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How to measure myofilament calcium sensitivity? Theory and practical applications

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In the heart as well in the smooth muscle (SM), contractility is determined mainly by the myoplasmic free calcium concentration. The more calcium that appears in the myoplasm, the more muscle contracts. However, between the appearance of calcium and the occurrence of contraction force lies a whole chain of biochemical reactions or the signaling pathways. This pathway is influenced by many intracellular factors that can facilitate or inhibit the passage of the calcium signal to the contractile apparatus, thereby determining the degree of muscle contraction in response to changes in intracellular calcium, or myofilament calcium sensitivity. Changes in myofilament sensitivity to calcium following changes in signaling pathway might be a very important in regulation muscle contractility [1–3].

One of the main dreams of pharmacologists and clinicians involved in cardio-vascular studies and disease treatment is based on the idea that the ideal positive inotropic agent (calcium sensitizer or so-called genuine cardiotonic) may be one whose sole action is to increase the sensitivity of contractile proteins to calcium. The problem is that almost all cardiotonics we know lead to Ca^{2+}- overload of myoplasm and thereat they are appeared to be rather toxic compounds.

In the case of vascular SM, clinicians usually need drugs with an opposite effect. Namely, they need compounds that possess the ability to decrease myofilament calcium sensitivity selectively and specifically, and hence relax SM and decreasing vascular tone under arterial hypertension.

Currently, it is this way of influencing calcium sensitivity/affinity that attracts the attention of pharmacologists, which represents itself a new, conditionally calcium-independent mechanism for vascular tone and cardiac contractility regulation. The data obtained in our and other laboratories may be a fundamental basis to design a new drug generation that possesses the ability to affect vascular tone or cardiac contractility via its direct effect on the contractile machinery or pathways regulating vascular tone. Finally, the role of changes in the affinity of regulatory proteins to calcium represents a common biological problem that has great importance in the understanding of cellular functions and communications.

That is why it appears to be important to know more about intracellular mechanisms underlying changes in calcium sensitivity of the contractile proteins, and that is why we must be able to evaluate this parameter properly and precisely.

Theoretical background. It is widely accepted that smooth muscle excitation evokes contraction via an increase in intracellular free calcium. In instance, in the beating heart, contractile activity is switched on and off in a most regular manner by the rise and fall of the myoplasmic free calcium ion concentration. In this work, we will discuss some issues related to the mechanisms of regulation of Ca^{2+} sensitivity and methods for its determination mainly for smooth muscle cells of blood vessels.

In the most simplified form, both contraction and relaxation of vascular SM develop along two main pathways [1, 4]. It is known that SM contraction follows an increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), the formation of complexes

with calmodulin, activation of myosin light chain kinase (MLCK), and phosphorylation of the 20 kDa light chain of myosin at Ser19 (first way). SM relaxation produced when the cytosolic Ca^{2+} concentration decreases (first way) or when the activity of myosin light chain phosphatases (MLCP) increases (the second way) (Fig. 1). Interestingly, several agonists acting via G-protein coupled receptors elicit a contraction with no increase in intracellular Ca^{2+} concentration but via inhibition MLCP and increased myosin phosphorylation [3].

It is well known how Ca^{2+}-entry and/or Ca^{2+}-release can be affected. Many pharmacological interventions appear to be involved in these calcium transients in both experimental conditions and clinics. It is important to concentrate our attention on that part of this scheme which operates mechanisms related to calcium – calmodulin interaction and phosphorylation/dephosphorylation of contractile proteins Any intervention in this path at any stage will lead to a change in the contractile state of the muscle, that is, it will change the ratio between $[Ca^{2+}]_i$ and the developing force of contraction [3].

Indeed, the contractile force depends on the calcium occupancy of the regulatory proteins (calcium «receptors») such as troponin C within cardiac myofilaments, or calmodulin in SM. However, specific calcium-binding sites are never fully occupied. At most they are half saturated with calcium during systole, so the contractility may be up- or down-regulated according to the variable calcium occupancy of troponin/calmodulin. In this way, the contractile activity of the myocardium and SM may be varied over a wide range to meet changing hemodynamic demands.

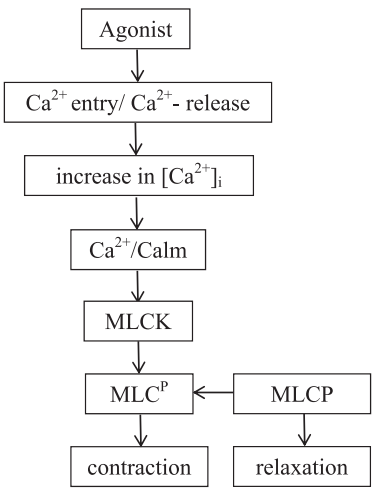

Fig. 1. Contraction-relaxation cycle in smooth muscle: Calm – calmodulin; MLCK – myosin light chain kinase; MLC^P – phosphorylated myosin light chain; MLCP – myosin light chain phosphatases

In principle, calcium occupancy of regulatory proteins may be increased, at least, in two ways. First, it may be increased by pharmacological interventions that increase $[Ca^{2+}]_i$. Norepinephrine, for instance, initiates a cascade of reactions that lead to a rise of $[Ca^{2+}]_i$. But this way of regulating the contraction is not associated with a change in the Ca^{2+}-sensitivity of the signaling contractile path, since the Ca^{2+}-regulatory protein binding constant do not change. However, amplitude modulation of calcium signal is not the only way to increase calcium occupancy of regulatory proteins and hence contractility. Another way is a modulation of the sensitivity of the calcium-signal receiver troponin or calmodulin. If, for instance, the affinity of proteins for calcium is enhanced, the calcium occupancy, as well as contractile force, increases at a given calcium ion concentration. Thus, we know that the contractile activity may also vary over a wide range without significant changes in $[Ca^{2+}]_i$ but there is a little known how it can be realized?

The second way to change Ca^{2+}-sensitivity is to influence the lower stages of the contractile signaling pathway, which determines and regulate the interaction of Ca^{2+}-troponin C or Ca^{2+}-calmodulin complexes with contractile proteins. Currently, some cardiotropic compounds (bepridil, levosimendan, EMD 57033 and others [5–7]), which realize their action by interacting with the components of the troponin complex, have already been found. As a result of this interaction, the sensitivity of thin filament to calcium increases without increasing in intracellular Ca^{2+}, which reduces the risk of

side effects due to calcium overload. It was a good and perspective idea to create drugs that modulate troponin C activity. This protein is a promising pharmacological target for the treatment of cardiac insufficiency.

The possibilities of creating new vasodilators whose the mechanism of action is based on the same principle, is also being intensively investigated and are aimed at activation or inhibition of specific signaling pathways, including the phosphatase signaling pathway, in vascular smooth muscle. Some potential pharmacological targets, the influence on which leads to relaxation of blood vessels and a lower of blood pressure, have already been identified. There is evidence of certain successes in this direction when using, for example, Rho-kinase inhibitors, endothelin inhibitors, G-protein receptor kinase-2 inhibitors, which modulates the dilatory ability of beta-adrenoreceptors, etc. [1]. The famous sildenafil, which has a relaxing effect through the inhibition of cGMP-specific phosphodiesterase type 5, regulating nitric oxide-dependent phosphatase signaling pathway, can also be attributed to this class [11].

It should be noted that the presented scheme is very simplified and the processes that regulate the transmission of the Ca^{2+} signal to contractile elements are much more diverse and complex [1–3]. However, for this article, we will based on simplification that Ca^{2+} sensitivity is determined by the balance between kinase phosphorylation and phosphatase dephosphorylation, which are the main ways of regulating contractility [8].

How is it possible to measure myofilaments calcium sensitivity? In the study of potential Ca^{2+} sensitizers or desensitizers, the main task is to prove that the change in contraction during their action occurred precisely without a change in Ca^{2+}, i.e. that a change in Ca^{2+} sensitivity has occurred. However, the question arises of how to measure the sensitivity, how to prove that it has changed, and what parameters need to be measured for this.

First of all, it is necessary to determine what we mean by the concept or term of «calcium sensitivity». We would like to express this concept with one parameter or number, which would characterize the relationship between the contractile force development and the concentration of free Ca^{2+} in the myoplasma. This would be an integral indicator, uniting the entire cascade of biochemical events between the appearance of Ca^{2+} and contraction, each of which can affect the transmission of the Ca^{2+} signal and change the magnitude of the contractile response, thereby changing the sensitivity of the contractile apparatus.

How are the concentrations of intracellular Ca^{2+} associated with the magnitude of the contractile response? The sequence of events between the appearance of Ca^{2+} in the myoplasma and the occurrence of the contraction force is a complex, multi-stage event and may be characterized mathematically on each stage with its own model and its own rate constants. The full mathematical expression describing the model of this process is very complex, cumbersome and not suitable for any practical use. However, fortunately, the dependence Ca^{2+} – contraction can be described with a good degree of accuracy by the Hill equation, which is quite enough determined by three parameters that are quite suitable for practical use [9]. (There is evidence that this dependence can be more accurately described by the two Hill equations [10], however, most researchers believe that one equation is a good approximation.). Under this assumption, the relationship between Ca^{2+} and contraction can be described by a simple scheme $Ca^{2+} + A \leftrightarrow F$, where Ca^{2+} is the intracellular calcium concentration, F is the contraction force resulting from this, A is a generalized summary parameter that includes all stages of biochemical enzyme-regulated reactions, beginning from Ca^{2+} interaction with calmodulin and ending with phosphorylation of myosin light chains.

Therefore, the force-calcium relation, which corresponds this simple model, should be described as Langmuirean isotherm (rectangular hyperbole or binding

isotherm) and force developed might be expressed as follows:

$$F = F_{max} \cdot [Ca^{2+}]_i / EC_{50} + [Ca^{2+}]_i;$$

where F is developed force, F_{max} is the maximal force at saturating $[Ca^{2+}]_i$, and EC_{50} is Ca^{2+} concentration needed to produce 50% of maximal response (Fig. 2A).

If you plot the same data on a semilog plot (the X-axis is log of $[Ca^{2+}]_i$ it becomes sigmoidal (Fig. 2B). The only difference between the left and right panel the graph is whether the X-axis is linear or logarithmic. However, on practice, we have deal with S-shape (sigmoid) curves if even linear X-axis is used, and that is why the force will be described with rearranged Hill relation. The Hill equation is as follows

$$F = F_{max} \cdot [Ca^{2+}]_i^n / EC_{50}^n + [Ca^{2+}]_i^n;$$

where n is the Hill coefficient. This dependence is definitely determined by three parameters – F_{max}, EC_{50}, and *Hill coefficient*.

How to characterize this dependence in a convenient way that would be suitable for practical purposes and would prove the existence of Ca^{2+} sensitization under any exposure? Imagine, for instance, you have decided to conduct the screening and bring to light potential Ca^{2+}-sensitizer and to compare it with a reference substance. Common sense suggests that it would be logical to unambiguously relate the value of the $[Ca^{2+}]_i$ to the force of contraction and introduce some comfortable coefficient of sensitivity, for example, $F/[Ca^{2+}]_i$, and the relation $F/[Ca^{2+}]_i$ should be calculated in both cases. These two values will be reflection of corresponding myofilament calcium sensitivity. To do this, we need to incubate vascular preparation with a potential Ca^{2+} -sensitizer and then act on him with any agonist. You should provide the measurements of two main parameters – force and intracellular calcium concentration. It seems very simple and attractive but it only seems that way. The use of such a coefficient is valid only with direct proportionality between Ca^{2+} and contractile force developed. But this dependence is non-linear, therefore, this method is incorrect, since the value of this coefficient will strongly depend on the value of $[Ca^{2+}]_i$ at which the measurement was performed.

Another way of expressing the Ca^{2+} sensitivity seems to be more correct, in which this parameter will be measured as follows: $\Delta F/\Delta[Ca^{2+}]_i$ or $dF/d[Ca^{2+}]_i$. To standardize the measurements, it seems correct to carry out measurements before and after exposure under the same conditions, therefore it is logical to choose for analysis similar points on the quasi-linear portion of the dose-response curve, for example, corresponding to EC_{50} values, where dF/dCa has maximum value. However, this method has its own drawbacks and does not seem correct in all cases. Before assessing the adequacy of this method, let's consider the possible options for changing the Hill curve, which can be realized with a change in Ca^{2+} sensitivity after any exposure. Theoretically, there are three possible reasons of a change in the Hill curve under any action (Fig. 3).

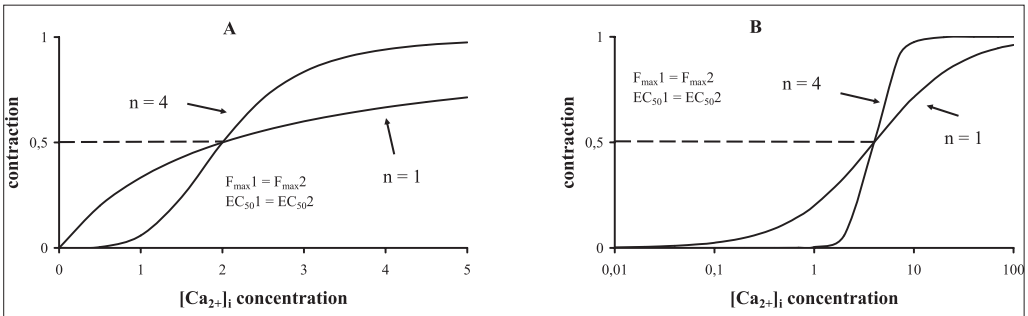

Fig. 2. A plot of the conditional contraction value as a function of the conditional $[Ca^{2+}]_i$ concentration value as described by the Hill equation. Hill curves parameters are as follows: $F_{max}1 = F_{max}2 = 1$, $EC_{50}1 = EC_{50}2 = 2$, $n = 1$ and $n = 4$ for two curves. The only difference between the left and right panel the graph is whether the X-axis is linear or logarithmic

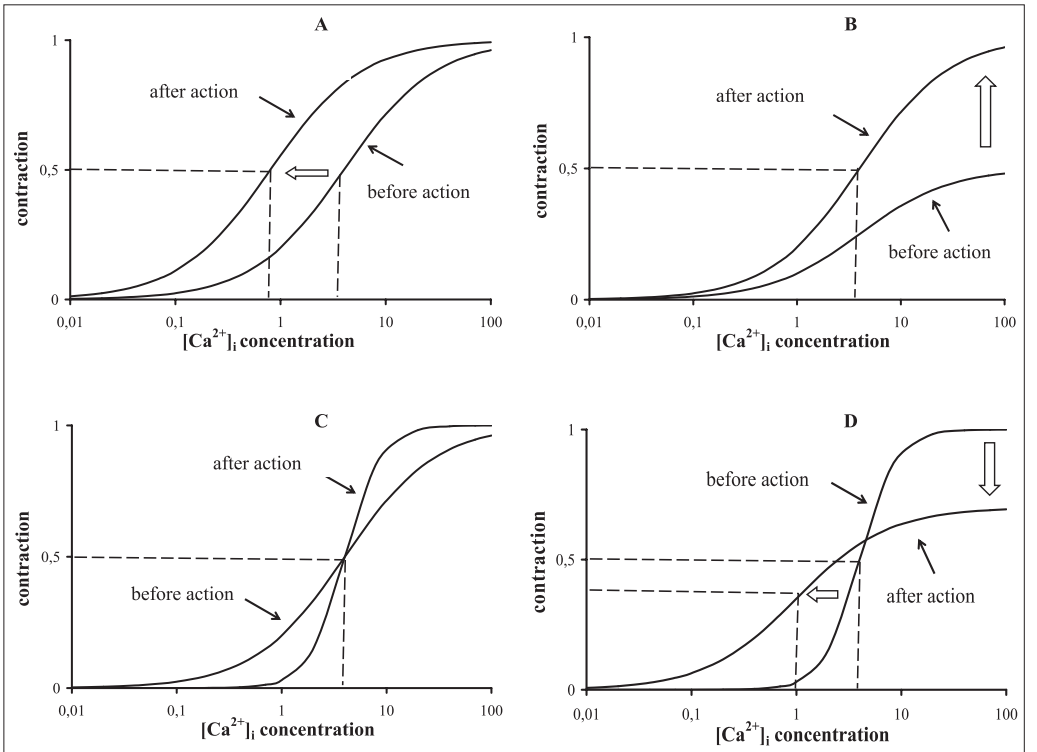

Fig. 3. A plots of the conditional contraction value as a function of the conditional $[Ca^{2+}]_i$ concentration value as described by the Hill equation for 4 cases of changing Hill curves parameters: A – EC_{50} changing; B – F_{max} changing; C – Hill coefficient changing; D – F_{max} and Hill coefficient changing

The first case is a change in the EC_{50} (Fig. 3A). We can assume that in this case, the EC_{50} will be a measure of muscle sensitivity to calcium. This description prevails among most researchers, and namely this parameter in most cases called sensitivity. Moreover, a change in muscle response to $[Ca^{2+}]_i$ is characterized by ΔEC_{50}. Indeed, in this case, the sensitivity to $[Ca^{2+}]_i$ changes, i.e. for each concentration of $[Ca^{2+}]_i$ after exposure, the amplitude of contraction becomes larger at the same $[Ca^{2+}]_i$ than before exposure. The second case is a change in F_{max} (Fig. 3B). In this a situation, F_{max} will already be a measure of sensitivity (in the literature this value is defined as responsiveness), and the change in sensitivity will be characterized by ΔF_{max}, since the EC_{50} value does not change, and the expression of sensitivity changes in terms of EC_{50} loses its meaning. In this case, an increase in the force of contraction after exposure will also be recorded with equal $[Ca^{2+}]_i$. And finally, the third case is a change in the Hill coefficient n (Fig. 3C). In such a situation, it is not clear at all how to characterize Ca^{2+} sensitivity and its changes, since the two previous definitions of sensitivity and responsiveness demonstrate the absence of changes, while it is obvious that the changes occure. Changes in the Ca^{2+} sensitivity, in this case, are ambiguous and more complex, since for large $[Ca^{2+}]_i$ an increase in the contraction force is observed, but for small values of $[Ca^{2+}]_i$ below the EC_{50}, it decreases.

In practice, of course, the real situation will be a combination of all three cases. It is theoretically possible to imagine the situation where ambiguous multidirectional changes in the contractile response to an increase in Ca^{2+} in the myoplasm can occur, for example, when EC_{50} and E_{max} increased simultaneously (Fig. 3D).

Let us return to the discussion of the second method of characterization Ca^{2+} sensitivity. If we try to characterize Ca^{2+} sensitivity as $dF/d[Ca^{2+}]_i$ at EC_{50},

a comparison of this value before and after exposure will lead to misconception of the real situation. The first derivative of the Hill equation at EC_{50} point is expressed by the following relation:

$$dF/d[Ca^{2+}]_i. = F_{max} \cdot n /4EC_{50};$$

Imagine a situation where, as a result of exposure, a simultaneous decrease in half both F_{max} and EC_{50}, occured i.e. F_{max} (after action) = F_{max} (before action)/2, and EC_{50} (after action) = EC_{50} (before action)/2. Then, $dF/d[Ca^{2+}]_i$. (after action) = $dF/d[Ca^{2+}]_i$. (before action).

It means that we can come to the wrong suggestion. The coefficient of calcium sensitivity, expressed in this way, states that there is no change in the dependence of F on $[Ca^{2+}]_i$, while even a visual comparison of the dose-response curves shows that such changes are obvious and significant. Thus, the value dF/dCa in the case of unidirectional changes in F_{max} and EC_{50}, or n and EC_{50} can give a wrong idea about the change in the SM cells reaction to calcium, or not show at all while dramatic changes in the dose-response curve occurred. In this scenario, at different $[Ca^{2+}]_i$ concentrations, both sensitization, and desensitization to calcium will be observed. The description of changes in $[Ca^{2+}]_i$ sensitivity using any one parameter will lead in this case to an incorrect interpretation of the data obtained and incorrect conclusions that do not correspond to the actual situation. The conclusion about calcium sensitization, based on a decrease in EC_{50}, will be valid only in case of small concentrations of intracellular Ca^{2+}, resulting from an excitatory effect of agonist, and will lead to an incorrect description of the contractile state of the muscle, since with strong stimuli a reduced contractile response to stimulation will be observed. Conversely, the conclusion about calcium desensitization based on a decrease in F_{max} will also give a distorted data of the contractile state of the muscle, since under small stimulating effects an increase in the contractile response will be observed. The data obtained in this case do not provide an answer to the question of whether this is due to one process, or different ones. In each case, other in-depth studies are needed to understand the mechanisms of this phenomenon.

So, the final goal of a screening study is to register and analyze the dependencies F- $[Ca^{2+}]_i$. Therefore, to describe vascular smooth muscle contractile machinery and to estimate myofilament calcium sensitivity properly, we need to measure at least three parameters:

- the affinity of myofilaments to Ca^{2+}, or sensitivity, which characterized by EC_{50} (PCa_{50}) which, in turn, is the concentration of ligand (calcium) needed to produce 50 % of maximal response;
- efficacy or myofilament calcium responsiveness, which characterized by maximal Ca^{2+}-activated force;
- the Hill coefficient (the Hill slope, or the slope factor).

The theoretical considerations presented, in principle, are valid for any type of muscle, including smooth, cardiac and skeletal muscles. However, all subsequent discussions on methods for determining changes in calcium sensitivity will concern only the SM cells of blood vessels.

In our opinion, for practical screening purposes, the following 3 methods can be considered as the most simple, adequate, relatively affordable and relatively cheap methods.

The optimal experimental design comprises:

- contractile recordings in intact smooth muscles;
- contractile recordings in chemically permeabilized (skinned) smooth muscles;
- simultaneous measurement of $[Ca^{2+}]_i$ and force development.

Also, it is desirable to use one more experimental technique as an additional method. Since the second main mechanism for changing Ca^{2+}-sensitivity is the change in the activity of phosphatase of the myosin light chains, which, in turn, is controlled by two enzymes, Rho-kinase and protein kinase C [8], it is important to determine the state of the phosphatase signaling pathway, i.e. determine the dilatory ability of the vessel under vasodilators action. For this aim nitroglycerin (if the muscle with removed endo-

thelium), and acetylcholine (if the endothelium is preserved) are suitable, or another substance the mechanism of action of which is involved Rho-and C-kinase activity.

In practice, none of the proposed methods gives a real complete description of the dependence of F on $[Ca^{2+}]_i$, however, the comparative results obtained using these techniques can, with a sufficient degree of validity, indicate the presence or absence of the properties of the sensitizer in the test compound.

So, in a first approximation, to assess the changes in the calcium sensitivity of the vessel under the influence of one or another factor (which may be external influences, for example, radiation, one or another pathological process or drug) to constrictor agents that cause an increase in Ca^{2+} in myoplasm, it is easiest to use contractile reaction to KCl. It is known that E_{max} and EC_{50} can serve as parameters describing the sensitivity of the vessel to constrictor influences. However, the data obtained will not serve as evidence of a change exactly of the Ca^{2+} sensitivity of the contractile cascade of events, i.e. an increase in the contractile response at equal $[Ca^{2+}]_i$, since the real increase in $[Ca^{2+}]_i$ with this technique cannot be accurately estimated for several reasons.

The effect of increased extracellular concentrations of K^+ is considered the most simple and understandable since it is associated with relatively simple physical processes. An increase in the concentration of external K^+ leads to a decrease in the potassium equilibrium potential following the Nernst equation and, as a result, depolarization of the cell membrane, the opening of L-type Ca^{2+} channels and Ca^{2+} entry into the cell. As a result of this, the dependence *KCl – contraction* will not correspond to the dependence $[Ca^{2+}]_i$ – F, it will rather reflect the dependence number of open calcium channels – the membrane potential, which obeys the Boltzmann equation. Also, processes such as Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum, Ca^{2+}-dependent K^+ channels of high conductivity and even, according to some data [1], a change in Ca^{2+} sensitivity under the influence of KCl will contribute to the resulting dependence. However, if any potential Ca^{2+} sensitizer changed the Ca^{2+} sensitivity of the muscles, this must necessarily affect the dose-response relationship for KCl and this way will give sufficient reason to assume that the test compound possesses calcium sensitizing properties.

A more specific answer to this question can be given by experiments with skinned muscles, where the EC_{50} will serve as a parameter for assessing Ca^{2+} sensitivity. However, this method has its own limitations, since the skinning procedure is rather traumatic for the cell. When working with skinned muscles there is a risk of missing due to a possible change in the intracellular enzyme content, any mechanisms that contribute to a change in the Ca^{2+} response, so the absence of an effect on the skinned muscle will not mean its absence in the intact vessel.

Let see how it usually operates in practice. The relation between contraction and myoplasmic calcium ion concentration may be studied on smooth muscle fibers which have been made hyperpermeable by membrane skinning. In skinned fibers, contractile structures within the cell can be exposed to an equilibrated with a given calcium ion concentration which has been stabilized with the calcium buffer calcium-EGTA which at neutral pH, has a stability constant of about 10^6. Since the skinned fibers rapidly lose soluble substances, the bathing medium must be complemented with ATP as an energy source, magnesium ions, and a pH-buffer.

Typically, the responsiveness of these preparations depends also on the type of skinning use. With saponin or β-escin for instance, internal membrane structures are left intact, so that the preparation may also be used to study the release of calcium from internal stores. If we are interested in removing the sarcoplasmic reticulum membrane, we must use 250 μg/ml of saponin, a concentration that has been reported to severely damage the sarcoplasmic reticulum and mitochondrial membranes. Skinning procedures involving the treatment by Triton X-100, on the other hand, destroy internal membrane

structures so that the sarcoplasmic reticulum and other calcium stores are no longer functional. Therefore, calcium uptake and release processes no longer interfere with the effect of added calcium on the contractile structures.

If you need to serve the functional properties of SM in a maximal degree you should select α-toxin as a permeabilizing agent. This compound makes pores in the plasma membrane too small to allow passage of proteins with a molecular weight larger than 1 kDa and allows preventing this way membrane-associated enzymes and ionic channels, and related loss of intracellular content.

The most adequate results can be obtained using fluorescent calcium indicator fura-2 AM combined with developed force measurement. The use of this method allows us to provide a direct measurement of the Ca^{2+}concentration in the myoplasm and to build to an accurate dependence of $[Ca^{2+}]_i$-force, which is the main task of experiments on the study of Ca^{2+} sensitivity. However, this method has its own limitations. For example, it is not always possible to obtain the complete $[Ca^{2+}]_i$- force curve, using physiological methods of increasing intracellular Ca^{2+}, i.e. an increased KCl concentrations or α-agonist phenylephrine. It is not always possible to obtain saturating concentrations of Ca^{2+} in the cell, which means that it is impossible to accurately measure EC_{50} and E_{max}. In this case, the parameter $\Delta Ca/\Delta F$ can be used as a characteristic of calcium sensitivity, which will determine the sensitivity of the contractile response to changes in Ca^{2+} in the area of physiological concentrations of Ca^{2+} in the pseudo-linear portion of the dose-response curve. This is precisely the goal to determine the sensitizing or desensitizing ability of the compound tested. At the same time, the comparison of contraction amplitude at equal Ca^{2+}concentration will give information about sensitivity of the vessel to constrictor influences.

And, finally, experiments with nitrovasodilators with a complete description of the Hill curve and determination of its parameters can give information about the dilatory ability of a vessel, at least when activating the phosphatase pathway. In this case, acetylcholine and nitroglycerin can serve as the most convenient dilator agents, when they were used as endothelium-dependent and independent vasodilators, respectively.

As an example, we present data on the determination of Ca^{2+} sensitivity changes obtained on the thoracic aorta of rabbits and rats that underwent to ionizing gamma radiation impact at a dose of 6 Gy (Fig. 4).

A study of the vessels reaction of healthy and irradiated animals to KCl showed that the contraction in irradiated vessels by different concentrations of KCl is significantly larger than the contraction of healthy vessels. (Fig. 4A) In this case, the analysis of the *concentration – contraction* dependences approximated by the Hill equation revealed an increase in the maximum contraction F_{max} and a shift of the curve to the left to the region of lower concentrations. No statistically significant changes of Hill coefficient were revealed. It is difficult to make any conclusions regarding changes in the calcium sensitivity of the contractile apparatus based on these data for the reasons already mentioned, but there are reasons to assume that such an irradiation effect takes place.

More in-depth information is provided by experiments with skinned vessels. The dependences Ca^{2+} – force (Fig. 4B) clearly show a shift of the curves to the left to the region of lower Ca^{2+} concentrations, which indicates an increase in Ca^{2+} sensitivity, which corresponds to the first case of smooth muscle contractile status described above (Fig. 2A). However, in these experiments, it is difficult to obtain data on changes in E_{max} due to a significant scatter in the contractile response of the skinned muscles. The procedure of normalization relatively to the maximum contraction allows identifying changes in the EC_{50}, but information about the changes in E_{max} is lost. No statistically significant changes in Hill coefficient were revealed.

The maximum information is provided by experiments with fluorescent calcium indicator fura-2 (Fig. 4C). A linear approximation of the Ca^{2+} – force dependence, which was obtained during muscle

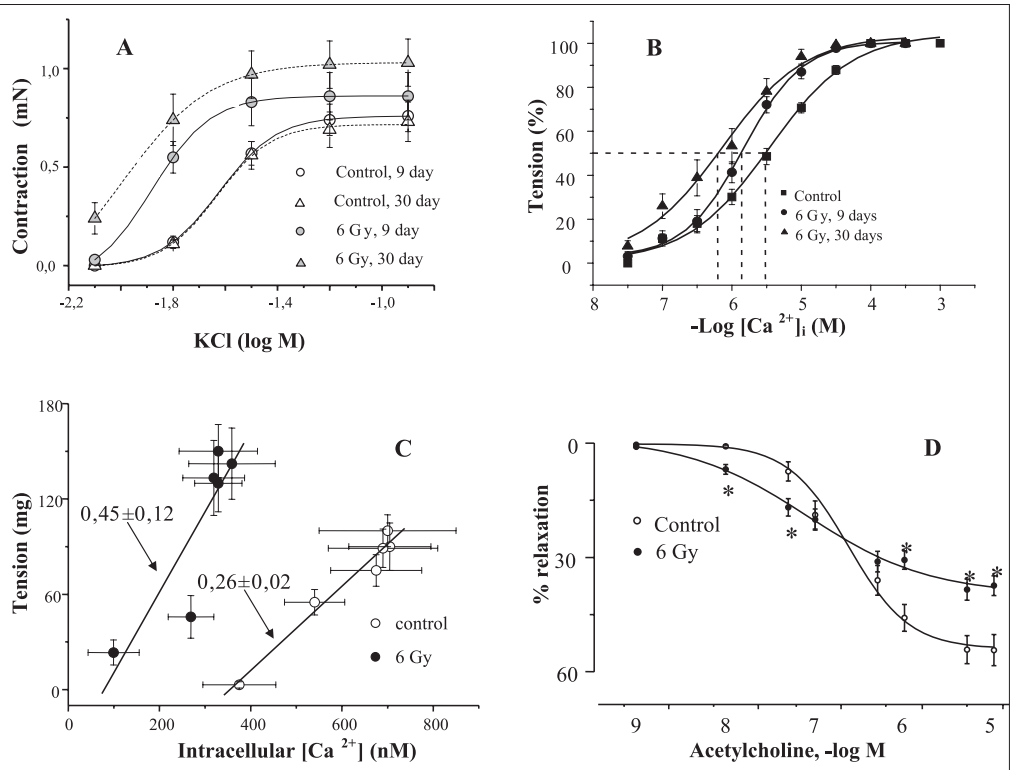

*Fig. 4. Effect of irradiation (6 Gy) on contraction and relaxation of the rat and rabbit thoracic aorta on the 9th and 30th days of post-irradiation period (for details see [12, 13]): A – KCl-mediated contraction of the intact rat thoracic aorta. Curves were fitted to Hill equation. Half-maximal effective concentration (EC_{50}) values, expressed as pD_2 (-Log EC_{50}), and maximal contraction values expressed in mN are as follows: (1,620 ± 0,003), (1,880 ± 0,001), (1,990 ± 0,005) and (0,760 ± 0,002) mN, (0,860 ± 0,001) mN, (1,030 ± 0,006) mN in control and on the 9th and 30th days respectively [13]; B – Force in β-escin permeabilized rat aortic rings as a function of calcium concentration in activating solution. Data are normalized to force at concentration at pCa 5.0. Curves were fitted to Hill equation. Half-maximal effective concentration (EC_{50}) values were expressed as pD_2 (-Log EC_{50}) and are as follows: control, pD_2 – (5,46 ± 0,07), 6 Gy, 9 days, pD_2 – (5,83 ± 0,03), 6 Gy, 30 days, pD_2 – (6,13 ± 0,14) [13]; C – $[Ca^{2+}]_i$-tension relationship was obtained by simultaneous measurement of $[Ca^{2+}]_i$ and tension induced by cumulative addition of KCL in aortic rings loaded with Fura–2 from healthy (closed circles) and irradiated (open circles) rats. The slope (g/tension/nM $[Ca^{2+}]_i$) was significantly increased in irradiated tissues, indicating that Ca^{2+} sensitivity of myofilaments had increased [13]; D – Concentration-relaxation curves to acetylcholine obtained on the thoracic aorta from healthy (open circles) and irradiated (closed circles) rabbits. Relaxations are expressed as percent decrease in the tension evoked by 10^{-5} M phenylephrine. *$p < 0.05$ compared with control responses to the same acetylcholine concentration [12].*

stimulation with increased concentrations of extracellular K^+ in the region of 30-120 mM, made it possible to obtain a pseudo-linear section of the Ca^{2+} – force curve and estimate the $\Delta F/\Delta Ca$ value in this region. The data obtained clearly demonstrate the phenomenon of increasing in calcium sensitivity of vascular SMC under radiation exposure. Based on the totality of the results obtained using all three methods, it can be argued that the effect of radiation leads to an increase in sensitivity in terms of EC_{50}, and an increase in responsiveness in terms of E_{max}. No statistically significant changes in calcium sensitivity were detected in terms of *Hill coefficient*.

Thus, it can be concluded that radiation exposure increases Ca^{2+} sensitivity in SMC in at least two ways: due to changes EC_{50} and E_{max}. Whether this effect is a consequence of radiation-induced changes in different parts of the biochemical contractile chain of events,

or whether these changes are interrelated, it is difficult to say based on the data obtained. The answer to this question requires other more detailed experiments with the study of the effect of radiation on individual known links in the contractile cascade of events.

Very interesting information about the contractile properties of irradiated vessels is given by experiments with dilatating agents that work along the second path. In experiments with acetylcholine, which causes endothelium-dependent relaxation mediated by the action of nitric oxide, it was found that the weakening of dilatation on acetylcholine also occurs in two ways. (Fig. 4D). A decrease in E_{max} and a decrease in EC_{50} were recorded. This is just the real case of complex combined changes of the Hill curve, similar to that presented in Fig. 3D. These results look paradoxical because there are two multidirectional processes – on the one hand, a decrease in sensitivity to NO due to the suppression of E_{max}, i.e. deactivation of the phosphatase pathway, which can lead to an increase in Ca^{2+} sensitivity, and, on the other hand, a decrease in Ca^{2+} sensitivity from a decrease in EC_{50} for NO. Similar results were obtained for other compounds whose action is carried out by the phosphatase pathway with nitric oxide as the active molecule – nitroglycerin, sodium nitroprusside, SIN-1. How are these changes related to changes in Ca^{2+} sensitivity, which effects are primary, and which are only a consequence of the former, are they related at all, or do these phenomena occur independently of each other? The answers to these questions are beyond the scope of the described experiments and require a different, more in-depth approach.

The described experiments can be considered as a step-by-step assessment method (from evaluative to more accurate) of the ability of any exposure, including pharmacological, to change the calcium sensitivity of the contractile apparatus of vessels SMC in terms of the parameters of the Hill curve. The presented methods can serve as tools for the initial screening of compounds that pretend to be calcium sensitizers or desensitizers.

Conclusions

1. The main goal of experiments to determine the changes in the Ca^{2+} sensitivity of the contractile apparatus of the vascular SMC during exposure of any external stimuli should be to build the dependencies $[Ca^{2+}]_i$- force before and after the exposure.
2. To estimate myofilament calcium sensitivity properly as a complex parameter, we need to measure at least three parameters: affinity or sensitivity of myofilaments to Ca^{2+} which characterized by EC_{50}, efficacy or myofilament calcium responsiveness, or maximal Ca^{2+}-activated force, and the Hill coefficient.
3. Optimal experimental design to estimate myofilament calcium sensitivity comprises: contractile recordings in intact smooth muscles, contractile recordings in chemically permeabilized (skinned) smooth muscles, simultaneous measurement of $[Ca^{2+}]_i$ and force development.
4. To assess the state of the phosphatase signaling pathway, which is the second main mechanism that determines the Ca^{2+}-sensitivity of SMC, it is necessary to determine the dilatory ability of the vessel and estimate the range of these changes.
5. The complex of these experiments will enable a step-by-step assessment of the ability of any exposure, including pharmacological intervention, to change the calcium sensitivity of the contractile apparatus of SMC and can serve as an effective tool for the initial screening of compounds that claimed to be calcium sensitizers or desensitizers.

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How to measure myofilament calcium sensitivity? Theory and practical applications

In the heart, as well as in smooth muscle cells (SMC) of blood vessels, contractility is mainly determined by the concentration of free calcium $[Ca^{2+}]_i$. in myoplasm. Changes in the sensitivity of myofilaments to calcium can be very important in the regulation of muscle contractility. Currently, it is precisely this way of influence on the contractile properties of the heart and vascular muscles that attracts much attention of pharmacologists. Studies of the nature of calcium sensitivity can be the fundamental basis for the development of a new generation of drugs that affect vascular tone or contractility of the heart through direct effects on the contractile apparatus or indirectly affecting signaling pathways that regulate vascular tone.

The dependence of the magnitude of the contraction F from $[Ca^{2+}]_i$ with a good degree of accuracy is described by the Hill equation $F = F_{max} * [Ca^{2+}]_i^n / (EC_{50}^n + [Ca^{2+}]_i^n)$, which is uniquely determined by three parameters: the maximum contraction F_{max}, the concentration of half contraction EC_{50}, and the Hill coefficient n. The main goal of the experiments to determine the changes in the calcium sensitivity of the contractile apparatus of the SMC during any external influences including conducting screening trials of potential drugs should be to obtain the dependencies of the contraction force F on $[Ca^{2+}]_i$. before and after its exposure. To correctly evaluate changes in the calcium sensitivity of myofilaments, it is necessary to measure at least changes in three parameters of the obtained dependences: the generalized affinity of myofilaments for $[Ca^{2+}]_i$, which is characterized as EC_{50}, the sensitivity of myofilaments to calcium, or F_{max}, and Hill coefficient.

Optimal experimental design for assessing changes in the calcium sensitivity of the vascular contractile apparatus includes: recording the contractile activity of intact smooth muscles, recording the contractile activity of chemically skinned vascular preparations, as well as simultaneously measuring $[Ca^{2+}]_i$, and contractile force using fluorescent calcium indicators.

The complex of these experiments will enable a step-by-step assessment of the ability of any external impact, including pharmacological intervention, to change the calcium sensitivity of the contractile apparatus of SMC and can serve as an effective tool for the initial screening of compounds claiming to be calcium sensitizers or desensitizers.

Key words: calcium sensitivity, smooth muscle, contraction, relaxation, Hill curves parameters, calcium sensitizers or desensitizers

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Як виміряти кальцієву чутливість м'язового скорочувального апарату? Теорія та практичні рекомендації

У серці, а також в гладеньком'язових клітинах (ГМК) судин скорочення визначається головним чином концентрацією вільного кальцію $[Ca^{2+}]_i$. у міоплазмі. Зміни чутливості міофіламентів до кальцію можуть бути дуже важливими в регуляції скоротливості м'язів. Натепер велику увагу фармакологів привертає саме цей шлях впливу на скоротливі властивості серцевих і судинних м'язів. Дослідження природи кальцієвої чутливості можуть стати фундаментальною основою для розробки нового покоління лікарських засобів, які впливають на тонус судин або скоротливу здатність серця за допомогою

прямого впливу на скорочувальний апарат або опосередковано шляхом впливу на сигнальні шляхи, які регулюють тонус судин.

Залежність величини скорочення F від $[Ca^{2+}]_i$. з високим ступенем точності описується рівнянням Хілла $F = F_{max} * [Ca^{2+}]_i.^n / (EC_{50}^n + [Ca^{2+}]_i.^n)$, яке однозначно визначається трьома параметрами: максимальним скороченням F_{max}, концентрацією половинного скорочення EC_{50} і коефіцієнтом Хілла n. Головною метою експериментів із визначення змін кальцієвої чутливості скоротливого апарату ГМК судин за будь-якого впливу або за проведення скринінгових досліджень потенційних лікарських препаратів такої спрямованості має бути побудова залежностей сили скорочення F від $[Ca^{2+}]_i$. до і після впливу. Щоб вірно оцінити зміни кальцієвої чутливості міофіламентів, необхідно визначити, принаймні, зміни трьох параметрів отриманих залежностей: узагальненої спорідненості міофіламентів до $[Ca^{2+}]_i$, що характеризується як EC_{50}, чутливості міофіламентів до кальцію, або F_{max}, і коефіцієнта Хілла.

Оптимальний експериментальний дизайн для оцінки змін кальцієвої чутливості скорочувального апарату судин включає: реєстрацію скорочувальної активності інтактних гладеньких м'язів, реєстрацію скорочувальної активності хімічно скінованих судинних препаратів, а також одночасне вимірювання $[Ca^{2+}]_i$ і сили скорочення з використанням флуоресцентних кальцієвих індикаторів.

Комплекс цих експериментів дасть можливість покрокової оцінки здатності будь-якого впливу, у тому числі й фармакологічного, змінювати кальцієву чутливість скорочувального апарату ГМК і може служити інструментом для первинного скринінгу сполук, які претендують на роль кальцієвих сенситизаторів або десенситизаторів.

Ключові слова: чутливість до кальцію, гладенькі м'язи, скорочення, розслаблення, параметри кривої Хілла, кальцієві сенситизатори або десенситизатори

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Как измерить кальциевую чувствительность мышечного сократительного аппарата? Теория и практические рекомендации

В сердце, а также в гладкомышечных клетках (ГМК) сосудов сокращение определяется главным образом концентрацией свободного кальция $[Ca^{2+}]_i$. в миоплазме. Изменения чувствительности миофиламентов к кальцию могут быть очень важны в регуляции сократимости мышц. В настоящее время большое внимание фармакологов привлекает именно этот путь влияния на сократительные свойства сердечных и сосудистых мышц. Исследования природы кальциевой чувствительности могут стать фундаментальной основой для разработки нового поколения лекарственных средств, которые влияют на тонус сосудов или сократительную способность сердца посредством прямого воздействия на сократительный аппарат, или опосредовано путем влияния на сигнальные пути, регулирующие тонус сосудов.

Зависимость величины сокращения F от $[Ca^{2+}]_i$. с хорошей степенью точности описывается уравнением Хилла $F = F_{max} * [Ca^{2+}]_i.^n / (EC_{50}^n + [Ca^{2+}]_i.^n)$, которое однозначно определяется тремя параметрами: максимальным сокращением F_{max}, концентрацией половинного сокращения EC_{50}, и коэффициентом Хилла n. Главной целью экспериментов по определению изменений кальциевой чувствительности сократительного аппарата ГМК сосудов при каком-либо воздействии или при проведении скрининговых исследований потенциальных лекарственных препаратов такой направленности должно являться построение зависимостей силы сокращения F от $[Ca^{2+}]_i$. до и после воздействия. Чтобы правильно оценить изменения кальциевой чувствительности миофиламентов, необходимо измерить, по крайней мере, изменения трех параметров полученных зависимостей: обобщенного сродства миофиламентов к $[Ca^{2+}]_i$, которое характеризуется как EC_{50}, чувствительности миофиламентов к кальцию, или F_{max}, и коэффициента Хилла.

Оптимальный экспериментальный дизайн для оценки изменений кальциевой чувствительности сократительного аппарата сосудов включает: регистрацию сократительной активности интактных гладких мышц, регистрацию сократительной активности химически скинированных сосудистых препаратов, а также одновременное измерение $[Ca^{2+}]_i$, и силы сокращения с использованием флуоресцентных кальциевых индикаторов.

Комплекс этих экспериментов даст возможность пошаговой оценки способности любого воздействия, в том числе и фармакологического, изменять кальциевую чувствительность сократительного аппарата ГМК и может служить инструментом для первичного скрининга соединений, претендующих на роль кальциевых сенситизаторов или десенситизаторов.

Ключевые слова: чувствительность к кальцию, гладкие мышцы, сокращение, расслабление, параметры кривой Хилла, кальциевые сенситизаторы или десенситизаторы

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