low free Ca^{2+} concentration (0.1 μM), while the extracellular space, on the other side of the plasma membrane, contains approximately 2 mM Ca^{2+}. Finally, the endoplasmic (sarcoplasmic) reticulum is capable of accumulating as much as 10–20 mM Ca^{2+} in the reticular stores. Such compartments may be schematized as shown in figure 1, where the plasma and reticular membranes are impermeable to Ca^{2+}. The steep Ca^{2+} gradients across membranes can only be maintained at the expense of ATP hydrolysis, which is either coupled directly to active Ca^{2+} transport by calcium pumps (Ca^{2+}-Mg^{2+}-ATPases) (Caroni & Carafoli 1981*a*; Moller *et al.* 1982) or used to build a Na^{+} gradient by the Na^{+},K^{+}-ATPase. The Na^{+} gradient is in turn the driving force for Ca^{2+} efflux through the Na^{+}–Ca^{2+} exchange in the sarcolemmal membrane (Pitts 1979; Philipson & Nishimoto 1981).

The steepness of the Ca^{2+} gradients allows Ca^{2+} to be used as a trigger in the excitation–contraction or excitation–secretion coupling provided that mechanisms exist that open Ca^{2+} channels through the plasma and reticular membranes. The rapid flow of Ca^{2+} ions along the concentration gradient into the cytosol increases the free Ca^{2+} concentration 100-fold up to 10 μM. The timing of the channel gate opening is precise enough to limit the Ca^{2+} increase to the above value, a prerequisite for the cell's ability to return to steady state by the ATP-dependent calcium efflux mechanisms described above. Negative feedback mechanisms exist

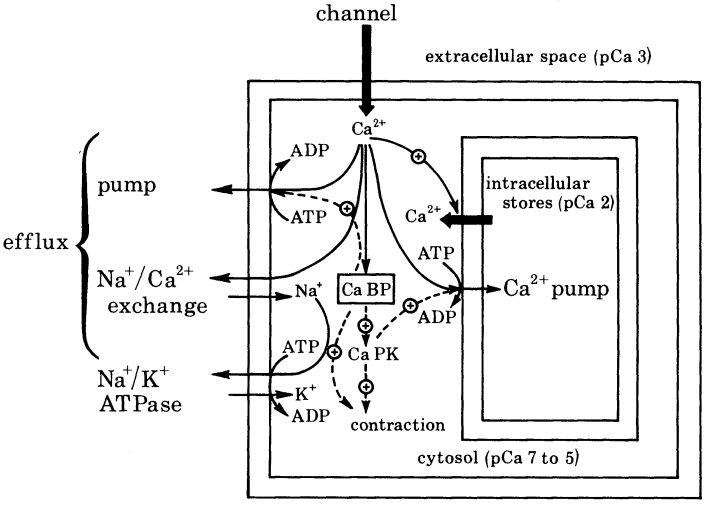

FIGURE 1. A scheme of the major cell compartments. Heavy arrows indicate Ca^{2+} channels in sarcolemmal and reticular membranes. CaBP, calcium-binding protein; CaPK, Ca^{2+}-calmodulin-dependent protein kinases; pCa = $-\log_{10}$[free Ca^{2+}]; + stands for activation. Solid arrows indicate ion binding or fluxes. (From Demaille & Pechère (1983), with permission.)

that increase the rate of Ca^{2+} efflux in response to the increase in cytosolic Ca^{2+}, e.g. by activation of the plasmalemmal Ca^{2+}–Mg^{2+}-ATPase by the Ca^{2+}-loaded calmodulin (Caroni & Carafoli 1981*a*). Calmodulin is indeed capable of binding Ca^{2+} ions with an approximate pK_d of 6, i.e. will be essentially Ca^{2+} free at pCa 7 and saturated by three Ca^{2+} ions at pCa 5, as shown by Haiech *et al.* (1981).

The above brief description of the Ca^{2+} movements into and out of the cytosol suggest that contractility or secretion may be regulated in a number of ways, for instance by phosphorylation of membrane proteins involved in either the influx or the efflux of Ca^{2+} ions.

Our attention will focus on the two classes of protein kinases that mediate the effects of the two major intracellular second messengers, namely Ca^{2+} ions and cyclic adenosine 3′:5′-monophosphate (cyclic AMP). It has indeed been shown that cyclic-AMP-dependent protein kinases catalyse the phosphorylation of membrane proteolipids that may be involved in the control of Ca^{2+} influx or efflux (Kirchberger *et al.* 1974; Kirchberger & Tada 1976; Rinaldi *et al.* 1981, 1982). Also a Ca^{2+}–calmodulin-dependent protein kinase has been described that catalyses the phosphorylation of phospholamban, the activator of the cardiac sarcoplasmic reticulum calcium pump (Le Peuch *et al.* 1979).

Such phosphorylations may help in explaining the following physiological facts. First, hormones and neurotransmitters are capable of modulating contractility. For instance, adrenalin, a β-adrenergic agonist, increases the cardiac contractile force and beat frequency, i.e.

induces positive inotropic and chronotropic effects. Second, the variations in the cytosolic free Ca^{2+} concentrations are strictly limited by built-in mechanisms that prevent a lethal Ca^{2+} overload that might occur, e.g. after prolonged adrenalin stimulation. This review addresses the above questions and describes the molecular mechanisms that underlie such physiological responses.

Phosphorylation modulates calcium efflux from cytosol

Ca^{2+} efflux to the extracellular space is carried out by Na^{+}–Ca^{2+} exchange (Philipson & Nishimoto 1981) and by the sarcolemmal Ca^{2+} pump (Caroni & Carafoli 1981*a*). It was recently reported that the regulation of the cardiac sarcolemmal Ca^{2+}-ATPase, which is activated by calmodulin through direct interaction, is also achieved by cyclic-AMP-dependent phosphorylation (Caroni & Carafoli 1981*b*). It is not yet known whether the 150 kDa pump itself or an associated protein is the substrate of cyclic-AMP-dependent protein kinases. Nevertheless, such a mechanism would account for the disposal of excess Ca^{2+} accumulated during β-adrenergic stimulation, which opens slow Ca^{2+} channels, as will be discussed below.

The covalent regulation of the cardiac sarcoplasmic reticulum calcium pump is now known in a much greater detail. One of the major effects of adrenalin on the cardiac muscle is indeed the abbreviation of systole, which is part of the positive chronotropic effect. The systole abbreviation results from the activation of the cardiac sarcoplasmic reticulum calcium pump and the subsequent acceleration of relaxation. An integral membrane protein, phospholamban, was identified by Kirchberger and coworkers (Kirchberger *et al.* 1974; Kirchberger & Tada 1976) as the substrate of cyclic-AMP-dependent protein kinase. Phospholamban phosphorylation is responsible for the pump activation.

There is now evidence from this laboratory (Le Peuch *et al.* 1979, 1980*a*, *b*) that the modulation of the cardiac sarcoplasmic reticulum pump activity is in fact more complex than initially foreseen. Phospholamban was indeed found to be phosphorylated at two different sites by the catalytic subunit of cyclic-AMP-dependent protein kinase and by the membrane-bound Ca^{2+}–calmodulin-dependent phospholamban kinase. The two enzymes are not part of a metabolic cascade similar to that found in glycogenolysis, where cyclic-AMP-dependent protein kinases activate phosphorylase kinase, another Ca^{2+}–calmodulin-dependent protein kinase.

Phospholamban kinase is entirely dependent on exogenous Ca^{2+} and calmodulin, with half-maximal activity at 70 nM calmodulin. Interestingly enough, phosphorylase kinase and phospholamban kinase are both capable of catalysing the phosphorylation of phospholamban and glycogen phosphorylase *b*, pointing to a similar substrate specificity.

Phospholamban phosphorylation in the presence of Ca^{2+} and calmodulin increases moderately but significantly the rate of Ca^{2+} uptake by cardiac sarcoplasmic reticulum vesicles, an effect that is amplified upon simultaneous cyclic-AMP-dependent phosphorylation.

There are some hints as to the organization of the Ca^{2+} pumping complex within the membrane plane. Indeed, phospholamban copurifies with the Ca^{2+}-ATPase when the latter is purified by the van Winkle *et al.* (1978) procedure, which includes detergent solubilization of vesicles and sucrose density gradient fractionation (Le Peuch *et al.* 1980*b*). Also, more recent experiments (C. J. Peuch & J. G. Demaille, unpublished results) point to the copurification of phospholamban kinase with the pump–phospholamban complex, leading to the hypothetical

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model described in figure 2. If this model were true, the cyclic-AMP-dependent site of phospholamban would be expected to be either empty or fully phosphorylated according to the β-adrenergic status of the heart, i.e. β-blocked or β-stimulated respectively. In contrast, the extent of phosphorylation of the Ca^{2+}–calmodulin-dependent site would reflect the average Ca^{2+} concentration of the myocardial cytosol during the previous beats. In other words, only half of the phospholamban phosphorylation would be susceptible to β-antagonists. This was indeed shown to be so when rat hearts were perfused by the Langendorff technique in the presence of either isoprenalin or propranolol (Le Peuch *et al.* 1980*a*). The Ca^{2+}–calmodulin-dependent phosphorylation may then be seen as a sensor device that may help in preventing Ca^{2+} overload.

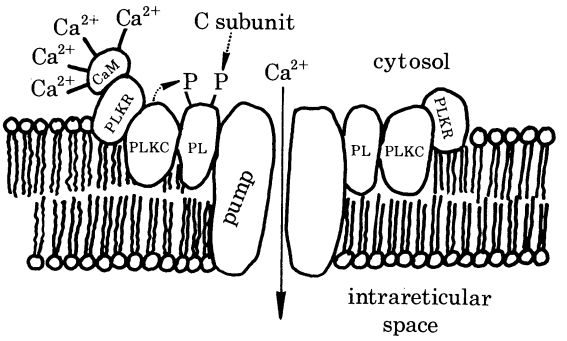

FIGURE 2. Hypothetical scheme of the calcium pump functional unit in cardiac sarcoplasmic reticulum. PL, phospholamban monomer; PLKC and PLKR, the catalytic and regulatory subunits or domains of phospholamban kinase, respectively; CaM, calmodulin; C subunit, the catalytic subunit of cyclic-AMP-dependent kinase. This scheme does not imply that all polypeptide chains are present in stoichiometric amounts. (From Cavadore *et al.* (1981), with permission.)

This proposal was recently confirmed and substantiated by Louis & Maffitt (1982) who showed that phospholamban kinase might only be activated at Ca^{2+} concentrations greater than those required for formation of the pump phosphoprotein intermediate, suggesting a role in the rescue from calcium overload.

Phospholamban kinase has not yet been purified and characterized. Though it has the same substrate specificity as phosphorylase kinase, it needs exogenous calmodulin for activation and is therefore inhibited by such calmodulin antagonists as fluphenazine, in contrast to phosphorylase kinase. Moreover, antibodies elicited against skeletal phosphorylase kinase, capable of inhibiting cardiac phosphorylase kinase, do not inhibit phospholamban kinase (C. J. Le Peuch & J. G. Demaille, unpublished results). The above findings argue in favour of the existence of two distinct enzymes.

PHOSPHORYLATION MODULATES CALCIUM INFLUX INTO CYTOSOL

The abrupt release of Ca^{2+} from the extracellular space or intrareticular stores into the cytosol is mostly triggered by membrane depolarization or by receptor-operated Ca^{2+} channels. It is also modulated by phosphorylation–dephosphorylation.

Campbell & MacLennan (1982) have recently proposed that the Ca^{2+}–calmodulin-dependent phosphorylation of a 60 kDa protein of skeletal muscle sarcoplasmic reticulum is involved in closing a gate of the Ca^{2+}-release channel, previously open in the absence of a proton gradient. Provided that it is kinetically compatible with the rate of the channel closing, this mechanism

would explain the observed higher limit of 10 μM in the cytosolic Ca^{2+} concentration through a negative feedback loop involving calmodulin.

Regulation of contractility by hormones, such as the positive inotropic effect of β-adrenergic agonists, is another well documented example of the modulation of Ca^{2+} influx by phosphorylation. It is well known that the slow inward current, mainly carried by Ca^{2+} in cardiac muscle, is stimulated by adrenalin (Pollack 1977) and by cyclic AMP synthesized in response to the β-adrenoceptor occupancy. Since cyclic AMP acts only through the dissociation and activation of cyclic-AMP-dependent protein kinase, it is plausible that β-agonists act through the cyclic-AMP-dependent phosphorylation of the Ca^{2+} channel. That has been elegantly confirmed by microinjection of the purified catalytic subunit of cyclic-AMP-dependent protein kinase, which increases the height of the plateau and the amplitude of the slow inward current, while the injected regulatory subunit shortens the action potential duration (Osterrieder *et al.* 1982). This explains why an extensive search for sarcolemmal substrates of cyclic-AMP-dependent protein kinases was conducted in several laboratories (Jones *et al.* 1979, 1980, 1981; Lamers & Stinis 1980; Lamers *et al.* 1981; St-Louis & Sulakhe 1979; Walsh *et al.* 1979). One of these substrates, calciductin, emerges as a major substrate (Rinaldi *et al.* 1981, 1982). It is present as a 23 kDa protein, together with an 11.5 kDa interconvertible form.

The sarcolemmal phosphorylation is accompanied by a 3.5-fold increase in the ATP-independent, voltage-dependent Ca^{2+} uptake into sarcolemmal vesicles, whereas in the absence of phosphorylation such uptake was still *ca.* 28 % of the maximal value, pointing to the possible existence of a low-conductance state of the sarcolemmal slow Ca^{2+} channel (Rinaldi *et al.* 1982). Obviously the possibility exists that the major substrate, calciductin, is not part of the Ca^{2+} channel, and that the correlation between calciductin phosphorylation and increase in the voltage-dependent Ca^{2+} entry merely reflects the phosphorylation of a channel protein distinct from calciductin. It has even been proposed that calciductin is not an intrinsic sarcolemmal protein (Manalan & Jones 1982).

Another cell in which cyclic AMP plays an important role is the blood platelet, in which Ca^{2+} ions control shape change, secretion and aggregation (Feinstein 1980). Calcium-accumulating vesicles prepared from sonicated platelets contain a 23 kDa 'phospholamban-like' protein that is phosphorylated by cyclic-AMP-dependent protein kinase (Kaser-Glanzmann *et al.* 1978, 1979). Phosphorylation of this protein was claimed either to activate the Ca^{2+} pump (Kaser-Glanzmann *et al.* 1979) or to accompany a doubling in the rate of Ca^{2+} efflux from the calcium-accumulating vesicles (Le Peuch *et al.* 1982). Though the above findings are discrepant, they both point to a modulation of platelet Ca^{2+} fluxes by cyclic-AMP-dependent phosphorylation.

Properties of phosphorylatable proteolipids

The above studies stress the importance of membrane phosphorylatable proteolipids, examplified by phospholamban, calciductin and the 'phospholamban-like' protein of platelet vesicles. Phospholamban (Le Peuch *et al.* 1980*b*) and calciductin (Rinaldi *et al.* 1982) are acidic proteolipids that can be extracted by chloroform–methanol mixtures provided that they are protonated in the presence of dilute acid. Both phospholamban and calciductin may be purified by high-performance liquid chromatography of organic solvent extracts on microparticulate silica (Capony *et al.* 1983). Their amino acid compositions are very similar and they comigrate upon two-dimensional gel electrophoresis, pointing to the same molecular mass and

isoelectric point. In fact they appear to be identical, a conclusion reached independently by Manalan & Jones (1982). Whether they belong to different membranes or represent a cross-contamination of sarcolemma by a sarcoplasmic reticulum protein is still a matter of controversy. At any rate calciductin is found in sarcolemmal vesicles as a major component, amounting to *ca.* 2% by mass of total sarcolemmal proteins.

Another interesting feature of membrane proteolipids is their behaviour in sieving gel electrophoretograms, where they migrate, according to their previous exposure to heat or to non-ionic detergents, with an apparent molecular mass of 22, 11 or even 5.5 kDa (Le Peuch *et al.* 1980*a*; Kirchberger & Antonetz 1982). This would mean that a short polypeptide chain of *ca.* 50 residues accommodates both the hydrophobic helix, by which the proteolipid is inserted into the membrane plane and probably interacts with the modulated protein, and the phosphorylatable head protruding into the cytosol.

Conclusion

The above examples, borrowed mostly from cardiac physiology, point to the importance of the covalent regulation of calcium fluxes. There is, however, evidence that other ion fluxes are also modulated by protein phosphorylation. For instance the α subunit of the sodium channel purified from rat brain is a substrate of cyclic-AMP-dependent protein kinase (Costa *et al.* 1982). Also the Ca^{2+}-activated K^+ conductance of snail neurons is modulated by cyclic-AMP-dependent phosphorylation (De Peyer *et al.* 1982), and the serotonin effects on *Aplysia* neurones are best explained in terms of cyclic-AMP-dependent phosphorylation, which regulates the neuronal electrical properties (Lemos *et al.* 1982; Siegelbaum *et al.* 1982). Membrane protein phosphorylation may therefore be a general mechanism for the modulation of Ca^{2+}, Na^+ and K^+ movements through the plasma membrane, i.e. of the cell response to external stimuli.

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