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Ca-exchange, Ca-channels and Ca-antagonists

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Introduction

Calcium ions play a pivotal role in the excitation-contraction coupling in smooth muscle cells, acting as the predominant messenger linking stimuli from extracellular origin to the intracellular environment. Direct evidence for the participation of Ca^{2+} in the contraction and relaxation of smooth muscle has been obtained with chemically skinned fibers. These preparations contract and relax in response to changes of the Ca^{2+} concentration in the perfusion medium. It has been shown that the threshold for mechanical activation of the contractile proteins is of the order of 10^{-7} M, and that full activation occurs at a free Ca^{2+} concentration of about 10^{-5} M[26,32,60]. Such an essential regulating role of Ca^{2+} implies that the cells must have the potential to adjust the cytosolic Ca^{2+} concentration rapidly. Smooth muscle cells have therefore a series of mechanisms to achieve that goal.

The most striking feature of the Ca-distribution in all cells is the large inwardly directed electrochemical gradient across the membrane, consisting of a 10,000-fold concentration gradient and of a 60 mV potential gradient. The maintenance of this Ca-gradient in the cells depends on the low permeability of the cell membrane to Ca^{2+}-ions under resting conditions, and on an efficient Ca extrusion pump which can easily compensate for this small inward leak and the increased Ca influx during stimulation.

In principle an increase of $[Ca^{2+}]_{cyt}$ can be obtained either by increasing the Ca-influx across the cell membrane or by decreasing the Ca extrusion. The former mechanism has been amply demonstrated, the second one remains uncertain and much less documented. In contrast to skeletal muscle, the contribution of Ca^{2+} supplied by intracellular stores is not very important for a maintained contraction.

The total exchangeable cellular calcium content ranges, depending on the preparation and the method of measurement, between 150 and 500 μmoles/kg tissue, values which are at least 1000 times higher than the free cytosolic Ca concentration. The largest part of the exchangeable fraction consists of Ca which is either bound to proteins (such as e.g. calmodulin) or which is sequestered in intracellular organelles such as endoplasmic reticulum or mitochondria.

We will discuss in this paper first the different sources of calcium which play a role in excitation-contracting coupling, and second the mechanisms by which the cytosolic calcium is regulated in relation to its extracellular concentration.

The plasma membrane as regulator of cellular calcium

Smooth muscle cells depend for a maintained force development on a continuous supply of calcium from the extracellular medium. On exposure to a solution containing no Ca^{2+} and with added 2 mM EGTA or in the presence of ions such as La^{3+} or Mn^{2+} which prevent the influx of Ca^{2+} across the cell membrane, smooth muscle tissue stimulated with an agonist do not develop a maintained force development. Depending on the type of smooth muscle tissue and on the time of exposure to Ca-free medium, only a phasic (transient) force development will occur due to the release of Ca from agonist-sensitive cellular Ca stores[4,14,25]. Also spontaneous action potentials and the concomitant contractions are abolished. K^+ rich solutions still depolarize the cells, but they fail to elicit a contraction.

These findings show that extracellular calcium is essential not only to obtain a contraction by K-depolarization, but also to either refill cellular calcium stores, or to cause further calcium release (Ca-induced Ca-release). In addition it can be concluded that K-depolarization is not an adequate stimulus to release Ca from cellular stores. In order to make relaxation possible and to maintain a steady Ca-distribution, the extra influx of calcium occurring during activation must be extruded out of the cell by active transmembrane Ca-extrusion.

1. Calcium entry

Calcium ions are believed to enter smooth muscle cells down their electrochemical gradient by several types of mechanisms; i.e. action potentials, membrane depolarization, agonist action and Na-Ca exchange. Spontaneous and evoked action potentials occur in most smooth muscle cells. These action potentials are insensitive to tetrodotoxin, but they are blocked by Mn^{2+} and organic Ca antagonists, such as D600 and verapamil[2,31,71,77,78,82]. For those reasons it is assumed that the inward current responsible for the upstroke of the action potential is mainly due to the influx of Ca^{2+} ions through potential dependent channels. However in some smooth muscle tissues action potentials have also been observed in Ca free solutions[49,52], suggesting that these channels are permeable both to Ca^{2+} and Na^+ ions. The relative contribution of each ion to the inward current depends on the extracellular concentration of these ions and on the relative selectivity of these channels for both cations. In some tissues the action potentials are rather abortive and in others they are completely absent. This may be due to an overlap in time of the inward and the outward currents, whereby the Ca influx is maintained but the net inward current is reduced or even completely inhibited. This is consistent with the observation that in the presence of substances which reduce the K-permeability, such as TEA or procaine, smooth muscle preparations, which are characterized by a stable membrane potential in normal physiological solutions, can generate action potentials either spontaneously or during electrical or pharmaco-

logical stimulation[25,37]. The difference between quiescent smooth muscle and those which present spikes is therefore not fundamental, but is most likely related to the different kinetic behavior of the ionic channels in different tissues.

It is theoretically possible to calculate from the amplitude of the action potential the amount of charge required to discharge the membrane capacity, and to estimate the transsarcolemmal flux of Ca. In this calculation it has to be assumed that the inward current is entirely carried by this cation and that the net inward charge movement is a reliable measure of this inward current. Such a hypothetical Ca current would increase the intracellular Ca by an amount which is one order of magnitude too small to fully activate the contractile apparatus. This has led to the postulation that either the transient depolarization of the membrane or the small amount of calcium ions entering the cell during the action potential triggers a release of calcium from intracellular Ca stores. However, until now there is no convincing experimental evidence in smooth muscle cells in favor of any of these hypotheses. Moreover it has been pointed out that the likely presence of an early outward current mainly carried by K^+ ions causes an underestimation of the inward charge movement and of the calculated calcium influx during the action potential.

A maintained depolarization of the smooth muscle membrane induced by increased concentrations of extracellular K also augments the Ca influx both in spike generating and quiescent tissues as indicated by the contractile response and by the increased uptake of ^{45}Ca. It is generally accepted that the opening of the same type of voltage operated Ca channel, which is responsible for the rising phase of the action potential, also constitutes the pathway for Ca influx and the concomitant maintained contraction during K-depolarization[50,73,75]. This hypothesis is supported by the observation that Ca antagonists inhibit both the action potentials and the accompanying contractions and the contractile response in K-depolarized tissues. The maintained force development in depolarized tissues is not compatible with the existence of an inactivation of the Ca-channels. Such inactivation could remain incomplete at a lower level of depolarization as suggested by the observation that the amplitude of the tonic component of the contraction induced in the guinea pig taenia coli by 42 mM K^+ is larger than that induced by 140 mM K^+[14].

Although the conductance of the voltage operated Ca channels might be relatively low for Na-ions, these ions might contribute significantly to the ionic current through these channels because of their high extracellular concentration as compared to Ca^{2+}. It is therefore not unexpected that Na-free solutions induce a contractile response, because the relative importance of the calcium influx through the voltage operated channels might become more important at low external Na concentrations. Because we have observed that in rabbit ear artery there exist similarities between the effects of K-depolarization and of Na-free solutions on the ^{45}Ca influx, contractile response and on the stimulation of the ^{45}Ca efflux, a common mechanism seems more likely than two different ones. The small depolarization of 10 to 20 mV, which we observed in Na free solutions could be sufficient for a partial activation of the voltage operated Ca channels[23]. The reduction of the competition between extracellular Na and Ca in Na-deficient solutions could further increase the influx of calcium and cause activation of the contractile proteins. In other tissues however it has been reported that the Na-free induced contractions and the concomitant increased ^{45}Ca uptake are not blocked with Ca-antagonists[47,54], and these observations are compatible with a role of Na-Ca exchange in the regulation of $[Ca]_{cyt}$.

An alternative pathway used by extracellular calcium to enter the cell is the so-called receptor operated Ca-channel[5]. Activation of receptors by an agonist can induce an increase of the Ca influx and a contractile response while causing depolarization, no depolarization at all or even hyperpolarization[12,25,35,36,45,67,69]. This type of activation most likely implies a direct effect of receptor activation on the Ca permeability of the membrane, without acting via a change of the membrane potential. There are several experimental arguments to differentiate in smooth muscle cells of large arteries these receptor operated channels from the voltage-dependent ones. First receptor-operated channels are mostly less sensitive to the organic calcium antagonists than the voltage-dependent ones[9,16,30]. Secondly the contractile responses induced by K-depolarization and by agonists present a different temperature sensitivity[3,24,55,56,63]. Also the finding that the stimulation of the ^{45}Ca-influx by K-depolarization and by noradrenaline are additive when the two procedures of activation are applied simultaneously support this hypothesis[51].

The limited effect of exogenously applied noradrenaline on the membrane potential of a number of vascular smooth muscle cells is in marked contrast with the electrical response to perivascular nerve stimulation. This is accompanied by excitatory junction potentials (e.j.p.) which summate and eventually give rise to an action potential[38-41]. These phenomena are rather insensitive to α-adrenergic blocking agents, and this observation has led to the postulation of a special type of adrenoceptor at the postjunctional smooth muscle membrane, the so-called γ-receptor[39]. This model relies on the classical view (Dale's principle) that a nerve releases only one type of transmitter substance. However this principle had recently to be modified to the co-transmitter hypothesis, because e.g. both noradrenaline and ATP can be released from the same nerve terminal. ATP could then be responsible for the e.j.p. and the subsequent firing of action potentials[8,65]. This co-transmitter hypothesis is also consistent with the observation that nerve stimulation has a biphasic effect on the membrane potential in some tissues, consisting of an e.j.p. and a more slowly developing depolarization[17,69]. The e.j.p. is insensitive to phentolamine, whereas the slower depolarization is blocked by phentolamine and potentiated by cocaine. These nerve mediated responses can be inhibited by Ca-antagonists, in contrast with the effect of these substances on the response induced by exogenous noradrenaline[44,47,48,68,70]. From our observation in rabbit ear artery it was evident that the Ca-antagonists have no effect on the release of ^3H-noradrenaline from the nerve terminals[44]. These substances would therefore exert their effect mainly on the voltage-dependent Ca channels of the postsynaptic

smooth muscle membrane. This indirect evidence in favor of these Ca channels has still to be supplemented with direct biophysical evidence obtained by patch clamping and with a biochemical isolation and characterization of the structure representing the Ca channel. For the latter procedure, the binding of Ca-antagonists of the dihydropyridine group to molecules present in the Ca channel might help to specify those membrane structures[28].

2. Ca extrusion across the plasmalemma

The important contribution of extracellular calcium to the maintained force development in smooth muscle tissue implies that in order to maintain the homeostasis of cellular calcium, Ca^{2+} ions must be extruded across the cell membrane against their electrochemical gradient. The two Ca extrusion mechanisms, ATP-dependent Ca pump and Na-Ca exchange, which have been investigated in other tissues have also been proposed for smooth muscle.

Linked movements of Na and Ca have been clearly demonstrated in squid giant axons[22], and it has been proposed that the Na gradient provides the energy for the Ca extrusion in this tissue. However the role of this Na-Ca exchange mechanism under physiological conditions has been questioned recently. In smooth muscle cells the electrochemical gradient for Na ions can be changed either by exposure to Na-free solutions or by inhibiting the Na-K pump by ouabain or K-free solutions[54,64]. Such procedures increase $[Na]_i$ and evoke a contractile response, whereas restoration of the Na-gradient induces relaxation. These observations are consistent with the Na-Ca exchange hypothesis but there is as yet no demonstration of a direct causal relation between these two phenomena in smooth muscle and as a consequence these observations should not be taken as prima facie evidence that Na-Ca exchange plays a critical role in the regulation of $[Ca]_i$. Moreover contractions and relaxations can still be induced by various procedures in the complete absence of a Na-gradient. Also after complete dissipation of the Na-gradient by a prolonged exposure to K-free solutions, smooth muscle may be completely relaxed[10]. On the other hand when this increased Ca influx is blocked either by perfusion with Ca-free solution or by using D600 or Mn^{2+}, Na-free solutions do not exert an effect on the ^{45}Ca efflux in rabbit ear artery. Also the contractile response which occurs in Na-free solution is inhibited by organic Ca antagonists in rabbit ear artery[23], but not in all other tissues[1,54]. Another observation which argues against Na-Ca exchange is that modifications of the Na-gradient do not affect the rate of extrusion of ^{45}Ca from the rabbit ear artery after its release by noradrenaline stimulation[23]. It was shown in guinea pig taenia coli[58] that the effect of Na-free solutions on the ^{45}Ca-efflux was a consequence of the increased diffusional pathway for Ca^{2+} ions caused by an increased Ca-binding capacity in the extracellular space. Such an increased binding capacity in the extracellular matrix in Na-free solutions has also been described for vascular smooth muscle tissue[76].

It has been possible to demonstrate in plasma membrane fractions from smooth muscle that a small component of the Ca uptake depends on the Na-gradient[33,34,53]. However this component is much smaller than the ATP-dependent Ca uptake and has a much lower affinity for Ca, suggesting that the Ca extrusion fueled by the hydrolysis of ATP is more important for Ca homeostasis than Na-Ca exchange. ATP-dependent Ca extrusion was first described in red blood cells[61]. The enzyme, a CaMg ATPase has been demonstrated and isolated from various smooth muscle tissues and its role in transmembranal Ca transport has been demonstrated[80,81] The problems arising from contamination with intracellular membranes are discussed in the papers by Daniel et al. (see pp. 905–913 of this issue) and Wuytack et al. (see pp. 900–905). It should be pointed out that a direct demonstration of an ATP-dependent Ca transport in intact smooth muscle preparations is as difficult as the demonstration of Na coupled Ca fluxes. There exist no specific inhibitors of this enzyme, and factors which interfere with the ATP production in the cells exert many secondary effects which render the interpretation of efflux data uncertain. Examples of such secondary action are the development of membrane leakiness [15], the release of Ca from mitochondria, and progressive accumulation of Ca^{2+} and Na^+ in the cytoplasma whereby the transmembrane ion gradients are running down. The increase of $[Ca^{2+}]_{cyt}$ may also increase the K^+ permeability of the membrane[11]. Therefore the main arguments in favor of an ATP-dependent Ca-extrusion across the cell membrane is provided by experimental evidence which is not consistent with Na-Ca exchange, and by the demonstration of a Ca transporting, ATP-splitting membrane enzyme in plasmalemmal microsomes.

Intracellular Ca-stores

The essential role of extracellular calcium in the long-term regulation of the excitation-contraction coupling in smooth muscle does not exclude that intracellular systems (organelles or Ca binding proteins) might play a role in parallel with the plasma membrane in the short-term regulation of this parameter to induce or overcome sudden fluctuations in $[Ca^{2+}]_{cyt}$.

The existence of intracellular Ca stores which play a role in excitation-contraction coupling in smooth muscle by releasing their calcium has been deduced from the observation that excitatory agonists are able to induce a transient contraction in tissues exposed to Ca free solutions or in tissues in which the entry of calcium is blocked by La^{3+}. The contractile response under these conditions is accompanied by a transient increase of the ^{45}Ca efflux, suggesting that the excitatory agonists induce a release of calcium from some intracellular store, and thereby activate the contractile apparatus[18,19,25]. This Ca is subsequently largely extruded out of the cell and a recycling to the sites from which it was released appears to be limited because a second activation by the agonist during the maintained exposure to Ca-free medium does not induce a second phasic contractile response, nor a measurable stimulation of the ^{45}Ca-efflux. Application of the agonist at various times after starting the Ca free superfusion has revealed that both the amplitude of the phasic contractile response and the amount of released ^{45}Ca decrease in an exponential manner. Both in rabbit aorta[18] and in rabbit ear artery[25] a half-time of approximately 30–40 min was found. In guinea pig taenia coli however, the force development elicited by carbachol was already abolished after exposure for a few minutes to Ca free solution[7,14]. This

rapid loss of contractile response is due to the fast loss of calcium from this store in Ca-free medium and not to its limited capacity, because a nearly maximal contraction can be induced after a short exposure to Ca free solutions. The maintenance of the contractile response in the latter tissue strongly depends on intracellular Na[6].

The amount of Ca which can be released by the agonist and which appears in the effluent has been estimated at 21 μmoles/kg in rabbit aorta[18,19] and at 60 μmoles/kg in rabbit ear artery[25]. The value found in the aorta is too small to induce a maximal contraction, whereas the second one found in the ear artery is sufficient to fully activate the myofilaments. In order to resolve the inconsistency between the contractile response and the amount of ^{45}Ca appearing in the effluent upon agonist stimulation, Van Breemen and co-workers[74] have postulated that the largest part of released calcium is not immediately eliminated from the cells after activation of the contractile proteins, but that at least part of it is taken up by another Ca sequestering organelle, from which it is extruded during relaxation by an as yet unidentified pathway. This model thus implies two separate Ca fractions: one close to the inner surface of the cell membrane from which the agonists are able to release calcium and which has to be replenished with Ca^{2+} bound to the outer membrane surface, and a second fraction, possibly the bulk of the SR, which removes calcium from the cytoplasm and the cell.

The estimate of 60 μmoles/kg is consistent with a simpler model, with only one type of sequestering organelle from which Ca can be released by an agonist to elicit contraction. The observation that the released Ca does not return to its release sites during exposure to Ca-free medium is not incompatible with the possibility that calcium is accumulated in these organelles during relaxation in normal Ca containing physiological solutions. This assumption is supported by the finding that pretreatment with K-rich solutions and β-agonists in the presence of Ca potentiate the subsequent agonist induced contractile response in Ca free solution, indicating that during such treatment more calcium is taken up in the cellular stores[14].

This agonist-sensitive Ca store might correspond to the sarcoplasmic reticulum. SR vesicles from smooth muscle can accumulate calcium in an ATP dependent way, and this has also been demonstrated for the SR in chemically skinned smooth muscle fibers[57]. Evidence has been provided showing that catecholamines and caffeine release Ca from the same intracellular store in intact smooth muscle[18,20]. Caffeine was shown to induce contraction in skinned smooth muscle fibers preloaded with calcium, by releasing Ca from intracellular Ca sequestering sites, probably the SR[42,43,66].

An interesting characteristic of the agonist sensitive Ca store in rabbit ear artery is that the refilling of this store by exposure to a Ca containing solution after its depletion with noradrenaline in Ca free solution, proceeds at a much faster rate than its depletion during exposure to Ca free solution[9]. Also the rate of filling and the final amount of calcium taken up in the store depend on $[Ca^{2+}]_0$. These observations suggest a direct pathway between the store and the extracellular space, allowing the rapid filling of that store. Consistent with such a direct pathway is the observation that the amplitude of the contraction induced by the agonist in Ca free solution is largely determined by the extracellular rather than by the intracellular Ca concentration during the preceding exposure to Ca containing solutions: the amplitude of the agonist induced contraction in Ca free solutions after an exposure to a solution containing 5.9 mM K and 10 mM Ca, during which the tissues remain relaxed, is higher than that following an exposure to a solution containing 59 mM K and 0.2 Mm Ca, which is accompanied by a contraction.

This direct pathway might be related to the close connection between the SR and the surface membrane observed in electron microscopic pictures[21]. These are specialized regions where the SR membrane is separated from the surface membrane by a 10–12 nm gap traversed by periodic electron opaque processes. An attractive implication of such direct connection is that it allows an alternative picture of receptor operated channels. Activation with an agonist releases Ca^{2+} ions from their storage sites and initiates the phasic component of contraction. The ensuing depletion of this store could increase the Ca permeability of the junction between agonist sensitive store and the extracellular space and create the continuous influx of calcium, which is responsible for the tonic component of the agonist-induced contraction in Ca containing solutions.

Ca channels and Ca antagonists

Several substances exert a blocking action on the Ca channels of smooth muscle cells. One of the striking aspects is the variety of chemical structures interacting with the permeation of Ca through channels. Besides metal ions such as Mn^{2+} and La^{3+}, there are natural substances such as batrachotoxin, veratridine and reserpine[13] and synthetized molecules such as the phenylalkylamines (verapamil, D600), the diphenylalkylamines (cinnarizine, flunarizine), the benzothiazepines (diltiazem) and the dihydropyridines (nifedipine, nitrendipine). Most of these compounds are not only very interesting research tools, but they have also proved to be effective therapeutic agents in the treatment of some cardiac arrhythmias and of coronary spasm.

It is an important observation that these antagonists are not uniformly effective in inhibiting the contractions occurring in vascular smooth muscle[16,72]. In most vascular smooth muscle tissues they strongly inhibit K-induced contractions, while their inhibitory effect on agonist induced responses is less[29,30,79]. In contrast, in most visceral smooth muscle the dose-dependency of the inhibition is more similar for agonist induced contractions and K induced contractions. In some tissues it has been observed that the agonist induced contractile responses are rather insensitive to some Ca antagonists, but are affected by others[46]. In addition some dihydropyridines act as Ca-agonists and enhance the influx of Ca rather than blocking the voltage sensitive Ca channels[62]. Because of these varied cellular responses a more direct and quantitative approach is necessary to identify and characterize the different types of Ca channels, as well as to investigate the mode of action of the different Ca antagonistic drugs.

The recently developed patch-clamp and whole-cell recording technique seem to be very promising in this regard.

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The Ca^{2+}-transport ATPases in smooth muscle

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Summary. A calmodulin stimulated Ca^{2+}-transport ATPase which has many of the characteristics of the erythrocyte type Ca^{2+}-transport ATPase has been purified from smooth muscle. In particular, the effect of calmodulin on these transport enzymes is mimiced by partial proteolysis and antibodies against erythrocyte Ca^{2+}-transport ATPase also bind to the smooth muscle $(Ca^{2+} + Mg^{2+})$ATPase. A correlation between the distribution of the calmodulin stimulated $(Ca^{2+} + Mg^{2+})$ATPase and $(Na^{+} + K^{+})$ATPase activities in smooth muscle membranes separated by density gradient centrifugation suggests a plasmalemmal distribution of this $(Ca^{2+} + Mg^{2+})$ATPase. A phosphoprotein intermediate in smooth muscle which strongly resembles the corresponding phosphoprotein in sarcoplasmic reticulum of skeletal muscle may indicate the presence in smooth muscle of a similar type of Ca^{2+}-transport ATPase.
Key words. Smooth muscle; calmodulin overlay; Ca^{2+}-transport ATPase phosphorylated intermediate.

1. Introduction

There is now ample experimental evidence for the existence in smooth muscle not only of an ATP-dependent Ca^{2+}-extrusion system in the plasma membranes, but also of an ATP-dependent Ca^{2+}-accumulation system in endo-(sarco)plasmic reticulum.

The relative contribution of both Ca^{2+}-transport systems to the removal of Ca^{2+} from the cytoplasm during relaxation remains unknown.