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The use of subcellular membrane fractions in analysis of control of smooth muscle function

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Introduction

Analysis of control of smooth muscle function has always been complicated by the diversity of the types and functions of smooth muscle. Thus the occurrence of smooth muscle primarily innervated by excitatory sympathetic nerves (e.g. some blood vessels and vas deferens), others primarily innervated by excitatory parasympathetic and inhibitory nonadrenergic, noncholinergic nerves (e.g. circular muscle of the gut), and yet others (e.g. urinary bladder detrusor) innervated by excitatory cholinergic nerves as well as by excitatory noncholinergic, nonadrenergic nerves[5,9,10,11,22] makes it clear that the postsynaptic receptors and their coupling via conductance channels and receptor-operated-Ca stores must differ vastly, as between various smooth muscles. Diversity in smooth muscle is not limited to its innervation and the effector mechanisms operated by neurotransmitters, it also occurs in the nature of the excitation-contraction coupling mechanisms and in the methods whereby relaxation from contraction is achieved. Muscles may be activated to contract by electromechanical means (opening of voltage-dependent Ca^{2+} channels), inhibited from contraction by opening of a variety of voltage- or Ca-dependent K^+-channels, excited by pharmacomechanical means (release of intracellular Ca-stores) or relaxed by other post- or presynaptic mechanisms. There is controversy about whether relaxation by postsynaptic mechanisms occurs by resequestration of internal calcium in endoplasmic reticulum or mitochondria or whether it is by pumping of calcium out of cells. This controversy will be considered further below in so far as evidence from isolated subcellular fractions bears on it. There is further controversy about the location of the internal stores of calcium which couple excitation to those contractions which occur (in some cases) without any external calcium. It is usually assumed that they are located in the endoplasmic reticulum, but a possible locus in the plasma membrane has not been excluded. Since studies with subcellular membranes do not provide much evidence about this question, it will be discussed only briefly.

The question arises: 'Are there levels of organization of smooth muscle at which common mechanisms operate to control contractile function?' Possibly one such level is the contractile apparatus itself, a matter not the focus of this chapter, but even here there appears to be diversity[23]. Another such level where common features of control may exist is the set of mechanisms for removal of elevated intracellular Ca^{2+}. Sites of Ca^{2+}-removal may also be sites of release of Ca^{2+} by pharmacomechanical means, but this turns out to be not necessarily or commonly the case. However, the question of how Ca is removed from the cell interior (transport to the extracellular space or resequestration internally in a cytoplasmic organelle or both) has also proved to be difficult to resolve, and it is unclear whether this is due to real diversity of smooth muscles, technical difficulties, conceptual limitations or other reasons.

The chapter will focus on the studies carried out in our laboratories on subcellular fractions in study of Ca-control systems. It is appropriate to point out in a volume dedicated to Dr Bülbring that findings she made or inspired using electrophysiological techniques about the function calcium in excitation of smooth muscle have provoked these as well as many other studies. There are many other ways to approach analysis of how smooth muscle cells control their internal calcium and the value, and limitations of study of membrane fractions need to be considered. Some general features will emerge: 1) from microsomal fractions of smooth muscles, it is always possible to obtain subfractions which are highly enriched in plasma membranes; 2) these plasma-membrane en-

riched membrane subfractions always contain some vesicles, presumably inside-out, which are capable of ATP-dependent, active transport of Ca into the vesicles; 3) in all cases studied so far the K_m for Ca^{2+} in this transport is in the range (~ 0.5 μM) appropriate for control of intracellular Ca^{2+} and the gradients of Ca^{2+} achievable are in the range of 1000- to 10,000-fold, comparable to those across the intact plasma membrane; 4) so far no comparable pure subfractions of endoplasmic reticulum have been obtained, but it is unclear whether this is due to the difficulty of separating it from plasma membrane which has a similar density combined with a low starting quantity of such membranes or to a differential loss of these membranes or their functional integrity during conventional isolation procedures; 5) however, evidence has been obtained that there may be two forms of ATP-dependent Ca-pumps in smooth muscle membranes, one further activated by Ca-precipation by anions such as oxalate or phosphate, the other not. Whether these are each located in separate membrane types (non-oxalate-activated in plasma membranes; oxalate-activated pump in endoplasmic reticulum) or whether they are both located in different subfractions of the plasma membrane is unclear and will be considered below.

The first motive for studying membrane subfractions was not to seek common mechanisms of calcium control in smooth muscle. Rather it was a response to difficulty in studying control of internal calcium in intact smooth muscle tissues. This difficulty arose first from the tiny fraction of intracellular calcium compared to total tissue calcium (200–500 μmoles/kg wet weight out of 2–5 mmoles/kg). Thus the signals related to intracellular calcium were usually lost in the measurement noise so that changes in it could not be reliably detected. Since then a variety of techniques (and modifications) have been put forward to improve the signal to noise ratio by eliminating extracellular calcium while attempting to hold intracellular calcium in place: the lanthanum method[15,81], the EGTA method[80], the cold (0 °C), Ca-efflux method[15] and combinations of these. Such methods have been discussed critically recently[12] and will not be considered further here.

Not only was signal to noise a major difficulty, but also when the signal was obtained it proved to be complex, not reflecting free intracellular calcium, the desired variable to be measured, but rather various intracellular compartments[12]. Also it was difficult to know and obtain with accuracy the relevant normalizing value for intracellular calcium, the concentration of intracellular water[12]. Some workers even regard that as an important variable in its own right, but it has proved difficult to measure accurately.

All these limitations suggested that simplification of the system by isolating the various cellular membrane components and studying their calcium-handling properties might provide independent insight into the operation of the control systems. To a considerable degree this has proved true but there have been significant failures still awaiting new solutions.

Insight into calcium-handling in smooth muscle from studies of subcellular membranes has been delayed by an inappropriate conceptual extrapolation. Studies of subcellular components has already become considerably advanced in skeletal muscle before such studies on smooth muscle began. In the skeletal muscle, the major intracellular membrane besides mitochondria, which can be isolated by differential contrifugation, was not plasma membrane but sarcoplasmic reticulum (SR). After removal of mitochondria from skeletal muscle, the remaining lighter membranes (the microsomes) are $\sim 95\%$ SR with only a small sarcolemmal component. This led to the assumption despite contrary evidence from quantitative electron microscopy showing only a small endoplasmic reticulum component in smooth muscle (e.g. Devine et al.[17], McGuffee and Bagby[60] and Popescu et al.[67]), that smooth muscle microsomes contained mostly endoplasmic reticulum like skeletal muscle. This assumption never had any supporting experimental evidence, but it has persisted despite much evidence directly contradicting it. Some of this evidence will be considered below.

Some methodology considerations

A variety of ways are conceivable to isolate subcellular membranes from smooth muscle. If one desires to obtain all classes of membranes the cells must be broken by one of several homogenization techniques[13]. None of these is capable of breaking the cells without also damaging intracellular organelles such as the nucleus or the mitochondria but clearly the objective is to choose a technique which optimizes cell breakage while minimizing damage to internal organelles. This improves yield while reducing cross-contamination[13]. A major problem with the widely used technique of breaking cells by shearing forces with a Polytron-type homogenizer is that some damage invariably occurs to mitochondria; often the external membrane is separated from the internal membrane and thereafter is difficult to separate from plasma membrane which has a similar buoyant density (e.g. see Matlib et al.[59]).

If one's objective is to obtain only one type of membrane in pure form at the expense of all other internal membranes and organelles and even at the expense of low yield, then a number of approaches are possible. To obtain pure plasma membrane, it would be sufficient to disrupt the cells slightly and pass them through an affinity column which has been made to have high selectivity for a component of the outer surface of the plasma membrane such as the lectin binding site (see Daniel et al.[13], p. 21). That method has not been tried in a vigorous way to isolate smooth muscle plasma membrane. Alternately, one could make holes in or otherwise disrupt the continuity of the plasma membrane and then using appropriate solutions to solubilize contractile proteins and/or microtubules one could empty the cells of their contents; but this has apparently not been tried in smooth muscle. Mitochondria in relatively pure fractions can be isolated by careful homogenization and differential contrifugation. The major problem is with separation of plasma membrane and endoplasmic reticulum. Whether the constantly observed failure to get a clean separation[13] is owing to the similar densities of the two membranes or to their mechanical coupling by some of the structural connections reported to link the two membranes[17,77] is unclear. However, electron microscopic examination of

plasma membrane and 'ER'-containing fractions fails to resolve whether the two membranes are mechanically coupled.

Once cells are broken, the various components are usually separated by centrifugation followed by further centrifugation of a microsomal fraction on a density gradient. Nuclear components, unbroken cells and some debris are brought down by low speed centrifugation (700–900 × g), for 10 min; most mitochondria and inner-mitochondrial fragments are bought down by higher speed centrifugation (7000 to 10,000 × g) for 10 min which is often best repeated to obtain more complete separation. Then a microsomal fraction is separated from soluble or very light materials by high speed centrifugation (100,000 × g) for 30 min or more. This is then layered on either a continuous or a discontinuous density gradient and centrifuged at about 100,000 × g for 120 min. First a continuous sucrose gradient is run from which fractions are taken and analyzed in detail for membrane markers for various membranes. This analysis is used to design an appropriate discontinuous sucrose gradient to improve yields in various fractions.

Exposure to sucrose (or any material forming a density gradient) and the associated osmotic shock, or the process of centrifugation itself may of selectively of nonselectively damage membranes and organelles. However, in our studies, starting with the contents of the post nuclear supernatent, most membranes as judged by their markers (but see below) were recovered in reasonable yield (60–70% or more). One exception is the membrane containing oxalate-activated ATP-dependent Ca-accumulation which is selectively lost[27,48]. This will be discussed further below. So far no alternate to sucrose for forming the density gradient has been used and evaluated sufficiently to know if an alternate material would perform as well or better.

Membrane markers

The above statements about recovery of membranes depended upon the use of markers of each membrane. An ideal membrane marker would be located *uniquely and uniformly* throughout a given membrane type and would be preserved throughout isolation of all membranes. Further, it would be an activity or a chemical easily determined with high sensitivity and with reliability. No markers have been established as ideal. In the absence of known ideal markers, it is best to use several markers for each membrane type, if possible, and compare their distributions throughout the process. If one desires to argue that a given activity (e.g. the ATP-dependent Ca-pump) is in a given class of membranes, it is essential to do a study correlating its distribution with a reliable marker or markers for that membrane[13].

If one wishes to know the degree of purity of fractions isolated, a first approach is to examine the degree of concentration or dilution of marker activity pert unit member protein. This will, however, not give absolute amounts of membrane but only the final composition relative to the initial homogenate. In the case of the commonly used plasma membrane marker 5'-nucleotidase activity, the existence of a soluble enzyme as well as the membrane bound one precludes use of this approach. Comparisons in this case must be made between membrane fractions. If absolute amounts (expressed in terms of protein content) of each membrane were known in the initial homogenate and concentrations and yields of membrane markers were followed at each stage of isolation, it should be possible to determine absolute amounts of each membrane in each fraction. This is attainable in principle by quantitative electron microscopy of membranes present in the intact smooth muscle tissue and by having knowledge of the protein content of various membrane systems. This information is very difficult to obtain in practice. Two major impediments have inhibited the application of this approach: 1) it is very difficult to determine activities accurately in the homogenate since they are low and the homogenate despite its name is not homogeneous; 2) the effort involved in the electron microscopy is enormous. I am unaware that anyone has attempted such a study.

As an alternative, we have adopted iterative approaches based on the use of markers for each membrane, on an assumption based on the best available evidence about the activity or concentration of a marker for each membrane type per unit of pure membrane protein, and the knowledge that the total of all membrane protein must in each fraction equal the sum of all the protein in its various membrane components. By adjusting the activity or concentration of marker assumed to be present in various pure membranes, we can arrive at a consistent solution as to the given amount of each present in each fraction[35]. Since highest enrichment of markers is likely to be attained during continuous sucrose density gradient centrifugation when a larger number of fractions is collected, Grover et al.[13,32,33,35] use this information to compute the expected membrane purity in the subsequently designed discontinous gradients.

According to such calculations, the purity of plasma membrane fractions so far achieved varies from 60% in some tissues to 90% in others (table 1). Rat myometrium plasma membrane fraction N1 isolated by the above methods has yielded the highest purity. Isolating mitochondria by techniques specially designed for the purpose has also been successful in achieving relatively pure preparations[13,78]. The lack of success with other membranes requires further consideration as already mentioned above. 60% or more of most markers are recov-

Table 1. Estimated purity of plasma membrane* fractions

		References
Rat myometrium	$N_1 > 95\%$ $N_2 \sim 60^{++}$	25, 59, 49
Rat mesenteric artery	F_1 70–80%	49
Rat fundus	F_2 70–80%	14, 56
Rat mesenteric vein	F_1 60–70%	53
Rat vas deferens	F_2 70–80%	14, 56
Canine Trachealis	F_2 60–70%	26a
Canine gastric corpus (circular muscle)	$F_2 \sim$ 60–70%	74

*No fractions of endoplasmic reticulum were so far obtained. > 50% purity. **Most of the impurity in this fraction appears to be attached contractile protein[25,29].

ered after isolation[49,53,54,56,59,74]. The clear exception is the oxalate-activated, ATP-dependent Ca-accumulation which is lost differentially compared to oxalate-independent, ATP-dependent Ca^{2+}-accumulation[27,50]. Recently, the loss of this activity was shown in one case[68] to be related probably to the mechanical stress of centrifugation. This activity is assumed to mark smooth muscle endoplasmic reticulum (ER) by many workers, as discussed above, but may be a subfraction of plasma membrane (PM). There is no comparable loss of other putative ER markers such as NADPH-cytochrome c-reductase[49,53,54,56,59,74] ^{3}H-leucine-uptake[59] or rotenone insensitive NADH-cytochrome c-reductase[59] or of PM markers including oxalate-independent ATP-dependent Ca^{2+}-accumulation[27,50]. This implies either that the membrane in question is not lost, but one of its acitivities is or that the activity is not really a component of this membrane[27,50]. There is, however, no membrane marker (and thus no membrane) lost in a degree comparable to this activity during the isolation procedure.

Conceivably there is a loss or inactivation of the Ca^{2+}-Mg^{2+}-ATPase assumed to be responsible for Ca-transport by ER or a subfraction of PM. A change of permeability properties of the membrane is an alternative hypothesis; e.g. loss of oxalate or phosphate permeability or increased leakiness to calcium ion. Use of antibodies to the transport ATPase responsible for this activity would help resolve these issues. So far no antibody to a transport ATPase derived for pure ER from smooth muscle is available. However, there are several which recognize the analogous Ca-transport ATPase for SR of skeletal muscle. There may be considerable homology between the enzymes from smooth and skeletal muscle. There is so far no published evidence demonstrating an antigen recognized by antibodies to Ca-transport ATPase from skeletal muscle SR in smooth muscle membrane fractions. There were, however, antigens to and a Ca-transport ATPase recognized by antibodies against the analogous, calmodulin-activated ATPase from red blood cells. Also, an antibody prepared against the similar enzyme from a plasma membrane-enriched fraction from pig antral muscle recognized the enzyme present in red blood cell plasma[70,85]. There thus seems to be clear evidence of a plasma membrane Ca-pump in smooth muscle. The evidence with regard to the ER-Ca-pump does not distinguish whether it is absent or present in very small quantities, lost during preparation of membranes or fails to share antigenic determinants with the Ca-pump from skeletal muscle SR. However, it seems likely that this problem will be resolved in the near future.

Raeymaekers and Hasselbach[69] isolated vesicles which they considered to be ER by loading microsomes from pig antrum with Ca^{2+} in the presence of oxalate and then centrifuging them in a sucrose gradient. Raeymaekers, Wuytack et al.[70] showed using a similar technique that a lighter vesicle fraction, which showed ATP-dependent Ca-accumulation but little oxalate stimulation could also be obtained. It had higher 5'-nucleotidase activity and less NADH-cytochrome c-reductase (rotenone-insensitive) activity than the fraction which was loaded in the presence of oxalate. However, there was little difference between the two fractions as regards substrate specificity for the Ca-transport or susceptibility to vanadate inhibition. The failure of Wuytack, Raeymaekers et al.[85] to find a distinct phosphorylated intermediate associated with a Ca^{2+}-ATPase activity other than the calmodulin-activated, plasmalemmal enzyme makes it difficult to decide between then possibility that the distinct vesicle fractions, from PM and ER, were present or the alternate hypothesis that two PM subfractions exist. When methods are found to isolate vesicles with the oxalate-stimulated Ca-pump without major losses, these different views can probably be resolved.

Properties of PM Ca-pump

Physiological Ca-transport properties of all PM vesicular systems isolated up to now are consistent with the hypothesis that the calcium transport system is capable of pumping intracellular calcium down to levels allowing relaxation (10^{-7} M). Table 2 summarizes a number of such studies. Note that the K_m for Ca^{2+} is usually about 0.5 μM. The pump velocity is usually maximal at about 1 μM. Since the range between 0.1 and 1 μM of $[Ca^{2+}_i]$ is the range between relaxation and maximal contraction the Ca-sensitivity of the system is clearly appropriate. The velocity of transport is usually approximately 20–70 μmoles/g protein/min at free Ca levels of 1–2 μM; this could remove 10^{-4} moles of Ca per l of cell water per minute from a typical smooth muscle cell (see p. 910). We lack sufficient information about Ca-transport, the rates of transport at lower free Ca^{2+} levels or the rate of fall of intracellular Ca^{2+} following a contraction to make an estimate of whether all or only part of the process should be attributed to the pump. There are additional complexities which must be resolved before the nature of the relationship between plasma membrane pump rate, $[Ca_i]$ and relaxation can be fully understood: e.g. are various Ca^{2+} channels fully inactivated when relaxation begins?; does internal sequestration by binding (see below) or pumping into ER occur simultaneously and at what rate?; and to what extent does Na^+-Ca^{2+}-exchange contribute to change in $[Ca^{2+}_i]$ during relaxation?

Even though vesicles of plasma membranes are likely to be somewhat damaged and therefore, more leaky as a result of the mechanical damage, osmotic and shear forces and loss of extrinsic proteins and soluble components during isolation, they still can create Ca-gradients estimated to be greater than 1000-fold (table 2). The

Table 2. Properties of Ca-pump in selected PM fractions*

Source	K_m app. μM	Hill cœfficient	V_o μmoles–g^+–min–1 (Ca^{2+} = 1 μM)	Max transport μmoles/g (Ca^{2+} = 1 μM)	Reference
Rat myometrium (N_1)	0.4–9.5	1.3–1.7	5–10	40–70	31
Rat fundus (F_2)	0.3–0.5	0.9–0.95	6–10	30–70	31
Canine corpus (F_3)	0.75–0.95	~ 1.0	6–8	20–25	73
Canine antrum (P_6)	0.25–0.3	2.0–2.5	5–10	20–25	34

* In all these cases, the Ca gradient produced was estimated to be > 1000-fold from 1 to 2 μM free Ca^{2+}.

actual values calculated are critically dependent upon the proportion of inside-out plasma membrane vesicles present in the mixture. Outside-out and broken vesicles are not expected to accumulate calcium. Unfortunately there has been considerable difficulty in determining this proportion but it appears that in the isolated rat myometrium PM about 20% of vesicles are broken and half the remainder are inside-out[13,25]. Preliminary data suggest that a similar distribution may exist in PM of rat mesenteric artery. The values quoted above, therefore, need to be considered as approximations.

So far the detailed molecular mechanisms and kinetics of the plasma membrane calcium pump have not been studied primarily because it has not been possible to demonstrate the existence of a Ca^{2+}-Mg^{2+} activated pump ATPase. The isolation of this ATPase has however been reported from one smooth muscle[84] and the reconstitution in lipid vesicles reported. The difficulty in most smooth muscles in studying the Ca-pump ATPase comes from an extraordinarily active basal Mg^{2+}-ATPase[13] which obscures any increased activity when Ca^{2+} is added in micromolar concentrations. It is noteworthy that the one tissue in which success has been reported[84,85] had a much lower than usual basal Mg^{2+}-ATPase activity. There have been occasional reports of the existence of a Ca^{2+}- and Mg^{2+}-activated ATPase, but careful examination of the protocols indicate that these studies almost invariably fail to take into account the existence[34,52] of a high affinity Ca^{2+}-activated ATPase requiring no Mg^{2+}. Thus an increase in ATPase activity after adding Ca^{2+} in the presence of Mg^{2+} is not sufficient to establish the existence of a Ca^{2+}-activated, Mg^{2+}-dependent ATPase. In studies carried out to date[34,52], the high affinity Ca^{2+}-ATPase has an appropriate K_m to function as a Ca^{2+}-pump but its properties differ from the Ca^{2+}-transport system in a number of respects; e.g. radiation inactivation property, susceptibility to inhibitors such as 4,4'-diisothiocyano-2,2'-stilbene disulfonic acids, etc. Although it is not equivalent to the active Ca^{2+}-transport system of PM, this high affinity Ca^{2+}-pump may be a component of it; but further analysis is required.

Even though the plasma membrane Ca^{2+}-pump has not been studied in depth, some of its properties are known. It is calmodulin activated[85] (Grover and Kwan, unpublished data), and in a number of systems, calmodulin antagonists inhibit Ca^{2+}-transport or its consequences[70,85] (Grover and Kwan, unpublished). The nature of the calmodulin activation has not been clarified and unpublished studies in our laboratory suggest that different smooth muscle plasma membranes may be activated in different ways, some by increased velocity of active transport (V_{max} increased), others by increase in affinity for Ca^{2+}, and others by increasing the Ca gradient.

The calcium pump may also be modulated by the levels of cycle nucleotides, since there are various hypotheses suggesting that elevation of cyclic AMP (c-AMP) or of cyclic GMP (c-GMP) may lead to relaxation[40,43]. There are some studies[2,3,41,61] with microsomes from smooth muscle suggesting that c-AMP may enhance ATP-dependent Ca-transport by microsomal vesicles; the results were usually interpreted as showing increased Ca-transport by ER. Not all investigators find such an effect; e.g. Allen[1]. However, studies with purified plasma membrane or ER are lacking. In one study in our laboratory[38], partially purified catalytic subunit of c-AMP-dependent protein kinase from rat mesenteric arteries activitated ATP-dependent Ca-transport by mesenteric artery plasma membranes slightly and only at high external Ca-levels (10^{-6} M). Further study of effects of cyclic nucleotides or the catalytic subunits of their protein kinases on Ca^{2+}-transport by purified PM and ER are needed.

Na-Ca exchange

In sarcolemma of cardiac muscle there is a counter-transport exchange mechanism whereby Na^+ and Ca^{2+}-ions can cross the membrane in opposite directions[4,65,71]. The system in heart appears to be electrogenic, exchanging 3 Na^+ for 1 Ca^{2+} and may function to regulate Ca^{2+}_i; its properties in cardiac sarcolemma are summarized in table 3. In intact smooth muscles, there have been several studies which could be interpreted as showing effects related to Na^+-Ca^{2+} exchange; they usually consist of demonstration that lowering external [Na^+] causes contraction while raising it causes relaxation (e.g. Brading[6], Brading et al.[7] and Masahashi and Tomita[58]). In some cases, the uptake of Ca^{2+} (using La^{3+} or EGTA method) was shown to increase when external Na^+ was lowered and to decrease when it was raised[79]. However, interpretation of such studies is difficult because: 1) the internal Na^+ concentrations are usually unknown and changing in a non-uniform fashion (over time and space) so the transmembrane gradients of Na^+ are uncertain (as are those of Ca^{2+}) and 2) alternate explanations of the data (such as competition of Na^+ and Ca^{2+} for a membrane channel) can be given.

The use of isolated plasma membrane vesicles has allowed unequivocal demonstration of the existence of Na^+-Ca^{2+}-exchange in rat myometrium[30,35], and some evidence has been obtained in mesenteric arteries[14]. These vesicles were prepared with a desired internal Na^+ and/or Ca^{2+} concentration by equilibrating them with known concentrations of the ion, and then diluted into different concentrations of a given ion to create a known gradient. When vesicles were loaded with 100 mM Na^+ and diluted 20- to 25-fold into Na-free Ca^{2+}-uptake solutions (an outward Na^+-gradient imposed) there was an extra uptake of Ca^{2+} (measured as $^{45}Ca^{2+}$) after 1–2 min. Similarly creation of an inwardly directed Na^+-gradient caused an extra loss of Ca^{2+} from vesicles preloaded passively or actively with Ca^{2+}. This extra uptake or loss of Ca^{2+} could not be produced by similar gradients of K^+, Rb^+, Cs^+ or Li^+ (Li^+ could partially substitute for Na^+), was abolished by Na or Ca ionophores, or by osmotic shock, and ceased after about one minute (presumably owing to collapse of the Na^+ gradient). The pH dependence and other properties of this Na^+-Ca^{2+} exchange in PM from rat myometrium are summarized in table 3 which also compares the properties of the system to those in cardiac sarcolemma. Both systems are capable of transporting Ca against a concentration gradient, even though the K_m values for Ca^{2+} are in the range of 1–20 μM, but there are important differences. The initial velocity of the system in smooth muscle PM is much less than those in cardiac PM ($1/100$) and there is no evidence so far that the system in smooth muscle is electrogenic. As well in smooth muscle PM, it

Table 3. Comparison of Na-Ca exchange-dependent Ca-uptake by uterine and cardiac muscle PM-enriched membranes

	Rat myometrium	Cardiac muscle
Uphill Ca^{2+} movement	Yes	Yes[4]
pH optimum	> 6.67	> 9[64]
v_o, μmoles · g^{-1} · min^{-1}		
− KCl	> 2[30]	246[8]
+ KCl	2–4	264[8]
Max value, μmol/g	2.11 ± 0.38	
− KCl	(n = 6)	
+ KCl	2.37 ± 0.30 (n = 16)	80[8]
Na specificity	Li^+ poorly substitutes[30]	Li^+ does not substitute[4]
K_m for Ca^{2+}, μM	7.0*	1.5, 20[8,64,71]
Stoichiometry		3.12 Na/Ca
Electrogenecity		Generates internal positive potential[8,64]
		Stimulation
Effect of valinomycin	None	Stimulation[71]
Effect of uncouplers	None	

Values are mean ± Se for n preparations. Rat myometrium was estrogen treated. *Concentration required for half-maximal activity.

has not been possible to demonstrate that a Ca^{2+}-gradient can produce net Na^+ transport. Probably this reflects the leakiness of these PM vesicles to Na and this leakiness may also cause underestimation of the velocity and capacity of the Na^+-Ca^{2+} exchange system in transporting Ca^{2+}. Future studies must aim at better understanding the Na^{2+}-leak from smooth muscle PM vesicles and development of a system in which the existence of the Na^{2+}-gradient or net Na^+-transport can be prolonged. It is possible that vesicles can be prepared in which the Na^+-K^+ pump is active (and can maintain an Na^+-gradient) or that the leak channels for Na^+ can be blocked. At present the contribution of the Na^+-Ca^{2+} exchange system to normal regulation of internal Ca^{2+} cannot be evaluated because the conditions under which it can be studied do not allow its optimal function. It must be emphasized that such an exchange system can move Na^+ or Ca^{2+} in either direction depending upon electrochemical gradients, the membrane potential (if exchange is electrogenic) and the stoichiometry of the exchange.

Ca-binding by PM vesicles

Ca-binding in this discussion refers to ATP-independent binding and probably represents passive binding to membrane sites. The usual method for studying it involves exposure of vesicles to $^{45}Ca^{2+}$ under various experimental conditions followed by dilution of the vesicles into an ^{45}Ca-free (EGTA-containing medium when removal of extravesicle Ca was desired), and by Millipore filtration. The Ca retained on the filter after an EGTA wash is Ca-bound and free inside vesicles; in the absence of such a wash, a small amount of additional Ca was bound to or trapped between vesicles. In PM from rat myometrium the amount of Ca-retained was markedly pH-dependent (between pH's 6.27 and 7.07) when external [Ca^{2+}] was in the micromolar range[32]. Most of this retained calcium was found to be bound to the inside of the membrane under these circumstances. The pH-dependently bound Ca (calculated as that found at ph 7.07 minus that found at pH 6.27) amounted to 15 to 20 μmoles/g membrane protein. The binding is half maximal at pH 6.8. This amounts to sufficient Ca (if all were released and assumed to derive from a cell of 3040 μm^3 volume with a surface area to volume ratio of 1.8), to contribute to raising [Ca^{2+}] to about 2×10^{-4} M*. Of course, some released Ca^{2+} will be sequestered in the cell. It also is sufficient Ca to imply binding occurs either to phospholipids or in a multivalent fashion to protein.

The affinity of pH-dependently bound Ca to its site is about 0.28 μM and the Hill coefficient for binding was 2.37 for Ca^{2+}. This implies a positive cooperativity in binding and a very high affinity. The nature of binding site which has these properties and a pK of about 6.8 is uncertain. However, the high affinity of this site for Ca^{2+} is confirmed by the fact that binding is not inhibited but accelerated by the calcium ionophore A23187, presumably because the ionophore enables Ca to cross the plasma membrane rapidly but the ionophore does not comp lete with the binding site for Ca^{2+}. Osmotic shock to break the PM vesicles also does not displace Ca from these binding sites.

Preliminary studies[26] indicate that this pH-dependent, high affinity binding to smooth muscle PM is a property of most smooth muscles. However, the capacity of these sites appears to vary markedly in different smooth muscles and owing to cell differences or to different methods of membrane isolation. It will be interesting to compare this property of a smooth muscle to its ability to support contraction by release of a sequestered store of Ca. In rabbit aorta, the microsomes were shown[26] to have pH dependent Ca-binding. Also the Ca, which is released from an intracellular store to support contraction by added norepinephrine in this tissue and has to be reloaded from the extracellular space, was shown to be bound in a pH-dependent fashion. In other words, reloading of this store at low pH, inhibited the subsequent contraction to norepinephrine carried out at a normal pH. Furthermore, the exposure to the tissue to low pH during efflux or $^{45}Ca^{2+}$ into EGTA-containing solutions released extra ^{45}Ca and inhibited the increase in efflux produced by subsequent norepinephrine. These results suggest that the Ca bound to the plasma membrane in a pH-dependent fashion may provide Ca^{2+} to support the contraction in some cases of pharmacomechanical coupling. This will require further study of pH-dependent properties of this store in a variety of tissues.

In addition to the high affinity Ca-binding there is a low affinity binding which has not been studied in detail. It may be of considerable importance to membrane stability, coupling of receptors to ion channels or binding sites. Certainly at the extracellular [Ca^{2+}] there will be considerable binding to the external surface of the membrane[12,32]. Whether this Ca also contributes to excitation-contractions coupling directly or only indirectly is unknown[12]. Its potential role in formation of caveolae or in interactions between plasma membrane dense bodies is also unknown.

Conclusions

1) Use of purified subcellular membranes from smooth muscle offers an approach to study mechanisms of Ca-

handling which may eliminate some problems which have delayed progress and confused interpretation of experiments with intact tissues.

2) With techniques available to date it has been possible to obtain satisfactorily purified plasma membrane vesicles and mitochondria, but endoplasmic reticulum vesicles have not been obtained in satisfactory purity.

3) Failure to isolate pure endoplasmic reticulum fractions may be caused by its similar densities to plasma membrane and to the larger quantity of plasma membrane as well as by differential damage to ER vesicles by the isolation techniques used. If oxalate-activation of ATP-dependent Ca^{2+}-transport proves to be a property unique to ER, the resultant increase in density of ER vesicles will aid their isolation. If so, and if the causes of differential loss of this transport function during isolation can be identified and avoided, then study of the properties of purified endoplasmic reticulum should be possible.

4) Purified plasma membrane vesicles have been shown to possess

a) an ATP-dependent pump capable of extruding Ca^{2+} from cell and lowering the internal Ca^{2+} to about 10^{-7} M;

b) a Na-Ca^{2+} exchange system which appears to have a limited velocity and capacity compared to heart and to nerve, but so far it has not been studied unter optimal conditions (e.g. with a maintained Na^+ gradient);

c) a pH-dependent, ATP-dependent, high affinity Ca binding which may participate in excitation-contraction coupling;

d) a pH-independent, low affinity Ca-binding which has not been studied in detail.

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* Membrane density is known to be about 1.1 g/cm^{3}h; protein-lipid ratio is 1:1; the partial specific density of protein and lipid are assumed to be 1.36 and 0.9 g/cm^3 respectively; this leads to 1.52 cm^3 of membrane 1 per g protein. If membrane is 8×10^{-7} cm thick, each g of protein will derive from 1.89×10^3 cm^2 of membrane.

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Full Papers

Titers of ecdysone, 20-hydroxyecdysone and juvenile hormone III throughout the life cycle of a hemimetabolous insect, the ovoviviparous cockroach *Nauphoeta cinerea*

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Summary. Titers of ecdysone, 20-hydroxyecdysone and juvenile hormone III were measured in whole body extracts or hemolymph of embryos, first, penultimate and last stadium nymphs, and adult females of *Naupoheta cinerea*. We used a gas-chromatography/mass spectrometry method for quantifying juvenile hormone and a radio-immunoassay for ecdysteroid determination. Juvenile hormone III is particularly abundant in the embryonic stage (up to 960 ng/g), at a low level in first and penultimate stadium nymphs (2–10 ng/ml) and almost absent in the last nymphal stadium; in the adult female the juvenile hormone titer rises to 180 ng/ml in hemolymph during rapid oocyte growth. The titers of ecdysone and 20-hydroxyecdysone undergo similar fluctuations in the embryonic and nymphal stages, being highest at the time of cuticle formation in the embryo and a few days before the nymphal and adult molts (around 100–200 ng/ml for exdysone and 2–4 µg/ml for 20-hydroxyecdysone).
Key words. Ecdysteroids; juvenile hormone III; developmental changes; cockroach; *Nauphoeta cinerea*.

Introduction

According to the classical model for the regulation of insect development and metamorphosis (fig. 1) molting is induced by ecdysteroids but the nature of the molt depends on the concentration of juvenile hormone (JH) in the hemolymph[30]. In holometabolous insects JH is assumed to be high before larval molts, low at the larval-pupal transformation and absent before metamorphosis. The classical model derives mainly from ligation, transplant and parabiosis experiments[30], whereas titer measurements of either JH or ecdysteroids have been made only in a few cases at selected stages[5, 6, 13, 17, 18, 28]. The embryo is not included in the classical model (fig. 1) because ecdysteroids[11, 19, 20, 22] and JH[3] have only recently been identified and measured during embryonic development and in only a few species. There is still uncertainty surrounding the function of these hormones in embryos[26]. Nevertheless, ecdysteroids seem to play a role in cuticle formation in the embryo similar to their function during nymphal development[10, 22, 26]. The same hormones are found also in the adult female of most insects where JH acts as a gonadotropic hormone by stimulating both oocyte growth and yolk protein synthesis in the fat body[10]. At this stage ecdysteroids are located mainly in the ovary[14] but in some insects they also circulate in the hemolymph[12, 15, 20, 32].

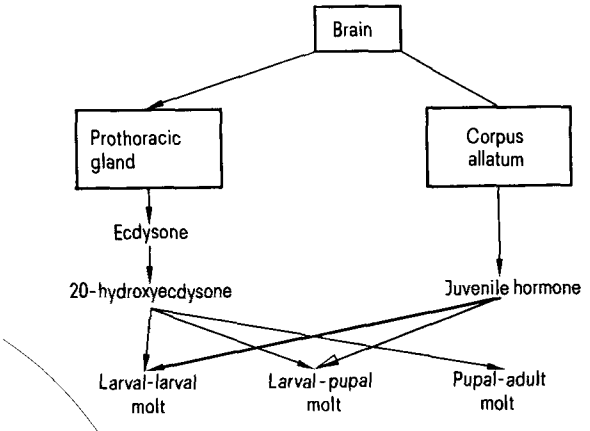

Figure 1. The classical model of the regulation of insect development and metamorphosis. The half-shaded arrow signifies a lower titer.