Membrane Phosphorylation in Vascular and Cardiac Muscles: Comparative Analysis and Functional Role

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Abstract

Advances in the investigation into the field of cytosol Ca^{2+} level regulation by second messengers in cardiomyocytes and smooth-muscle cells are reviewed with special reference to the major differences in this regulation between heart and blood vessels. The effects of cAMP, cGMP, Ca^{2+}, calmodulin, diacylglycerol and polyphosphoinositides on the Ca^{2+}-channel, Ca^{2+}-ATPase of plasmalemma and sarcoplasmic reticulum as well as cell-membrane Na^{+}-Ca^{2+}-carrier activities are considered. Special emphasis is placed on the necessity of taking into account the compartmentalization of second messengers, enzymes of second messenger metabolism, protein kinases and protein phosphatases in cardiac and smooth-muscle cells during extrapolation of *in vitro* data to an *in situ* situation as well for the choice of therapeutic methods based on the modulation of strength and duration of intracellular signals. Possible role of impaired membrane-bound protein phosphorylation in cardiac insufficiency and atherosclerosis is discussed.

Key words: heart, vascular smooth muscle, Ca^{2+}, second messengers, phosphorylation, sarcoplasmic reticulum, plasmalemma

BAM 1 (3): 209-235, 1991

It is known that cAMP, cGMP, calmodulin and phospholipids, either by activating the corresponding protein kinases or by modulating the activities of phosphoprotein phosphatases and enzymes of second messenger metabolism, participate in a rapid and reversible phosphorylation of a considerable number of intracellular protein substrates. Thus, the regulation of cardiomyocyte (CMC) and vascular smooth-muscle cell (SMC) contractility is realized mainly via the cyclic nucleotide-dependent and Ca^{2+}-dependent phosphorylation of target proteins in the sarcolemma (SL), sarcoplasmic reticulum (SR) and myofibrils of these cells.

Contraction of myocardium and vascular smooth-muscle cells is triggered by Ca^{2+}. Trigger properties of Ca^{2+} are caused in particular by a low concentration of these ions in the myocyte cytoplasm, as compared with that in the extracellular medium. Therefore, it is possible for CMCs and vascular SMCs to use the injection of relatively small amounts of Ca^{2+} into the cytoplasm as a signal that leads to the initiation of contraction.

Myocardial contraction results from the Ca^{2+} binding to troponin C, whereas the regulation of myofibrillar complex activity with the use of second messengers is realized mainly with the participation of troponin. The low phosphate turnover rate in the light chains of cardiac myosin seems to rule out the regulatory role of phosphorylation of these proteins [70]. It is generally believed that cAMP-dependent phosphorylation of cardiac troponin I leads to the decrease in myofibril responsiveness to Ca^{2+} [3, 172]. Troponin has not been found in smooth muscle, in contrast to myocardium. The functional roles of cardiac troponin C and troponin I are close to the regulatory importance of smooth-muscle calmodulin and caldesmon [192], notwithstanding the fact that troponin I: actin ratio in the heart is equal to 1:7 whereas caldesmon:actin ratio in smooth-muscle thin filaments is 1:28 [131]. Calmodulin is a main Ca^{2+} intracellular receptor in the smooth muscle, activating in a complex with Ca^{2+}, myosin light chain kinase. It is believed that myosin light chain phosphorylation regulates the contractility of vascular smooth muscle [191]. Caldesmon inhibits the activity of actomyosin ATPase in the absence of Ca^{2+}. It has been suggested that caldesmon phosphorylation hinders this inhibition [145]. In the present paper, the role of troponin I, myosin light chain and

caldesmon phosphorylation in the regulation of CMC and vascular SMC functional activities is not discussed, since this important question is worth a separate consideration.

This review briefly summarizes recent advances in the research into CMC and SMC cytosol free Ca^{2+} level regulation by second messengers, putting a special emphasis on the basic differences in this regulation between the myocardium and vascular smooth muscle. It is known, for example, that cAMP and cGMP regulate Ca^{2+} flow in both directions through the CMC sarcolemma and SMC plasmalemma; at the same time, biochemical mechanisms of cyclic nucleotide intracellular action in these tissues are still far from being completely understood. Thus, it has been determined that, in CMCs [208] and SMCs [178] stimulated with depolarization or various agonists, cyclic AMP performs the regulation of free Ca^{2+} levels. However, it is still unclear why does cAMP, while both promoting the contraction and accelerating the relaxation of CMCs, influence only the contractility of SMCs reducing their contraction, whereas the effect of this nucleotide on the relaxation of vascular smooth-muscle cells is rather problematic [52, 206]. In general, it should obviously be accepted that second messengers such as cAMP, cGMP, Ca^{2+}, polyphosphoinositides and diacylglycerol act in a coordinated manner as regards not only the mutual regulation of levels of these signal molecules but also the similar metabolic transformations in CMCs and SMCs [10, 165].

Calcium Channels of the Cell Membrane

Phospholipid bilayer of the cell membrane contains the protein and glycoprotein molecules including those participating in Ca2+ transport. Three ways via which Ca2+ is transported through the cell membrane can be distinguished as follows: Ca2+-ATPase, Na+-Ca2+ exchange and slow channels.

At the moment of contraction, free Ca^{2+} concentration in the CMC cytosol grows from 0.05-0.2 μM at rest to 1-10 μM [20, 75], while in the SMC cytosol under the same conditions, it increases from 0.1 μM to 0.7-1.0 μM [227]. Myocardial contraction is initiated by the penetration of Ca^{2+} into the cell via voltage-dependent Ca^{2+} channels of the SL. This amount of Ca^{2+}, sometimes called a "trigger" one, equals according to different estimates from 2-3% [229] to 20-30% [45] of the amount required for a contraction response to manifest itself. Slow Ca^{2+} channel blocking and, consequently, the blocking of Ca^{2+} penetration into CMCs with antagonists verapamyl, diltiazem, Co^{2+}, La^{3+} etc. suppresses the contraction completely without exerting any significant influence on the action potential; in other words, the contraction is dissociated from the excitation. These data provide a support for the hypothesis [49] that Ca^{2+} entering the cell stimulates the release of a much greater amount of these ions from the SR as compared with the "trigger"' amount.

It has become known by now that β-adrenergic agonists increase the myocardial contractility by opening Ca^{2+} channels during depolarization [4, 174, 194, 213]. It has been established that this effect results from the activation of cAMP-dependent protein kinase (A-kinase) that phosphorylates channel proteins [194, 213].

Modulation of Ca^{2+} flow through cardiac slow channels can be achieved by means of cAMP injection in liposomic envelopes or else by pressure-injecting cholera toxin (an adenylate cyclase activator) or thermostable A-kinase inhibitor [194] . The effect of β-adrenergic agonists can be also reproduced by the injection of methyl xanthins e.g. of theophyllin, cAMP phosphodiesterase (PDE) inhibitors [174]. All these findings confirm the hypothesis about Ca^{2+} channel phosphorylation.

Fluoride, a phosphatase inhibitor, also renders calcium channels capable of electrodependent activation, probably by hindering their dephosphorylation [174, 229]. Hence, the decrease in dephosphorylation rate exerts the same influence on slow Ca^{2+} channels as does the acceleration of phosphorylation. It has been shown that protein phosphatase 2B (calcineurin) and phosphatase 2A inhibit the slow Ca^{2+} flow through the myocardial SL [92]. However, the leading role in such inhibition seems to belong to protein phosphatase I, a main phosphatase of the myocardium [92, 132].

Catecholamines, while influencing the protein phosphorylation, do not change the parameters of ion permeability of those slow channels in the cardiac SL that are already in action but they increase the time during which the channels are open or they open some new Ca^{2+} channels [194]. Phosphorylation can activate slow Ca^{2+} channels by changing their conformation, which allows the channel either to open itself during depolarization or to effectually increase the diameter of a pore filled with water, providing the possibility for Ca-ions to pass through the channel. Dephosphorylated channels are electrically inactive. Depending on cAMP level and protein phosphatase activity, there is a balance in CMCs between the phosphorylated and dephosphorylated channels. Substances that increase myocardial contractility by means of raising the cAMP level can also increase the proportion of phosphorylated Ca^{2+} channels [114]. As a substrate for phosphorylation, one of the proteins forming the slow channel itself may serve, as well as an adjacent regulatory protein closely connected with the Ca^{2+} channel. A similar picture can be found in the complex of phospholamban with Ca^{2+}-ATPase in the SR [194].

A 11.7 kDa protein isolated from the cardiac SL and containing a considerable amount of Ca^{2+}-binding sites was reported to provide a possible substrate for a cAMP-dependent protein kinase of the slow channels [213]. According to several authors [88, 226] it is the cAMP-dependent phosphorylation of cardiac SL's low-molecular proteins that may be directly associated with the regulation of slow Ca^{2+} channel function. Velema et al. described a 17.5 kDa glycoprotein from the cardiac SL that could be phosphorylated by cAMP-dependent protein kinase [213]. This finding is of especial interest since glyco-

proteins take part in the Ca-ion binding in the glycocalix.

Cardiac SL protein phosphorylation can be also activated by insulin. Thus, a proteolipid with molecular mass of 15.5 kDa contains a protein subunit 3.6 kDa which can be phosphorylated at Ser sites in the presence of insulin [219]. The authors suggested that this proteolipid could perform a transport function in the membrane, while forming the channels permeable for ions and certain water-soluble compounds.

In the cardiac SL preparations, a considerable amount of protein substrates for A-kinase has been found by now [4, 88, 110, 111, 174, 213, 226]. Such a variety of results is often due to the differing conditions under which membrane preparation specimens are processed before electrophoresis. For instance, a 24 kDa protein heated at up to 95-100°C will produce 9 kDa polypeptide fragments, which is not the case when incubating this protein at 30°C in the presence of sodium dodecyl sulphate [110]. Non-optimal conditions of pre-phoretic SL membrane treatment can as well lead to the appearing of rapidly aggregating high-molecular protein complexes.

Among SL proteins, of special interest is calciductin discovered and described by Rinaldi et al. [175]. Calciductin, a protein with M_r 23 kDa, is, in the authors' opinion, a main target protein for A-kinase in the cardiac SL. Calciductin phosphorylation in the presence of cAMP does not change the properties of Na^+-Ca^{2+} carrier but is accompanied by a more than twofold increase in electrodependent Ca^{2+} absorption in SL vesicles [176]. This process probably reflects the functioning of slow Ca^{2+} channels. The total combination of data thus obtained led the authors to a conclusion that β-agonists realize their regulating action on the myocardium via the phosphorylation of calciductin which both may itself form a channel or serve in its phosphorylated form as a Ca^{2+} channel activator [175, 176].

Calciductin phosphorylation can be highly efficient. In the first place, A-kinase is localized in submembrane compartments in close vicinity to calciductin [89]. In the second place, calciductin is an excellent phosphate acceptor. Thus, submaximum calciductin phosphorylation and Ca^{2+} absorption activation can be achieved with the dissociation of only 6% of the total amount of cAMP-dependent protein kinase holoenzyme [176]. Hence, calciductin can be efficiently phosphorylated *in vivo* under a minimum CMC stimulation by β-agonists.

Calciductin is phosphorylated virtually with the participation of A-kinase alone [193], although the involvement of Ca^{2+}-calmodulin-dependent protein kinases (B kinases) also cannot be excluded [89]. In this case, it is possible to suggest that the increase in low-molecular substrate phosphorylation as calcium enters the myocyte may lead to the maintenance of this process for a longer time due to the positive feedback effect. Indeed, cardiac SL preparations contain endogenous Ca^{2+}-dependent protein kinase whose activity will grow several times higher upon addition of calmodulin [214]. Maximum Ca^{2+} transport stimulation was observed in the SL in the presence of catalytic A-kinase subunit as well as of calmodulin, the effect being more than additive [214]. Stimulation of Ca^{2+} transport through the SL by means of protein phosphorylation takes place under both low and saturating concentrations of this ion, whereas in the SR this is the case only for Ca^{2+} concentrations of not more than 1 μM.

Ca^{2+} penetration into the phosphorylated vesicles is inhibited by verapamyl, lanthanum and bivalent cathions. These agents do not influence Ca^{2+} penetration through the nonphosphorylated channels [176]. Thus, in the authors' opinion, calciductin phosphorylation promotes the opening of new channels, which become identical with those already acting in the SL. However, it is noteworthy that the above results were obtained, during the study of SL preparations probably significantly contaminated with SR vesicles. This suggestion is based on the fact that a high level of oxalate-dependent Ca^{2+} absorption (300-400 nmole Ca^{2+} per mg protein) was observed in this study. In addition, a significant proportion of the protein studied could have been represented by phospholamban [130]. Indeed, phospholamban appears in SR preparations at relatively high concentrations of 1.5-2.0 nmole per mg protein [202]; at the same time, 23 kDa protein contents in SL preparations is about 0.1 nmole/mg [95]. Therefore, even an insignificant contamination of SL preparations with SR fragments can result in the appearing of additionally assayed amounts of 23 kDa protein. Nevertheless, it is believed that the presence of calciductin in the SL is not caused by the contamination with SR fragments and that a protein identical with phospholamban really belongs to the SL [80]. Calciductin and phospholamban significantly differ from each other in their capacities of being phosphorylated under the influence of various protein kinases [176]. These differences may be associated both with the specific structure of phosphosubstrates themselves and with different protein and lipid environments in the SL and SR membranes.

Undoubtedly, further investigation using highly purified SL preparations is required in order to evaluate the influence of 23 kDa protein phosphorylation on Ca^{2+}-transporting function of the SL. It was even recommended to avoid the usage of the term "calciductin" as regards 23 kDa protein until the participation of this protein in the regulation of slow Ca^{2+} activity is finally proved [154]. In the meanwhile, the search for regulatory proteins that can be phosphorylated by cAMP-dependent protein kinase in the slow SL channels is continuing. This search is stimulated by promising results obtained in this field. Thus, injection of catalytic A-kinase subunit into the CMCs is accompanied by the increase in action potentials duration and amplitude of slow Ca^{2+} flow into the myocyte at the plateau phase of action potential. Injection of regulatory subunits of this enzyme into the CMCs leads to an opposite effect, which is probably due to the binding of free catalytic subunits [150].

The data available on the regulatory role of Ca^{2+}-calmo-

dulin-dependent phosphorylation in the SL contradict each other. For instance, as the intracellular Ca^{2+} level rises up to 10^{-5}M, Ca^{2+}-calmodulin-protein kinase complex is being formed and certain protein substrates of the SL such as 54 kDa and 44 kDa proteins are being phosphorylated [209]. These events are accompanied by the closing or reduction in number of Ca^{2+} channels, which stops the penetration of Ca^{2+} into the cell [36]. At the same time, it has been demonstrated that calmodulin inhibitors such as trifluoperazine reduce the slow Ca^{2+} flow in the heart [149, 194]. Alpha adrenergic agonists, in their turn, increase the penetration of Ca^{2+} into the cell via slow channels [194]. As it is known, α-adreno-reception stimulates phospatidyl inositol (PI) cycle to generate inositol trisphosphate (IP_3) and diacylglycerol. IP_3 promotes Ca^{2+} release from the SR depot; diacylglycerol and Ca^{2+}, in their turn, activate C-kinase, a Ca^{2+}-phospholipid-dependent enzyme. However, there is still no direct evidence for the participation of C-kinase in the regulation of slow Ca^{2+} channel permeability, although a considerable amount of proteins that can be phosphorylated by this kinase was found in the myocardial SL [216].

cGMP and cAMP exert two opposite effects on the slow Ca^{2+} flow in the heart. Thus, in the frog heart ventricles, cAMP (1-5 μM) stimulates a 10-13-fold increase in the entering slow Ca^{2+} flow, whereas cGMP (0.3-20 μM) inhibits this process [216]. cGMP pressure-injection into the CMCs also rapidly and efficiently inhibits the slow entering Ca^{2+} flow. Similar result was also obtained with the injection of cGMP in liposomic envelopes [194]. Acetylcholine inhibits slow Ca^{2+} flow probably by activating guanylate cyclase associated with muscarinic receptors, which leads to a subsequent increase in cGMP level. There are three different points of view on the mechanism of inhibitory cGMP action on the slow Ca^{2+} flow in the heart. Thus, it has been determined that cGMP can inhibit slow Ca^{2+} flow only after the rise in cAMP level in the CMC and does not influence the baseline, non-stimulated by cAMP, entering Ca^{2+} flow through the slow channels [66]. The authors believe that inhibitory cGMP action on the slow Ca^{2+} flow is associated with PDE stimulation, which leads to the decrease in cAMP level. To support this hypothesis, the authors adduce the following experimental results: 1) cGMP does not influence slow Ca^{2+} flow stimulated by 8-Br-cAMP, a PDE-resistant nucleotide analogue, and 2) IBMX, an inhibitor of cGMP-stimulated PDE, reduces cGMP effect on slow Ca^{2+} flow in the heart [66]. Since cGMP-stimulated cAMP PDE was found in many tissues, cAMP hydrolysis activation can form an universal mechanism underlying cGMP's regulatory action. This statement blends well with the known conclusion on opposite biological effects produced by cAMP and cGMP.

Discovery of cGMP-binding proteins differing from G-kinase, (a cGMP-dependent enzyme) may indeed testify to the suggestion that cGMP not always realizes its action via the cGMP-dependent phosphorylation. At the same time, there is an evidence that it is cGMP-dependent phosphorylation that influences Ca^{2+} flow in the CMCs. Thus, 8-Br-cGMP was shown to inhibit Ca^{2+} flow without producing the corresponding decrease in cAMP level [194]. 8-Br-cGMP also inhibits the baseline non-cAMP-stimulated slow Ca^{2+} flow in chicken CMCs [218]. It should be noted that this cGMP analogue capable of penetrating through the membrane does not stimulate PDE but can activate G- kinase. The authors believe that cGMP inhibits slow Ca^{2+} flow directly, by phosphorylating a specific protein with G-kinase. This protein is closely connected with slow Ca^{2+} channels in the SL and differs from a protein that can be phosphorylated by A-kinase. The existence of this protein that can be phosphorylated by G kinase in the myocardial SL has already been reported [194]. It cannot be excluded that targets for A- or G-kinase action are formed by different amino acid residues within a single protein molecule that forms the channel and/or is closely connected with it.

Finally, the third way via which cGMP exerts its influence on slow Ca^{2+} flow in the heart is probably associated with A-kinase activity modulation. Thus, cGMP causes the increase in A-kinase's regulatory subunit phosphorylation in guinea-pig cardiac SL and the corresponding change in cAMP-dependent protein kinase activity [37]. It is believed that cGMP-dependent phosphorylation of A-kinase regulatory subunit is an universal regulation mechanism of physiological importance [37].

In the heart muscle, fast Ca^{2+} channels were also found. These channels can be inactivated much more rapidly than the slow ones [25]. The function of these channels is not yet clear, and cAMP and calcium antagonists exert virtually no effect on the performance of these channels [194].

Permeability of Ca^{2+} channels in the vascular smooth-muscle cell plasmalemma, similarly to that in the cardiomyocyte sarcolemma, is regulated with the participation of second messengers. However, certain significant differences have been found as to the cAMP-dependent and Ca^{2+}-dependent regulation of CMC and vascular SMC slow Ca^{2+} channels *in vitro*. For instance, it was demonstrated that cAMP reduced the entering Ca^{2+} flow induced by membrane depolarization in the vascular SMCs [177]; the mechanism of such nucleotide's action remains unknown. Unusually high cGMP and G-kinase levels in the vascular SMCs testify to an important role that belongs to this second-messenger system in the smooth muscle, which in particular concerns the regulation of Ca^{2+} channel permeability [34, 52].

Probably, the different effects exerted by cAMP and cGMP on Ca^{2+} flow in the CMCs and vascular SMCs can be explained by the difference between these tissues in cGMP-dependent PDE activation [195]. Thus, in the myocardium and vascular smooth muscle, two different PDE forms were found as follows: PDE I, an enzyme stimulated by calmodulin (Recently, it has been shown that brain [184] and heart [183] PDE I is formed by two subunits with molecular masses of 60 kDa and 63 kDa respectively. These subunits are respectively phosphory-

lated by cAMP-dependent and Ca^{2+}-calmodulin-dependent protein kinases [185]. Phosphorylation of both PDE isoforms leads to a decrease in enzyme's affinity to Ca^{2+}-calmodulin complex, whereas their dephosphorylation by calcineurin, a Ca^{2+}-calmodulin dependent protein phosphatase, restores this affinitg [184]. It is likely that one of the ways of cyclic nucleotide level regulation is realized in the similar manner in other cell types.) and catalysing the hydrolysis of both cAMP and cGMP, and PDE IV, an enzyme form competitively inhibited by cGMP and catalysing the hydrolysis of cAMP with low K_m [44]. By now, it has been determined that, in the heart, hormones and neurotransmitters exert mainly a stimulating influence on PDE activity with low K_m. However physiological inhibitors of this enzyme form also exist [134]. In particular, glucagon is one of these inhibitors, glucagon receptors being coupled with PDE IV by G-proteins. Inhibition of PDE IV seems to cause a positive inotropic glucagon effect in the heart of those animal species e.g., guinea-pigs, mice and monkeys in which this peptide does not stimulate the adenylate cyclase activity [134]. At the same time, PDE II, an enzyme form hydrolyzing both cAMP and cGMP and activated by cGMP, was found in the heart. This enzyme form cannot be observed in the vascular SMCs. On the contrary, in SMCs of aorta but not in those of other vessels such as coronary arteries G-PDE was discovered, this enzyme form specifically hydrolyzing cGMP but not cAMP [195]. The latter enzyme could not be found in the CMCs. Thus , the two different PDE isoenzyme spectra in the CMCs and vascular SMCs cause two different modes of regulation for both cyclic nucleotide levels in these cells and certain cyclic nucleotide dependent processes including Ca^{2+} through the slow channels. In the heart but not in the vascular smooth muscle, cGMP can inhibit cAMP-stimulated Ca^{2+} flow through the voltage-dependent channels with PDE II [66]. cGMP can also inhibit β-adrenergic CMC activation via PDE mechanism [50]. It should be noted that slow Ca^{2+} flow inhibition in the presence of cGMP could be as well observed in the vascular SMCs [195]; however, it remains unclear which of the two, phosphodiesterase or cGMP-dependent protein kinase activity with subsequent phosphorylation of slow-channel proteins, is modulated in this case.

It is believed that, at least in model systems, cGMP causes the decrease in Ca^{2+} flow in aortal SMCs to a greater extent through the receptor-dependent plasmalemma channels than through the voltage-dependent ones [35, 133]. This conclusion is based on the results obtained during the studies on the influence produced by 8-Br-cGMP on the relaxation of aorta preparations preconstricted with noradrenalin and high extra-cellular K^+ concentration. In these studies, the relaxing effect of cGMP caused by reduced excitation Ca^{2+} flow into the SMCs [129, 205] was significantly higher in the former case as compared with the latter case. Relaxing effect achieved in the preparations of aorta preconstricted with high extra-cellular K^+ concentration was probably associated with another cGMP-dependent process differing from that connected with Ca^{2+} inflow inhibition [35].

In general, it is believed that cGMP acts as a main SMC relaxant primarily via the cGMP-dependent phosphorylation of membrane proteins [45]. cGMP-dependent phosphorylation of SMC myofibrillar proteins also seems to play a certain role. Thus, the increase in cGMP level was associated with myosin light chain dephosphorylation [41]. It is believed that such cGMP effect in the SMCs may be caused by the inhibition of myosin light chain kinase activity and/or by the increase in phosphoprotein phosphatase activity [142]. For instance, in SMC membranes derived from rabbit aorta, a 150 kDa protein specifically phosphorylated by G-kinase was found [121]. On the contrary, heart perfusion with compounds increasing the cGMP level leads to a slight negative inotropic effect, that is, to a slight decrease in contraction rate. Cholinergic agonists' action in the CMCs seems to be associated with the activation of dephosphorylation [65] rather than with cAMP hydrolysis stimulation by cGMP-activated PDE [45]. It should be noted that Hartzell and Titus [65] did not present any data on the direct cGMP influence on phosphoprotein phosphatase activity. In their experiments, these authors could only determine that carbamylcholine, a muscarinic cholinergic agonist, inhibited ^{32}P incorporation into cardiac C-protein.

Regulation of slow Ca^{2+} flow through the cell membrane of CMCs and SMCs may be associated with the phosphorylation of 15 kDa protein and 16kDa protein respectively. The two proteins are phosphorylated by A-and C-kinases, the effect of both phosphorylation types being additive [158, 179]. In the heart, 15 kDa protein phosphorylation is accompanied by a positive inotropic effect which may be caused by both α- and β-agonists. β-adrenergic stimulation also results in phosphorylation of 16 kDa protein of the aortal myocyte plasmalemma [72]. During C kinase activation with phorbol esters and the interaction between vasopressin and V_1 receptors, phosphorylation of this protein could be also observed [180]. In the latter case, phosphatidyl inositol 4,5-bisphosphate (PIP_2) hydrolysis resulted in the formation of C-kinase-activating diacylglycerol and IP_3 which stimulated Ca^{2+} release from intracellular stores. In this process, a rapid Ca^{2+} release was followed by the phosphorylation of 16kDa protein in the aortal SMCs; phospholipid-dependent phosphorylation, in its turn, was followed by the contraction response in these cells [180].

It has been suggested that 15 kDa and 16 kDa protein phosphorylation in CMCs and vascular SMCs is associated with the increase in Ca^{2+} flow through slow channels of the cell membrane [125]. However, there are no at least slightly convincing data as yet concerning the spatial coupling between the voltage-dependent channels or other Ca^{2+}-transporting systems of the cell membrane and these proteins. In the case of SMCs, a functional association between cAMP-dependent phosphorylation and increase in slow Ca^{2+} is as much as doubted [45]. In particular, it

has been established that, in SMCs of the bovine trachea, L-type calcium current is regulated via β_2-adrenergic receptors without involving cAMP cascade [225]. Evidently, β_2-adrenergic receptors are directly coupled with Ca^{2+} channels through G protein. This coupling may result in the growth of cytosol calcium level as these receptors are stimulated.

It should be noted that, in certain SMC types, action potential is developed and calcium ions enter the cells through voltage-dependent Ca^{2+} channels. In these cells, Ca^{2+} release from SR is caused by Ca^{2+}-induced mechanism of Ca^{2+} release, which presents a parallel to that in the CMCs [79]. In other SMC types, action potential does not develop at all and calcium ions enter the cell through receptor-dependent channels in response to different hormones [15]. In the CMCs as well as in the vascular SMCs, intracellular Ca^{2+} release in response to a hormonal signal is regulated with IP_3 [79]. This statement is not always correct. Thus, muscarinic agonists induce phosphoinositide hydrolysis but do not cause any increase in cytosol Ca^{2+} level in the CMCs [19]. A negative inotropic effect in this case is due to a decrease in intracellular cAMP concentration [76]. It is believed that long-term muscarinic effects on the myocardium in association with phospholipase C activation result not from the IP_3 synthesis but rather from the stimulation of C-kinase by diacylglycerol [68]. In this case, phosphoinositides do not probably play the role of second messengers but act, for instance, as regulators of catabolic enzyme activities [27]. Inositol trisphosphate at concentrations ranging from 1 μM to 10 μM induces Ca^{2+} release from the cardiac SR in the preparations obtained from SL-free fibers [147]. Agonists acting via α_1-adrenoreceptors stimulate tension development in a contracting heart without increasing the relaxation rate. As it has been determined, in atria and ventricles, α_1-agonists cause a rapid 50% rise in IP_3 level, which is followed by the increase in contractility [182]. Although α_1-receptors could not be found in the heart of many animal species and man, there is no doubt that the increase in myocardial contractility induced by α_1-agonists is associated with IP_3 action. Thus, this second messenger produces similar effects on intracellular Ca^{2+} concentration changes in the CMCs and vascular SMCs in response to the stimulation with different agonists.

Calcium ATPase of the Cell Membrane and Na^+-Ca^{2+} Carrier

Besides the cell-membrane channels, there are at least two more systems for calcium transport through this membrane in the myocardium and vascular smooth muscle: a specific ATPase and Na^+-Ca^{2+} carrier. Whereas slow Ca^{2+} channels participate in the initiation of systole by allowing trigger amounts of calcium to enter the CMC sarcoplasm, which subsequently leads to Ca^{2+} release from the SR, SL Ca^{2+}-ATPase characterized by a high affinity to calcium ions but low catalyzed reaction rate [23, 25] seems to remove calcium from the cell during diastole. Lately, a direction in which does the third system, Na^+-Ca^{2+} carrier, act has been determined. Thus, although it was believed earlier that the carrier only removes Ca^{2+} from the myocyte [229], the direction of carrier-stimulated ion flow seems to vary during the relaxation-contraction cycle in the myocardium [23, 42]. The carrier is characterized by a relatively low affinity to Ca^{2+}, whereas the maximum carrier-stimulated Ca^{2+}-transport rate is approximately 30 times as high as that of the Ca^{2+}-ATPase-stimulated one [23, 25]. The carrier is electrogenous, and the stoichiometry of 3 Na^+ vs 1 Ca^{2+} exchange is probable [25]. Electrogenousness of the carrier allows to suggest that the carrier's performance is directed towards Ca^{2+} inlet into the myocyte during the plateau phase of action potential in the heart [8]. The rate of Na^+ vs Ca^{2+} exchange seems to depend not only on concentration gradients but also on the membrane voltage. In this manner, another way for Ca^{2+} inflow into the myocyte is provided in addition to that via calcium channels. Na^+ vs Ca^{2+} exchange allows to remove from the sarcoplasm not only those calcium ions that were brought into the cell during depolarization by the carrier itself but also those that enter the cell through the calcium channels [157].

There is still little information available concerning the influence of phosphorylation and dephosphorylation on Na^+-Ca^{2+} carrier function in the heart. For instance, cAMP-dependent phosphorylation could not be observed to produce any effect on the carrier function [176]. Calmodulin does not influence the carrier directly. Experiments using adenosine 5'-[γ thio] trisphosphate allowed to determine that calmodulin carrier-activating effect was associated with Ca^{2+}-calmodulin-dependent protein kinase which increases both carrier affinity to Ca^{2+} and V_{max}, whereas calmodulin-induced inactivating influence was due to the processing of SL preparations with phosphorylase phosphatase [25]. Phosphorylase phosphatase is a sarcoplasmic enzyme, and it is therefore unlikely that it could dephosphorylate SL proteins *in vivo*. Phosphorylase phosphatase-induced effect on the carrier activity can be reproduced with the incubation of SL vesicles with Mg^{2+}, Ca^{2+} and calmodulin. It was shown that membrane-bound Ca^{2+}+(Mg^{2+})-calmodulin-dependent protein phosphatase dephosphorylated phosphoproteins without the obligatory involvement of the Na^+-Ca^{2+} carrier itself, which led to a reduced Na^+ vs Ca^{2+} exchange activity [22].

Calmodulin-dependent process inhibitors, trifluoperazine [116] and R24571 [176], inhibit Na^+ vs Ca^{2+} exchange activation by approximately 90%. SL Na^+-Ca^{2+} carrier completely activated by Ca^{2+}-calmodulin-dependent phosphorylation is characterized by a significantly higher affinity to Ca^{2+} (K_m about 2 μM) as compared with that of the nonphosphorylated carrier (K_m about 12 μM), the initial rate of Ca^{2+} in the former case being 2 times as high as that in the latter case. Coordinated action of protein phosphatase and protein kinase may be caused by the difference in their Ca^{2+} responsiveness affinity of protein phosphatase to Ca^{2+} (K_m > 1μM being lower than that of

protein kinase $K_m \leq 1\mu M$) as well as by Ca^{2+} and calmodulin levels in the sarcoplasm [22]. Thus, the carrier probably can be activated via the stimulation by Ca^{2+}-calmodulin-dependent protein-kinase during the growth of Ca^{2+} level in the CMC. As soon as Ca^{2+} concentration in the sarcoplasm has grown to a sufficiently high level, a negative-feedback mechanism comes into action as follows: the carrier becomes inactivated (dephosphorylated) and hinders excessive Ca^{2+} accumulation in the CMC [25]. During this process, phosphorylation and dephosphorylation may involve both the carrier itself and an unknown regulatory protein closely associated with the former [22]. It should be noted that the authors [22, 25] present no data concerning the correlation between Na^+-Ca^{2+} carrier phosphorylation/dephosphorylation time and systole/diastole phases in the heart. Undoubtedly, this lack of information makes it difficult to evaluate the physiologic role of the carrier.

The fact that Ca^{2+}-ATPase is present in the cardiac SL was established in 1980 [24]. Purified enzyme has M_r = 140 kDa. SL Ca^{2+}-ATPase is vanadate-sensitive and has high affinity to Ca^{2+} (K_m about 0.5 μM) and relatively low as compared with Na^+ vs Ca^{2+} exchange rate of Ca^{2+} transport (V_{max} about 0.5 nmole per mg membrane proteins at 30°C) [22, 23, 25]. Unlike that performed by Na^+-Ca^{2+} carrier, Ca^{2+}-ATPase-induced Ca^{2+} removal from the sarcoplasm seems to be stimulated by cAMP-dependent process [22, 114, 233]. Na^+ vs Ca^{2+} exchange probably regulates CMC cytosol Ca^{2+} level during excitation, whereas specific Ca^{2+}-ATPase performs this function at rest [22, 23]. It is likely that the influence of cAMP on the SL Ca^{2+}-ATPase is mediated via the change in membrane-bound Ca^{2+} amount. This hypothesis finds support in the data concerning the increase in Ca^{2+} binding with the cardiac SL in the presence of cAMP and exogenous A-kinase [4, 128]. Under conditions of high protein kinase concentration, on the contrary, the inhibition of SL Ca^{2+} pump activity could be observed [213]. It is likely that high phosphorylation level in the SL is associated with the lowering of Ca^{2+}-ATPase-mediated Ca^{2+} transport rate. This lowering may lead to a prolonged persistence of activating myoplasm Ca^{2+} concentration, which may be of great importance for the development of positive inotropic effect caused by catecholamines [109]. It is not yet clear which particular mechanism underlies the phosphorylation-dependent diversity of directions in which Ca^{2+} ATPase activity is regulated: whether it is associated with the existence of several, activating and inhibiting, centers of phosphorylation in Ca^{2+}-ATPase molecule or, for instance, with consecutive phosphorylation of different regulatory proteins closely connected with ATPase.

It has been established that the activation of SL calcium pump is induced not only by exogenous A-kinase but also by an endogenous, membrane-bound cAMP-dependent enzyme [109, 113]; a disactivating phosphatase has not been found yet. In the cardiac SL, a 9 kDa protein associated with SL Ca^{2+} pump was identified, which was phosphorylated by A-kinase and calmodulin-dependent protein kinase at different sites respectively [114, 223]. This protein is a monomer of a phospholamban-like 23-24 kDa protein. Molar ratio of 9 kDa protein: Ca^{2+}-ATPase monomer is greater than 5 : 1 [112]. 9 kDa protein as well as 15 kDa protein took up labelled phosphate during heart perfusion with catecholamines [72]. It was demonstrated that 9 kDa protein had been phosphorylated 5-10 s before the inotropic effect appeared [114]. These findings provide an evidence for the fact that 9 kDa and 15 kDa protein phosphorylation in the cardiac SL may modulate the processes of Ca^{2+} transport.

Phospholamban-like protein subunit phosphorylation in the cardiac SL resembles the situation in the SR where cAMP-dependent phosphorylation and calmodulin-dependent phosphorylation produce an additive effect while stimulating Ca^{2+} pump. It has been established that phosphorylated 9 kDa protein is not bound with SR membranes but belongs exclusively to the outer cell membrane [21, 72, 112]. Under conditions of low Ca^{2+} concentrations (0.3 μM), 9 kDa protein is probably phosphorylated by A-kinase since calmodulin-dependent protein kinase displays minimum activity under these conditions [111, 114]. In general, it could be suggested that *in situ* activation of cardiac Ca^{2+}-ATPase in the presence of Ca^{2+}-calmodulin complex is not associated with Ca^{2+} calmodulin-dependent SL phosphorylation [40]. It is also known that cardiac SL 9 kDa protein is phosphorylated by C-kinase [80]. Physiological importance of this phosphorylation, however, remains unclear.

Calmodulin can manifest its regulatory activity in the SL not only as an activator of corresponding protein kinases. Thus, it has been established that SL Ca^{2+}-ATPase is closely connected with calmodulin which directly (without the participation of calmodulin-dependent protein kinase) regulates the responsiveness of this pump towards calcium ions. In the presence of calmodulin, K_m for Ca^{2+} becomes 28 times lower, whereas enzyme's V_{max} undergoes 9-12-fold growth [40]. In addition, SL Ca^{2+}-ATPase activation with A-kinase catalytic subunit exerts virtually no influence on the pump's affinity to Ca^{2+}, and V_{max} values grows 1.8 and 2.4 times higher in the absence and in the presence of calmodulin respectively [40]. It was shown that certain calmodulin antagonists such as calmidazol effectively inhibit SL Ca^{2+} pump by lowering Ca^{2+}-ATPase affinity to calcium ions [9, 112]. On the contrary SL calcium channel blockers, niphedipine and phelodipine, at relatively low concentrations not only do not inhibit but even stimulate SL Ca^{2+}-ATPase [38]. There is an evidence that calmodulin or calmodulin-like protein is associated not only with SL Ca^{2+} ATPase but also with "inner gates" of slow calcium channels of this CMC membrane [85-87]. The "inner gates" are open at low and closed at high intracellular Ca^{2+} levels [87]. Calcium channel blockers can interact with calmodulin-like protein to increase the affinity of this protein to Ca^{2+} [85]. As a result, slow calcium channels become closed more rapidly. Thus, Ca^{2+}-

antagonistic effect of slow calcium channel blockers may be caused both by the stimulation of SL Ca^{2+}-ATPase via the increase in calmodulin's or calmodulin-like protein's affinity to Ca^{2+} and by the closing of slow calcium channels due to the similar mechanism.

It should be noted that, in the myocardium, besides calmodulin, second messengers cAMP, cGMP and Ca^{2+} and protein kinases activated by all of them, G proteins, which interact directly with ion channels or with yet unidentified regulatory components of these channels, directly participate in the modulation of transmembrane ion flows [33, 127, 169]. Thus, stimulation of CMCs from the chicken atria with muscarinic cholinergic receptor agonists resulted in the increase in K^+ current into the cells and hyperpolarization [127]. It has been established that for the coupling between muscarinic receptors and K^+-channels GTP is required. Pretreatment of CMCs with pertussis toxin blocked this coupling and led to ADP-ribosylation of G proteins with 39 kDa and 42 kDa α subunits [127]. It is known that pertussis toxin prevents GTP from binding with G_i, an inhibitor G protein, to suppress the action of adenylate cyclase inhibitors; during this process the toxin ADP-ribosylates G_i α-subunit [169]. Infusion of $G_{pp}NH_p$, a non-hydrolyzable GTP analogue, into CMCs from the frog atria caused a prolonged activation of K^+ current induced by acetylcholine [127]. Finally, a direct evidence was obtained for the functional coupling between G proteins and ion channels. Thus, a purified pertussis toxin-responsive G protein with 40 kDa α-subunit activated K^+ channels in the atrial cells [127]. G proteins probably directly participate also in the modulation of CMC Ca^{2+}-channel activity [33].

In the smooth muscle, as well as in the heart, transport Ca^{2+}-ATPases are associated with the membranes of plasmalemma and SR. Thus, a 100 kDa protein from the bovine main pulmonary artery reacted with polyclonal antibodies against Ca^{2+}, Mg^{2+}-ATPase from the porcine myocardial SR [43]. As it is known, SR Ca^{2+}-ATPase from both SMCs and CMCs does not bind calmodulin. On the contrary, Ca^{2+}-ATPase activity in the smooth muscle plasmalemma preparation is manifested by a 130 kDa protein that binds [^{125}I] calmodulin with high affinity [17].

In different smooth muscle types different proportions of phosphorylated Ca^{2+}-ATPase intermediates with M_r = 100 kDa and Mr = 130 kDa can be observed. For instance, in the plasmalemma and SR from the bovine pulmonary artery this ratio is significantly higher than that in the membranes of porcine gastric smooth muscle [43]. Hence, 130 kDa transport ATPase's contribution to the Ca^{2+} transporting capacity of the SMCs in the bovine pulmonary artery is much lower than that in the visceral smooth muscle. It should be noted that these differences depend both on the type of smooth-muscle tissue and on the species of animal [43].

In the bovine aorta, phosphorylated 130 kDa intermediate either could not be observed at all [43] or was found in only insignificant amounts [54]. It is likely that in bovine aorta Ca^{2+} transport through SR membranes is of primary functional importance. An important role of intracellular Ca^{2+} in the development of vessel wall's tension is evidenced by the fact that muscles of certain major vessels are able to contract for some time in the absence of Ca^{2+} from the surrounding solution. At the same time, in the aorta the main role in the regulation of this vessel's tone seems to belong to plasmalemmal Ca^{2+}-ATPase. This suggestion is supported by an observation that vanadate inhibits plasmalemmal Ca^{2+}-ATPase and simultaneously causes a maximum contractility in the rat aorta [163].

In general, plasmalemma's contribution to Ca^{2+}-transporting activity in vascular SMCs is lower than that in visceral SMCs [43]. These observations correspond well with morphological data as to a high development of the SR in major arteries (up to 7.5% of the cell volume excluding nuclei and mitochondria). In visceral SMCs, SR forms only 2% of the cell volume [190].

Influence of second messengers on the cell-membrane Ca^{2+}-pump activity in the CMCs differs significantly from that in the vascular SMCs. Thus, unlike those in the myocardium, cAMP and A-kinase do not stimulate Ca^{2+}-ATPase activity in the plasmalemma of rat [122] and porcine [217] aorta. However, SMC relaxation caused by both exogenous (nitroglycerol and nitroprusside) and endogenous (atrial natriuretic factor and endothelium-derived factor) vasodilators is associated with guanylate cyclase activation and corresponding increase in cGMP level. It has been established that under the influence of endothelium-derived factor and exogenous vasodilators (Relaxing effect of these compounds on the blood vessels varies significantly in different animal species. Thus, soluble guanylate cyclase is much more responsive to these agents in rat aorta as compared with rabbit aorta [35]) the soluble form of the enzyme is activated [35, 217, 228], and in the former case, in addition, phospholipase C activation occurs followed by the formation of second messengers [162]. Acetylcholine's influence on the endothelium is associated with the release of a special factor that activates guanylate cyclase and thus leads to the relaxation of vessels [142]. Atrial natriuretic factor relaxes isolated bovine tracheal smooth muscles contracted under the influence of carbachol, histamine or serotonin more efficiently than does isoprenalin but to a lesser extent than does sodium nitroprusside [78]. In this case, insoluble guanylate cyclase becomes activated (whereas the activity of soluble enzyme remains unchanged), and cGMP grows 3-5 times higher (cAMP level does not change) [78].

It is suggested that the relaxing effect of cGMP is realized via the phosphorylation of specific proteins by G-kinase [152]. Many authors explain this effect with the decrease in cytosol Ca^{2+} concentration [164, 217]. cGMP may cause the lowering of Ca^{2+} level in the sarcoplasm of vascular SMCs by several mechanisms as follows: by lowering the entry and/or by increasing the outflow of Ca^{2+} through the plasmalemma as well as by changing Ca^{2+} in- and out-flow as regards SR depot. An evidence for the existence of the

latter mechanism was obtained by Raeymaekers et al. [160] who showed that phospholamban phosphorylation by G-kinase stimulated the accumulation of Ca^{2+} in the SR of smooth muscle and myocardium (see below).

The most convincing data supporting the suggestion that cGMP's relaxing effect on vascular SMCs is caused by the stimulation of plasmalemmal Ca^{2+}-ATPase are already available [100, 164, 210]. A stimulating effect of cGMP and G-kinase on plasmalemmal Ca^{2+}-ATPase of vascular smooth muscle is additive in relation to that of calmodulin [122] and it was found in both crude [164] and purified [156] membrane preparations. An attempt to demonstrate the enzyme activation with G-kinase using a partly purified Ca^{2+}-ATPase preparation from the bovine aorta proved to be unsuccessful [122]. The enzyme, derived from bovine aorta and purified with affine chromatography using calmodulin, also lost its responsiveness towards C-kinase [77], this finding providing an evidence that this protein kinase realized its action via other plasmalemmal components different from Ca^{2+}-ATPase.

How does G-kinase cause the increase in affinity to Ca^{2+} and the activation of plasmalemmal Ca^{2+}-pump in the vascular SMCs? A report on the fact that the mechanism of such activation was realized via direct cGMP-dependent phosphorylation of Ca^{2+}-transporting protein [55] was doubted by Vrolix et al. [217]. Thus, it was shown that cGMP-dependent stimulation of purified Ca^{2+}-ATPase, derived from the porcine aorta and built in into liposomes, depended on the phospholipid composition of liposomes and could be observed only in the presence of PI. In purified Ca^{2+}-ATPase preparations G-kinase, unlike A-kinase, stimulated PI phosphorylation to PIP [217]. It is known that Ca^{2+}-ATPase of the SL from aorta is activated only by PIP but not by the latter's dephosphorylated form. Hence, it is suggested that G-kinase stimulates plasmalemmal Ca^{2+}-pump in vascular SMCs not directly but via phosphorylation of phosphatidyl inositol kinase closely connected with Ca^{2+}-transporting ATPase. Such kinase activity may be manifested by 55 kDa and 45 kDa proteins which present a preferable substrate for G-kinase. These proteins have been found only in Ca^{2+}-ATPase preparations obtained from vascular SMCs but could not be observed in red blood cells in which PI phosphorylation is not stimulated by G-kinase [217]. Further phosphorylation of PIP to PIP_2 in SMC membranes is less effective as regards the stimulation of Ca^{2+}-ATPase activity [217].

As mentioned above, cAMP-dependent mechanism of vascular smooth muscle relaxation does not find any unequivocal experimental confirmation as yet. Thus, on the one hand, a correlation could be demonstrated between adenylate cyclase activation, increase in cAMP level and intensification of A-kinase activity and the relaxation of blood vessels induced by isoprenaline, PDE inhibitors, adenosine, forskolin and PGE_2 [63, 102, 108, 189]. Dibutyryl cAMP form's ability to cause the relaxation of different smooth muscle types was also demonstrated by several authors [189, 206]. However, using bovine [144] and rat aorta preparations precontracted under the influence of epinephrine [120], it was established that only 8-Br-cGMP could cause the relaxation of these vessels, where as 8-Br-cAMP did not produce such effect. A possible explanation of this contradiction may be as follows. In the first place, as it is known, a whole number of cAMP and cGMP analogues (including, for example, dibutyryl cAMP form) can activate both A-kinase and G-kinase. Therefore, the physiologic effect produced by these analogues cannot be surely identified with the activation of a certain enzyme type. It is also necessary to take into account the difference in the resistence of cAMP and cGMP analogues against phosphodiesterase-induced hydrolysis and their ability to penetrate through the cell membranes. In the second place, if the stimulation by adenylate cyclase and guanylate cyclase agonists is sufficiently pronounced, high levels of endogenous cAMP or cGMP cause the activation of both kinases. Thus, in order to elucidate the physiological importance of A-kinase or G-kinase activation in the relaxation of vascular smooth muscle, cAMP and cGMP analogues highly specific with respect to A-kinase and G-kinase activation must be employed. Francis et al. [52], having tried to follow this imperative in their study in which 24 nucleotide analogues were used, came to a conclusion about the exclusive participation of G-kinase in the relaxation of blood vessels (porcine coronary arteries). No doubt, further investigation is required in order to draw a final conclusion about the participation of cAMP system in the relaxation of vascular smooth muscle.

It should be noted that cAMP causes the decrease in cytosol Ca^{2+} concentration in visceral SMCs. Thus, in the presence of β-agonists or dibutyryl cAMP, phosphorylation of ouabain-responsive Na^{2+}, K^{+}-ATPase of gastric SMCs occurs. As a result, the enzyme becomes activated, and cytosol Ca^{2+} level in these cells decreases due to the modulation of Na^{+}-Ca^{2+} exchange [181].

In conclusion of this part of our study, we shall consider the role of C-kinase in the regulation of SMC cell membrane's permeability for Ca^{2+}. As agonists that activate phospholipase C are being bound, PIP_2 is hydrolyzed to increase IP_3 level. Subsequent mobilization of Ca^{2+} from the SR depot leads to the increase in Ca^{2+} concentration both in the SMC cytosol $[Ca^{2+}]_c$ and in the submembrane region $[Ca^{2+}]_{sm}$ [168]. The growth of $[Ca^{2+}]_c$ is only transient and accompanied by calmodulin-dependent activation of myosin light chain kinase. The increase in IP_3 is likewise short-lived since this phosphoalcohol can be phosphorylated to IP_4. During the prolonged, tonic response phase, characteristic of activated SMC, when IP_3 concentration [67] and intracellular Ca^{2+} level [16] are low, IP_4 concentration remains increased. IP_4 interacts with plasmalemmal Ca^{2+}-channels, which leads to a prolonged acceleration of Ca^{2+} entry into the cell [167]. As a result, a $[Ca^{2+}]_{sm}$ zone is formed with high ion concentration. Recently, the mechanism of Ca^{2+} recirculation in this zone has been clarified. This mechanism is associated with the acceleration of Ca^{2+} entry as well as with the acceler-

ation of Ca^{2+} outflow from the SMC [168]. The latter is caused by the activation of C-kinase due to the increase in the affinity of this enzyme to the cell membrane as diacylglycerol level, $[Ca^{2+}]_c$ and $[Ca^{2+}]_{sm}$ are increasing. During this process, C-kinase remains in a membrane-bound state for at least 50 minutes [62]. Whereas the initial growth of $[Ca^{2+}]_{sm}$ results in calmodulin-dependent activation of plasmalemmal Ca^{2+}-ATPase (a direct transient negative feedback), the prolonged increase in Ca^{2+}-pump activity, required for the realization of Ca^{2+} transmembrane recirculation, seems to be caused by C-kinase-induced ATPase stimulation (an indirect long-term feedback) [167]. Such mechanism of C-kinase's action on plasmalemmal Ca^{2+}-ATPase was confirmed in the course of experiments with cultured vascular SMCs [56] including those derived from the aorta [122]. It is noteworthy that the enzyme, purified by means of affinity chromatography using calmodulin, lost its ability to be activated by G-kinase [77], this fact, as was the case with G-kinase (see above), indicating the existence in SMC plasmalemma of other, different from Ca^{2+}-ATPase, targets for phosphorylation by these protein kinases.

It should be noted that during the positive phase of muscle contraction, as a result of $[Ca^{2+}]_{sm}$ increase and activation of cell membrane-bound C-kinase, desmin and caldesmon phosphorylation occurs, which seems to cause the prolongation of contraction response. Membrane-bound C-kinase probably phosphorylates desmin and caldesmon, localized in SMC compartments distant from the cell membrane, not directly but rather via a protein-kinase cascade. It is known that C-kinase phosphorylates caldesmon *in vitro* [211]. At the same time, caldesmon molecule sites that are phosphorylated by C-kinase and B-kinase *in vitro* differ from those phosphorylated in caldesmon derived from cultured cells [2].

The above data provide an explanation for the endothelium-derived factor-induced decrease in vascular smooth muscle tone during the tonic phase of contraction. During the initiation of contraction the endothelium-derived factor as well as nitroprusside inhibit the formation of IP_3, during the prolonged phase, however, the influence of cGMP on IP_3 synthesis is slackening [117]. During this period, a vasodilating cGMP effect is probably associated with the inhibition of diacylglycerol formation [117] whose increased level can be observed during the tonic phase of vascular smooth muscle contraction [59]. Inhibition of diacylglycerol synthesis results in the decrease in membrane-bound C-kinase activity and in vascular tone.

Thus, long-term changes in vascular tone depend at least on two factors: the amount of membrane-bound C-kinase and Ca^{2+} or $[Ca^{2+}]_{sm}$ circulation rate. Since in this case C-kinase seems to be not completely activated and its prolonged association with the membrane does not depend on $[Ca^{2+}]_{sm}$ significant tone changes induced by corresponding agonists may be caused by relatively slight changes in Ca^{2+} inward and outward currents through the cell membrane [167]. Hence, the circulation of $[Ca^{2+}]_{sm}$ plays a role of a main messenger in the development of prolonged SMC response.

Na^+-Ca^{2+} carrier was found in the majority of animal and human blood vessel types [12, 139, 173]. A probable stoichiometry of Na^+ vs Ca^{2+} exchange in the vascular SMCs is similar to that in the heart, that is 3 Na ions per 1 Ca ion [91], and differs from the electroneutral Na^+ vs Ca^{2+} exchange in other smooth muscle types such as myometrium [61] and intestinal muscle [138].

The importance of Na^+ vs Ca^{2+} exchange for the regulation of $[Ca^{2+}]_c$ level and contractility of major blood vessels such as the aorta is doubtless [14, 186]. As well as in the heart, in the SMCs of these vessels in the case of blocking of Ca^{2+} accumulation in the SR, the main mechanism of Ca^{2+} removal from the cytosol is associated with Na^+ vs Ca^{2+} exchange but not with plasmalemmal Ca^{2+}-ATPase activity [166]. Data on the physiological importance of Na^+ vs Ca^{2+} exchange in blood vessels are contradictory enough, ranging from a report on the central role of this exchange in the regulation of vascular tone [115] to a statement that Na^+ vs Ca^{2+} can be physiologically important only under extraordinary conditions [140, 187]. At any rate, elucidation of the role of Na^+ vs Ca^{2+} exchange in the regulation of peripheral vascular tone is undoubtedly required to confirm or to refute a hypothesis that this ion-transporting system presents a necessary link in the association between salt attack and development of hypertension [12, 13].

At present, there is a lack of evidence in favor of the regulation by second messengers of vascular SMC Na^+-Ca^{2+} carrier activity. At the same time, the treatment of invertebrate smooth muscle with cAMP analogues causes muscle relaxation which seems to be associated with Na^+ vs Ca^{2+} exchange stimulation [181]. In the vascular SMCs, as well as in the CMCs, regulation of Na^+-Ca^{2+} carrier activity by second messengers may be realized in an indirect way via the Na^+ vs H^+ exchange system found in the cell membrane of these cells [91]. Thus, it has been established that amiloride, a Na^+- H^+ exchange inhibitor, inhibits Na^+ vs Ca^{2+} exchange in arterial SMCs [119, 187]. In SMCs from the rat aorta, Ca^{2+} ionophore A23187-dependent stimulation of Na^+ vs H^+ exchange in the presence of C-kinase-activating phorbol ester (TPA) was demonstrated [74]. These data on the synergism between A23187 and TPA may provide an evidence that, on the one hand, TPA-induced C-kinase activation requires an increase in $[Ca^{2+}]_c$ and, on the other hand, phorbol ester-induced C-kinase activation causes the manifestation of Ca^{2+}-calmodulin-dependent regulation. As a result, Na^+ vs H^+ is stimulated, $[Na^+]_c$ value increases and the rate of Na^+ vs Ca^{2+} exchange through vascular SMC plasmalemma grows higher. In aorta endothelial cells, C-kinase-activating phorbol esters, in addition, induce the increase in intracellular pH value, whereas C-kinase inhibitors suppress this process [99].

Phosphorylation of Sarcoplasmic Reticulum Proteins

As mentioned above, in the SR and SL, under the participation of different second-messenger systems, phosphorylation of regulatory target proteins is realized, which results in the change in ion permeability of myocyte membranes. Thus, it has been already shown that, in the presence of cAMP and protein kinase, cardiac microsomes enriched with SR fraction are to a greater extent capable of ATP-dependent accumulation of calcium ions. Although in these works membrane orientation of vesicles was not studied, Ca^{2+}-accumulating activity in the SR was compared with Ca^{2+} removal from the cytoplasm *in vivo* [98, 201, 230].

The influence of catecholamines on the heart contractility is caused by a number of biochemical reactions which couple excitation with contraction. As a main intermediate link in this chain of reactions, cAMP-dependent phosphorylation of a SR membrane-bound protein was distinguished. This protein, referred to as phospholamban by Katz et al. [94], forms about 4-5% of the total protein content in the cardiac SR. A-kinase catalytic subunit phosphorylates purified phospholamban up to the level of 5 moles P_i per mole protein [82] (phospholamban's molecular weight was assumed to be 27 kDa). Phospholamban is preferably phosphorylated by type II A-kinase from the heart [204]. Introduction of a β-agonist, isoprenaline, resulted in a 5-fold [135] increase in phospholamban phosphorylation by A-kinase in the heart or a 6-fold increase within 60 s [224]. β-antagonists such as propanolol blocked this process, phospholamban being rapidly dephosphorylated [135]. cAMP-dependent phospholamban phosphorylation led to a 2-fold to 3-fold increase in Ca^{2+} accumulation in SR vesicles without changing the transport stoichiometry (2 moles Ca^{2+} per mole hydrolyzed ATP) [201]. Thus, in response to even insignificant adrenergic agonist concentrations, for instance 3×10^{-9} M isoprenaline, rapid (within 20 s after cathecolamine introduction) phosphorylation of myocardial phospholamban occurs [123]. During this process, phospholamban phosphorylation and SR Ca^{2+}-ATPase activity undergo changes parallel with the acceleration of relaxation. Upon isoprenaline removal from the perfusate, certain differences could be observed in the kinetics of changes between biochemical (phosphorylation) and mechanical parameters [123]. It is likely that phospholamban phosphorylation initiates the relaxing effect produced by catecholamines, whereas the end-phase of relaxation is associated with another mechanism which differs from phospholamban phosphorylation.

Phospholamban is absent from fast skeletal muscles but has been found in slow ones. Recent data evidence that phospholamban consists of 5 identical subunits [82], each of them containing 52 amino-acid residues and having molecular weight of 6,080 Da [30]. It is believed that phospholamban forms an ion channel consisting of 5 identical noncovalently bound monomers subjected to phosphorylation [188]. An hydrophobic site at the C-end of the molecule, which is probably incorporated into the SR membrane and can interact with the hydrophobic Ca^{2+}-ATPase domain, imparts a typically lipophylic properties to phospholamban. In contrast, at the N-end of the molecule there is an hydrophilic domain consisting of about 30 amino-acid residues, which projects over the SR membrane. Ser-16 and Thr-17 residues of this domain are selectively phosphorylated by A-kinase and B-kinase respectively [224]. A serine residue is phosphorylated initially, followed by phosphorylation of a threonine residue [224]. In authors' opinion, these observations may suggest that: 1) A-kinase, which is activated in response to β-agonists, phosphorylates Ser-16 directly and 2) increase in intracellular Ca^{2+}, resulting from adrenergic activation of the slow inward Ca^{2+} channel of the SL, subsequently activates B-kinase, leading to phosphorylation of Thr-17 and a further increase in the rate of Ca^{2+} uptake. Such sequence of events, however consistent it may seem at first sight, is unlikely to reflect the situation *in situ* (see below).

At the N-end of phospholamban molecule, several positively charged amino-acid residues are situated (3 Arg and 2 Lys per subunit), which impart a basic character to the molecule. Indeed, phospholamban pI value is about 10 [90]. Phospholamban phosphorylation is accompanied by a strong acidic shift of pI value which reaches 6.2-6.4 after phosphorylation of this protein by A-kinase or B-kinase. Simultaneous phospholamban phosphorylation by both kinases leads to a further acidic shift in the subunit pI values up to 5.2 [30]. Ser and Thr phosphorylation blocks the inhibiting phospholamban effect on Ca^{2+}-ATPase due to the interaction of negatively charged phosphate groups with Lys and Arg. The latter event reduces hydrophobic interaction between phospholamban and Ca^{2+}-ATPpase [97].

Ca^{2+}-Mg^{2+} ATPase in the SR membranes is closely connected with phospholamban, the stoichiometry of this complex approaching 1:1. Upon SR vesicle solubilization with either Triton X-100 or deoxycholate, phospholamban remains firmly bound to Ca^{2+}-pump as demonstrated using isoelectric focusing or sucrose-gradient centrifugation [153]. A direct evidence was obtained for the interaction between ATPase and phospholamban as follows: regulatory influence of protein kinases could be reproduced with the use of anti-phospholamban specific monoclonal antibody [199]. This strong interaction between ATPase and phospholamban corresponds well with the hypothesis about the changes in Ca^{2+}-pump hydrophobic microenvironment during phospholamban phosphorylation [71].

Phosphorylation of phospholamban derived from the canine myocardial SR by A-kinase leads to a change in the surface membrane voltage by 7 mV. During this process, an apparent K_m value of the calcium pump grows higher [31]. Data thus obtained suggest the participation of electrostatic forces in the regulation of the myocardial SR calcium pump by phospholamban. It should be noted that the study on the influence of cAMP-dependent phospho-

lamban phosphorylation on the rotating mobility of canine cardiac SR ATPase provided support to a conclusion about the association of both dephosphorylated and phosphorylated phospholamban with ATPase [51].

Regulatory complex isolated from the myocardium after solubilization with deoxycholate consists of a polypeptide chain of Ca^{2+}-ATPase, phospholamban, A-kinase and phospholamban kinase. The latter enzyme is Ca^{2+}-calmodulin dependent. Phospholamban kinase is in its properties closer to multifunctional type II B-kinase than to specific calmodulin-dependent type I protein kinases [84]. Addition of Ca^{2+} and exogenous calmodulin to cardiac microsome preparations results in phospholamban phosphorylation to the same level as achieved with the incubation with catalytic A-kinase subunit [26]. These two types of phosphorylation are characterized by an additive effect, which indicates the existence of different sites phosphorylated by cAMP-dependent and Ca^{2+}-calmodulin dependent membrane-bound protein kinases. Maximum phospholamban amount phosphorylated by type II B-kinase under conditions of optimal Ca^{2+} concentration (5-10 μM) is equal approximately to the amount of protein phosphorylated by A-kinase (203).

Additive and isolated action of A-kinase and B-kinase on Ca^{2+} transport in the myocardial SR may be caused by different lipid and/or protein microenvironments of phospholamban in different reticulum fractions. Thus, calmodulin-dependent phospholamban phosphorylation can be observed mainly in terminal SR cisterns, whereas in longitudinal SR tubules phospholamban is phosphorylated mostly by A-kinase [57].

Evidently, these reticulum sections contain different phospholamban pools and are controlled by different regulation systems. Indeed, although phospholamban subunits have certain sites to be phosphorylated by either A-kinase or B-kinase, SR phosphomembrane analysis could not reveal any phospholamban molecules in the reticulum that could be simultaneously phosphorylated by the two kinases. This fact may provide an evidence for the uneven intracellular distribution of phospholamban kinase and A-kinase. Phospholamban kinase, a membrane-bound enzyme, phosphorylates its own phospholamban pool.

cAMP-dependent phosphorylation of another phospholamban pool is to a great extent caused by a soluble form of exogenous A-kinase. Phospholamban domains phosphorylated by endogenous B-kinase may be inaccessible for exogenous A-kinase [57]. Therefore in the presence of both protein kinases phospholamban phosphorylation is usually additive, and different pools of this protein are phosphorylated. When A-kinase is present at very high concentrations together with calmodulin, the level of phosphorylation becomes more than additive, which indicates that new phosphorylation sites are appearing [30]. It is likely that phospholamban pool interacting with phospholamban kinase, upon calmodulin-dependent phosphorylation, can undergo additional phosphorylation by exogenous A-kinase. In this case, pI 6.4 subunits disappear and a protein emerges which is phosphorylated by the two protein kinases with pI 5.2 [30]. These findings may provide an explanation for the additional stimulating effect of A-kinase on Ca^{2+} transport in the SR, which can be observed in vitro [153]. Phospholamban phosphorylation in the presence of calmodulin and cAMP causes in particular the increase in resistance to proteolysis in SR Ca^{2+} transport [6]. It should be noted that synergistic A- and B-kinase effect in the SR has been shown only under conditions of high A-kinase concentration (not less than 500 IU/ml), which seems to rule out the physiological importance of this effect [30]. From the other hand, cardiac SR Ca^{2+} pump activity can be regulated by different kinases owing to the compartmentalization of phospholamban [57].

It has been established that phospholamban contains a considerable number (up to 10) of phosphorylation sites [223]. A wide variety of phospholamban isoforms in the CMCs also makes this protein a convenient substrate for different protein kinases. Thus, in the guinea-pig heart, 11 phospholamban forms could be found, which differed from each other in their electrophoretic mobility and corresponded with protein pentamers in which from 0 to 10 sites were phosphorylated [224]. cGMP-dependent phospholamban phosphorylation stimulated Ca^{2+} accumulation in the SR vesicles *in vitro* [160]. Recently, cardiac phospholamban phosphorylation by G-kinase was demonstrated, which was performed at the same Ser sites as in the case of cAMP-dependent phosphorylation [68]. Interestingly, phospholamban appeared to be even better substrate for G-kinase than for A-kinase [73]. At the same time, under the influence of compounds causing the increase in intracellular cGMP level (guinea-pig heart perfusion with carbamylcholine or 8-Br-cGMP) no increase in phospholamban phosphorylation *in situ* could be demonstrated [73]. One possible explanation for this phenomenon may be provided by the fact that heart A-kinase contents is about 10 times as high as that of G-kinase[143]. cGMP-dependent phospholamban phosphorylation in the heart can be hindered also by the presence in the close vicinity of phospholamban of a specific phosphatase or by localization of PDE, which degrades cGMP and blocks G-kinase activation, in the same compartment.

It was also found that phospholamban could be phosphorylated by Ca^{2+}-activated phospholipid-dependent protein kinase at a site that differed from those phosphorylated by cAMP-dependent and Ca^{2+}-calmodulin-dependent protein kinases [80]. Phospholamban phosphorylation by C-kinase, in authors' opinion, might be activated by β-adrenergic agonists which caused Ca^{2+} entry into the cell. At the same time, α-adrenergic stimulation, which could result in C-kinase activation caused by the increase in diacylglycerol level, did not lead to phospholamban phosphorylation in the intact heart [124]. There is also no unanimous opinion as yet about the physiological role of Ca^{2+}-calmodulin-dependent phospholamban phosphorylation system.

It is believed that permanent Ca^{2+} sensor that activates SR Ca^{2+}-pump as cytosol Ca^{2+} contents grows to μmolar

concentrations during systole is identifiable with Ca^{2+}-calmodulin-dependent phospholamban kinase [26]. At first sight, a permanent character of Ca^{2+}-pump activity in the myocardium may be caused by heart muscle function itself (it is in the myocardium that Ca^{2+}-calmodulin-dependent regulation via phosphorylation is present, which is not the case with fast skeletal muscles). It is suggested that a group of phospholamban molecules can remain phosphorylated even in the absence of β-adrenergic stimulation, when cAMP concentration is fixed at a low basal level. In this case, Ca^{2+} entry into the SR and cardiac rhythm may be controlled by calmodulin-dependent phospholamban phosphorylation [26]. However, the coupling of contraction cycles with phospholamban phosphorylation and dephosphorylation according to kinetic parameters is hardly possible in the heart of homoiothermal animals [123]. It should be noted that this coupling has been established for regulatory proteins of the myofibrils. At very low pulsation rate of the tortoise heart (4 beats/min at 9°C), the degree of myosin light chain (19 kDa) phosphorylation correlates with the development of tension [7].

Karczewski et al. observed a simultaneous increase in cAMP-dependent and calmodulin dependent phospholamban phosphorylation in response to isoprenaline injection [93]. The authors suggested that in this case cAMP-dependent phosphorylation of regulatory membrane proteins led to the increase in cytosol Ca^{2+} level and phospholamban kinase stimulation. Thus, calmodulin-dependent phospholamban phosphorylation can only enhance the cAMP-dependent response to β-agonists and does not play any independent role in the heart [93]. Moreover, doubted is not only the "independence" but also the regulatory role of Ca^{2+}-calmodulin-dependent phospholamban phosphorylation *in vivo* in general. Thus, in the intact guinea-pig heart [124] as well as in the rat heart [7] compounds such as ouabain and Ca^{2+} ionophores, which increased intracellular calcium concentration and induced a positive inotropic effect but, unlike β-agonists, did not increase cAMP level, could not phosphorylate phospholamban. It has been establisheed that Ca^{2+}-calmodulin dependent phospholamban phosphorylation can be reproduced *in vitro* and that SR membranes contain phospholamban kinase. At the same time, phosphate turnover rate is low at the site phosphorylated by this kinase and, *in vivo*, this site remains phosphorylated even in the absence of those compounds that increase the contractility. Therefore, a statement by Wegener et al. [224] about β-agonist-stimulated phospholamban phosphorylation by Ca^{2+} β-calmodulin-dependent protein kinase seems to be problematic. It is likely that phospholamban phosphorylation and myocardial relaxation can change under the influence of intracellular Ca^{2+} concentration changes and with the participation of calmodulin only when intracellular cAMP concentration is high [141].

In the myocardial SR preparations, endogenous phosphoprotein-phosphatase activity was found, which participated in phospholamban dephosphorylation [105, 107]. Dephosphorylation rate did not depend on the way in which phospholamban had been phosphorylated either by cAMP-dependent or by Ca^{2+}-calmodulin-dependent protein kinase [105]. Phospholamban phosphatase activity does not depend on Ca^{2+} or calmodulin. This protein phosphatase is closer in its properties to phosphatase I [132] but not to phosphatase 2A, as it was suggested earlier [107]. An important role that is performed in the heart by protein phosphatase I has become evident only recently and was underestimated earlier since this enzyme rapidly lost its activity in hearts left *in situ post mortem* [132]. Phosphoprotein phosphatase activity found in the SR supplements the regulatory cycle of Ca^{2+}-pump function in the heart, which is caused by phospholamban phosphorylation and dephosphorylation. Thus, it has been established that, upon phosphorylation by A-kinase, inhibitor 1 completely suppresses phosphoprotein phosphatase 1 activity. In the heart, inhibitor 1 is present in the cytosol, and its phosphorylation is stimulated by isoprenaline and inhibited by β-antagonists. Phosphorylated inhibitor 1 efficiently suppresses phospholamban dephosphorylation in the SR [81]. These data suggest the following sequence of events under conditions of β-adrenergic hormone action on the heart: A-kinase activation - inhibitor 1 phosphorylation - protein phosphatase 1 inhibition - slowing down of phospholamban dephosphorylation - increase in Ca^{2+} uptake rate in the SR. Recently, it has become evident that A-kinase performs an additional (as regards the direct phospholamban phosphorylation) regulation of Ca^{2+} transport in the cardiac SR not only by modulating the activity of inhibitor 1 but also via the change in phospholamban phosphatase compartmentalization [132]. Thus, it has been established that protein phosphatase 1 associated with the SR in the heart and skeletal muscle closely resembles or is even identical to protein phosphatase 1_G, an enzyme associated with glycogen particles. Phosphatase 1_G, probably a main phospholamban phosphatase of the heart, consists of a catalytic subunit (about 37 kDa) and G-subunit (about 160 kDa), the latter being responsible for binding to the SR and glycogen particles [132]. G-subunit is phosphorylated by A-kinase. Phosphorylation triggers phosphatase 1_G dissociation and free catalytic subunit translocation from the SR or glycogen particles into the cytosol. Released catalytic subunit is 5-10 times less active than the non-dissociated enzyme. Hence, in the heart, cAMP-dependent G-subunit phosphorylation appears to be an important mechanism of phosphatase 1 inhibition and corresponding additional increase in phosphorylation of Ser-16 on phospholamban in response to β-agonists.

Muscarinic receptor agonists, on the contrary, in some as yet unknown way activate phospholamban dephosphorylation. Thus, acetylcholine significantly suppresses phosphate uptake into phospholamban induced by isoprenaline and simultaneously blocks a positive inotropic effect produced by this catecholamine [220]. Such antagonistic action of muscarinic receptor agonists in respect to catecholamines is most likely not associated with the de-

crease in cAMP level or A-kinase activity inhibition [220] and seems not to depend on cGMP level [65]. It is very likely that muscarinic agonist action in the cardiac SR is realized through a cyclic nucleotide-independent mechanism.

Increase in Ca^{2+} uptake rate in the cardiac SR in the presence of cAMP allows to explain two main effects exerted by catecholamines on myocardial mechanical activity as follows: systole shortening and contractility increase. Under conditions of cAMP-dependent phospholamban phosphorylation, the acceleration of Ca^{2+} uptake in the SR may cause the shortening of systole since Ca^{2+} will be removed from troponin C at an increased rate. Acceleration of calcium uptake leads to an increase in Ca^{2+} accumulation in the SR, thus allowing the cell to keep a certain additional amount of Ca^{2+}. This additional Ca^{2+} may be delivered to myofibrils during the systole, thus causing the increase in myocardial contractility.

The influence of cAMP on ATP-dependent Ca^{2+} transport in the myocardial SR is actually generally accepted, perhaps with the only exception of the work by England et al. who did not observe phospholamban phosphorylation during heart perfusion with catecholamines [46], the data on the participation of calmodulin in the regulation of Ca^{2+} pump activity in the cardiac SR, however, are quite contradictory. Thus, on the one hand phospholamban is reported to bind Ca^{2+}-calmodulin complex, this binding hindering phospholamban phosphorylation [136]. The authors believe that such mechanism is acting during the systole, promoting the contraction under conditions of increase in $[Ca^{2+}]_c$ and blocking the stimulation of SR Ca^{2+}-pump. According to this opinion, cAMP dependent phospholamban phosphorylation is realized only when $[Ca^{2+}]_c$ value is low, that is during the diastole [125, 136]. On the other hand low calmodulin contents in the SR membranes may rule out calmodulin's regulatory role in Ca^{2+} transport by the cardiac sarcoplasmic reticulum *in vivo*. Thus, experimental study of canine cardiac SR membranes treated with [^{125}I] calmodulin allowed to reveal both 120 kDa complex of calmodulin with Ca^{2+}-ATPase and calmodulin complexes with phospholamban (40 kDa) and its subunits (26 kDa and 28 kDa) respectively [28]. At the same time, calmodulin contents in the membranes isolated in the presence of Ca^{2+} was only 1 mole calmodulin per 250 moles ATPase [64]. In the *in vitro* experiments, under conditions simulating the physiological ones (100 mM KCl), addition of saturating calmodulin concentrations to SR membrane preparations resulted in only insignificant (1.2-1.4-fold) increase in oxalate-dependent Ca^{2+} accumulation [231]. In addition, the participation of calmodulin in the regulation of myocardial SR Ca^{2+} pump activity *in vivo* can be doubted in connection with the fact that the affinity of this enzyme to calmodulin is about 10-100 times lower than that of most calmodulin-dependent enzymes [149].

In order to make it clear whether calmodulin can directly interact with cardiac SR Ca^{2+}-ATase or whether it activates Ca^{2+}-pump only via phospholamban phosphorylation by endogenous Ca^{2+}-calmodulin-dependent protein kinases, a comparative analysis of calmodulin effects on phospholamban-containing and phospholamban-free reticulum preparations was carried out [30]. In the former case, calmodulin exerted no influence on Ca^{2+}-ATPase at a wide range of Ca^{2+} concentration values, whereas in the latter case calmodulin activated Ca^{2+}-ATPase and Ca^{2+} transport, this activation being maintained if calmodulin was washed after phospholamban phosphorylation. The activation was accompanied by the growth of Ca^{2+}-ATPase affinity to Ca^{2+} [30]. The data thus obtained provide an evidence for the fact that the effect of calmodulin on cardiac SR Ca^{2+}-pump *in vitro* is mediated via phospholamban phosphorylation by B-kinases.

Problematicity of the regulatory role performed by such phosphorylation *in vivo* has been already mentioned above. It should be also mentioned that the slow kinetics of phosphorylation and dephosphorylation processes seems to rule out the participation of calmodulin in the regulation of contraction and relaxation cycles in the heart [30].

As it has been established recently, purified phospholamban isolated from the canine heart contains about 0.6 micromole of lipid P_i per mg protein [82]. Main phospholipids associated with phospholamban are phosphatidylserine (34%), phosphatidylcholine (22%), sphyngomyeline (17%), phosphatidylethanolamine (9%) and PI (13%) which stimulates phosphorylation of purified phospholamban [200]. These phospholipids forming a complex with phospholamban are phosphorylated by catalytic A-kinase subunit, up to 4 nmole of phosphate being incorporated per mg protein [82]. Main phosphorylated phospholipids are phosphoinositides, PIP and PIP_2. Phosphoinositide phosphorylation ky A-kinase was demonstrated also in rabbit cardiac SR [48]. It is thus far unclear whether A-kinase itself directly phosphorylates phosphoinositides or whether A-kinase regulates the activity of endogenous phosphatidylinositide kinases. At any rate, catalytic subunit of A-kinase phosphorylates purified PI to form only PIP_2 [82]. At the same time, phospholamban phosphorylation is associated with the formation of not only PIP_2 but also of PIP. Polyphosphoinositide phosphorylation is reversed by phospholamban phosphatase and inhibited by thermostable A-kinase inhibitor [82].

Phospholipid phosphorylation, like protein phosphorylation, can also take part in the regulation of membrane function. Thus, for instance, it is believed that phosphoinositide phosphorylation regulates membrane permeability for ions [11] as well as SR Ca^{2+}-ATPase activity in the skeletal muscles [212] (The authors demonstrated that SR calcium ATPase activity undergoes a considerable increase during phosphorylation of enzyme associated PI to PIP) and plasmalemmal Ca^{2+}-ATPase activity in the red blood cells [170]. Although polyphosphoinositides are minor components as compared with PI, their phosphorylation and dephosphorylation seem to be able of changing

phospholamban's polarity and thus of modifying the conformation of this protein. Changes in phospholamban's conformation, in their turn, seem to play a key role in phospholamban phosphorylation by protein kinases and/or in the interaction between phospholamban and Ca^{2+}-ATPase.

It is possible that the effect of β-adrenergic agonists on the mammalian heart is at least in part caused by the increased formation of polyphosphoinositides and by corresponding changes in the properties of the SR. Thus, β-adrenergic stimulation of a contracting heart together with phospholamban phosphorylation at the peak of the inotropic response [106] also leads to the increased phosphate incorporation into phosphoinositides, resulting in the formation of PIP and PIP_2 [83]. Influence of protein phosphorylation on Ca^{2+} transport into the SR is sufficiently well-studied, regulation of Ca^{2+} release from the SR through reticulum channels in response to an electric stimulus and/or to the action of intracellular second messengers such as Ca^{2+} or IP_3, however, is largely unclear. It is known that the "light" reticulum contains large amounts of Ca^{2+}-ATPase and calsequestrin, a calcium-binding protein. On the contrary, "heavy" SR fraction with high specificity binds calcium channel blockers, which indicates the preferential participation ot this fraction in Ca^{2+} release into the cytoplasm in response to electric excitation of the SR or to nicotinic receptor agonists [149]. It was shown that phospholamban phosphorylation by Ca^{2+}-calmodulind-dependent protein kinase did not influence the passive Ca^{2+} release from canine cardiac SR vesicles preloaded with this ion [96]. Obviously, the mechanism of Ca^{2+} release from the SR is not caused by calmodulin-dependent phosphorylation. It is believed that cAMP-dependent phosphorylation in the myocardial SR is associated with Ca^{2+} release stimulation [96]. However, Kovacs et al. reported that dephosphorylated phospholamban could operate as a calcium channel [101]. In the "heavy" SR fraction, high-molecular proteins were found, their molecular weights being 305 kDa and 320 kDa respectively; both proteins could be phosphorylated by endogenous calmodulin-dependent and exogenous cAMP-dependent protein kinase [149]. Functional role of these proteins in Ca^{2+} transport regulation remains unknown.

cAMP-dependent and calmodulin dependent stimulation of Ca^{2+} transport in the SR of vascular smooth muscle as well as the presence of phospholamban in this structure were doubted even quite recently [29]. However, up to now, data have been obtained that provide a convincing evidence for the presence of phospholamban in the SR of many smooth muscle types including that of the blood vessels [159], which might throw the light on calcium transport regulation in the SR of these muscle types. Such regulation is carried out in particular using cAMP-dependent Ca^{2+} transport stimulation in the SMCs [39, 146].

It has been established that in aorta microsome preparations both A-kinase and its catalytic subunit phosphorylate an 11 kDa protein [221]. Contents of this protein, as determined by the authors, is about 17 pmole/mg microsomal protein, which virtually corresponds with the contents of Ca^{2+}-pump (18 pmole/mg protein). Hence, the stoichiometry between 11 kDa phosphoprotein and 110 kDa phosphoenzyme may be 1:1, that is exactly the same as in the case with phospholamban and cardiac SR Ca^{2+}-ATPase. Like in the heart, phospholamban phosphorylation in the aorta smooth muscle increases SR Ca^{2+}-pump's affinity to Ca^{2+} without changing the maximum Ca^{2+} transport rate [221].

cAMP-dependent Ca^{2+} transport stimulation in the aorta microsomes is, however, significantly lower than that in the myocardial SR [164]. Since phospholamban amino-acid sequence in the smooth muscle is identical to that in the heart, the above difference cannot be associated with the difference in phospholamban structure between these two muscle types but seems rather to be caused by the simultaneous presence of several (up to 3) Ca^{2+}-ATPase isoforms in the SMC SR [161]. In addition, phospholamban may either interact in different ways with these isoforms or be bound only with one of them. Furthermore, the difference in the degree of cAMP-dependent Ca^{2+} transport stimulation in the SR between CMCs and SMCs may reflect the different basal phospholamban phosphorylation levels in these muscle types [221] and, as argued by Raeymaekers et al., a lower phospholamban/Ca^{2+}-ATPase ratio in the smooth muscle as compared with that in the heart [161]. Preincubation of aorta microsomes with A-kinase inhibitor did not exert any influence on the oxalate-dependent Ca^{2+} accumulation at Ca^{2+} and oxalate concentrations of 0.3 μM and 10 mM respectively [221]. It is likely that quantitative difference in A-kinase effect on Ca^{2+}-transporting systems in the SR of the heart and smooth muscle reflect the difference in phospholamban's interaction with Ca^{2+}-pump between these tissues. The qualitative effect of cAMP-dependent phospholamban phosphorylation is, however, the same in the aorta SMCs as in CMCs, that is the acceleration of blood vessel and heart relaxation.

Unlike the cAMP effect, cGMP effect on Ca^{2+} transport in the SMC SR could not be observed at first [197]. This fact was probably connected in particular with the difficulties in experimental discrimination between cGMP influences on the SR and plasmalemma. However, recently applied method of chemical plasmalemma skinning with the use of saponin allows to distinguish the role of cGMP in Ca^{2+} transport regulation in the SR of vascular SMCs. Addition of cGMP at concentration ranging from 10^{-8} M to 10^{-5} M to the preparations of the SL-free cells leads to a considerable Ca^{2+} transport stimulation which may be compared with that achieved by the effect of cAMP on this process [210]. Subsequent introduction of 10 μM G-kinase into the incubation medium does not enhance cGMP's stimulating influence, which may provide an evidence that endogenous G-kinase was not washed out during the skinning procedure. The influence of cGMP on calcium transport in the aorta SMC SR manifests itself both when

$[Ca^{2+}]_c$ value is equal to that characteristic of the SMCs in the relaxed state (0.1 μM) and that characteristic of the contraction (0.3-1 μM) [210]. Thus, in authors' opinion, vasodilators that influence the vascular smooth muscle via the cGMP system can cause the relaxation of blood vessels, in particular by accelerating Ca^{2+} accumulation in the SR. It is believed that such influence of cGMP on the vascular tone may be associated with the activation of G-kinase and subsequent phospholamban phosphorylation by this enzyme at the same Ser residue as in the case of phospholamban phosphorylation by A-kinase [160].

However, it should be noted that conclusions on the physiological importance of cGMP-dependent phospholamban phosphorylation for the induction of a decrease in vascular SMC $[Ca^{2+}]_c$ under the influence of vasodilators causing the increase in intracellular cGMP contents are based on the *in vitro* experimental findings. As regards the *in situ* situation, phospholamban phosphorylation could not be observed in rabbit aorta preparations precontracted using K^+ depolarization, upon addition of Na nitroprusside to these preparations [73]. The absence of any effect of nitroprusside on phospholamban phosphorylation in this study was not associated with the dephosphorylation of this protein during the procedure of SMC membrane isolation. The absence of cGMP-dependent phospholamban phosphorylation *in situ* may indicate in particular the difference in phospholamban, G-kinase and/or cGMP compartmentalization in the intact SMCs.

It should be noted that cyclic nucleotide and protein kinase compartmentalization in the myocytes makes it difficult to extrapolate the data obtained *in vitro* to the situation in the live cell. For instance, it is known that PGE_1 induces the increase in cAMP level in the CMCs but, unlike isoprenaline, does not cause any positive inotropic effect [207]. In addition, under the influence of isoprenaline, cAMP level and A-kinase activity grow higher both in the membrane compartments and in the cytosol of CMCs, whereas under the influence of PGE_1 these parameters increase only in the soluble CMC fraction [69]. Obviously, the difference in the effects on myocardium between isoprenaline and PGE_1 is not associated with the presence of different A-kinase isoforms in the CMC compartments. Thus, it is known that in the myocardium of many animal species type II A-kinase is associated with the CMC cell membrane. At the same time, isoprenaline and PGE_1 exert similar effects in the rat (80% type I A-kinase) and guinea-pig (90% type II A-kinase) heart [69]. The absence of a positive inotropic effect is ascribed to the lack of phosphorylation of a considerable number of regulatory target proteins in the CMCs during A-kinase activation [69]. Similar effect on the myocardium is exerted also by rolipram, a phosphodiesterase inhibitor [171]. It may be suggested that these compounds induce the increase in cAMP level and A-kinase activity in those compartments that do not contain target proteins. Rat heart perfusion with forskolin and isoprenaline leads to equal increases in cAMP level in the CMCs; however, A-kinase activation and protein phosphorylation level are significantly higher in the case of isoprenaline perfusion [47]. This fact provides an evidence that forskolin increases cAMP level in those compartments that are inaccessible for A-kinase.

Total combination of the data thus obtained formed a basis for an hypothesis that cAMP level increase in the submembrane compartments of the CMCs results in a translocation of a dissociated catalytic A-kinase subunit from the insoluble into the soluble fraction [143]. Such translocation can be observed in the presence of isoprenaline but not of PGE_1, indicating that this process is necessary for the development of positive inotropic effect. However, it means that target proteins can "choose" between the catalytic subunit translocated into the cytosol and soluble A-kinase already present there. Thus, PGE_1 induces a 65% activation of soluble A-kinase without increasing the contractility [171]. Hence, it can be concluded that a considerable portion of soluble A-kinase in the CMC is spatially isolated from its potential substrates [143]. This hypothesis is also supported by the finding by Aass et al. that submembrane cAMP pool is the most important factor in the regulation of heart contractility [1].

The data presented in this review on the compartmentalization of second messengers, protein kinases and protein phosphatases in SMCs and CMCs again and again remind us of the caution with which results obtained in the experiments *in vitro* should be extrapolated to the situation *in situ*. Compartmentalization of second messengers, enzymes of second messenger metabolism, protein kinases and protein phosphatases should taken into account also when choosing therapeutic methods based on the modulation of strength and duration of intracellular signals. As it is shown by practical experience, the use of compounds such as dibutyryl cAMP, other cyclic nucleotide analogues penetrating through the cell membrane etc. is far from being always efficient. Indeed, it has already become clear that, in order to obtain a functional response, the increase or the decrease in second messenger as well as the modulation of corresponding protein kinase activities are required not in the entire cell but in its certain specific compartments. This effect can be achieved, for example, by specifically inhibiting or activating different cyclic nucleotide PDE forms localized in corresponding intracellular compartment.

At present, the investigation into the influence of second messengers on the permeability of smooth muscle SR ion channels is underway. Thus, it has been established that cAMP can stimulate not only accumulation but also the release of Ca^{2+} from the SR of vascular smooth muscle [18, 177]. A biphasic character of the latter process suggests the heterogeneity of SR Ca^{2+} channels [222]. cGMP does not exert any influence on the IP_3-induced or caffeine-induced Ca^{2+} release from the aorta SR [210]. Hence, the previously observed inhibitory effect of human atrial natriuretic factor on Ca^{2+} release from the SR depot [53] was probably associated with a decrease in IP_3 synthesis rather

than with the cGMP influence on calcium permeability of SR channels.

In summary, it should be noted that the regulation of free calcium level in the cytosol of CMCs and SMCs, which determines the functional activity of the myocardium and vascular smooth muscle, is controlled by different second-messenger systems. According to the data obtained thus far, in the heart as well as in the smooth muscle, the main role in this control *in situ* belongs to cyclic nucleotide-activated protein kinases as compared with the role performed by Ca^{2+}-calmodulin-dependent and Ca^{2+}-phospholipid-dependent kinases. First of all, this is the case with the regulation of Ca^{2+}-channel and cell-membrane Ca^{2+}-ATPase activities as well as SR Ca^{2+}-ATPase activity. In the vascular smooth muscle, a priority belongs to cGMP-dependent membrane phosphorylation regulation, whereas in the heart the leading role is performed by cAMP-dependent regulation. This difference may be connected in particular with higher cGMP and G-kinase levels in the vacscular SMCs as compared with those in the CMCs. At the same time, an important role of C-kinase in the regulation of plasmalemmal Ca^{2+}-ATPase activity in the smooth muscle is evident. Similarly important is the role of Ca^{2+}-calmodulin-dependent protein kinase in the regulation of Na^+ vs Ca^{2+} exchange in the heart. However, it should be emphasized that these conclusions cannot be regarded as final ones, since regulatory phosphorylation in the membranes of cardiovascular system cells arouses the growing interest in investigators into this field of biochemistry of intracellular signals and new data can be expected to appear in the next future.

The Role of Calcium Overload in Vascular and Heart Lesions

It has been established that a critical factor in the development of profound changes in damaged CMCs is the accumulation of excessive calcium ion amounts in the sarcoplasm [32, 155]. Pronounced arrhythmia is one of the consequences of this event [60]. High $[Ca^{2+}]_c$ level in myocardial ischemia causes CMC depolarization resulting from membrane ion permeability change [32], the shortening of action potential and the decrease in myocardial contractility [118]. It may be suggested that overloading of the cytosol of damaged CMCs with calcium ions and corresponding disturbances in heart activity are associated in particular with the disturbance in protein phosphorylation in the membranes of these cells [5].

It is also known that virtually all atherosclerosis-inducing processes such as changes in membrane permeability, protein secretion and cell proliferation, migration and death are regulated by intracellular Ca^{2+} [104]. Significantly increased cytosol calcium level can be observed in experimental atherosclerosis. At the same time, calcium antagonists that inhibit Ca^{2+} entry into the cell do not reduce cholesterol and low-density lipoprotein contents in the plasma but prevent the development of atherosclerotic lesions in the blood vessels of rabbits receiving cholesterol-rich diet [196, 198]. On the contrary, vitamin D_3 shows atherogenic action since it promotes Ca^{2+} uptake into the cells [104]. It is very likely that cholesterol is only one of the risk-factors in the development of atherosclerosis since, being incorporated into the plasmalemma, it interferes with the functions of ion channels and enzymes responsive to lipid microenvironment. Thus, the increase in red blood cell membrane cholesterol leads to an increase in Ca^{2+} entry in these cells [232], which correlates with the progress of hypertension. Similar correlation can be observed between $[Ca^{2+}]_c$ level in the vascular SMCs and the increase in blood pressure [196], which results in the hypercontractility of SMCs.

High intracellular Ca^{2+} level causes the most important atherogenetic events such as SMC migration into the intima, SMC proliferation in the intima in response to platelet growth factor, mast cell formation [104], thrombosis [151] (Increase in platelet cGMP level can reduce intracellular calcium level, prevent thrombogenesis and hinder atherogenesis [137]) and elastin and glycosaminoglycan secretion by the SMCs [58].

The increase in Ca^{2+} concentration causes also the exposure of hydrophobic domains on elastin molecule, which promotes cholesterol binding [103].

It should be noted that atherogenesis is characterized by increased $[Ca^{2+}]_c$ not only in vascular SMCs but also in the endothelial cells. As a result, the increased endothelial cell contractility contributes to the increase in endothelial barrier permeability for platelets and low-density lipoproteins [148]. Interestingly, morphologic endothelial lesions and the increase in low-density lipoprotein transport in the perfused aorta can be caused by a simultaneous increase in cAMP and diacylglycerol levels [215]. It is very likely that the synergism between β-adrenergic adenylate cyclase activators and α-adrenergic (or muscarinic cholinergic) activators of phosphoinositide metabolism may determine one of the ways of endotheliocyte Ca^{2+} homeostasis disturbance in vascular lesions of different origins.

Hence, the maintenance of Ca^{2+} homeostasis in the cells of blood vessels and myocardium is of utmost importance for the prevention of damage to these tissues. Thus, it is possible that, basing on the use of compounds that regulate Ca^{2+} level in the arteries and in particular in the SMCs, alternative methods will be developed to combat atherosclerosis even in those cases when high plasma cholesterol and low-density lipoprotein levels cannot be corrected. In turn, targets for these compounds may be represented by ion channels and pumps of the cell membranes, whose participation in the maintenance of Ca^{2+} homeostasis as well regulation of Ca^{2+} homeostasis by second messengers in the CMCs and vascular SMCs have been the subject of the present review.

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