Several abstracts for the conference on 'Regulation of transport mechanisms'

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Abstracts for the conference on 'Regulation of transport mechanisms'

1. CHANGES IN CATION COMPOSITION OF BOVINE AORTIC ENDOTHELIAL CELLS IN CULTURE

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The content of the two main electrolytes, Na and K, was determined in an endothelial cell clone of bovine origin (BFA-1c) after a different number of passages, or in cultures of bovine endothelial cells from calf aortas obtained at a local abattoir. The addition of penicillin, streptomycin and mycostatin at concentrations of 100 U/ml, 100, and 0.25 μg/ml, respectively, to the culture medium did not affect the Na and K content of these cells after seven days. The effect of a longer period of culture with antibiotics has not been tested. Likewise, Na- and K-free washing solutions of different ionic strength and electrolyte composition did not affect the cation composition of endothelial cells. Cell Na and K content was determined daily in cultured cells for up to 30 days in order to establish the time at which a steady state for cations was reached. In this type of experiments the protein content/35 mm diameter dish reached values of up to 1.355 $\pm$ 0.023 mg, n = 4. In addition, the time at which the protein content/dish became constant was dependent on the seeding density. The intracellular K content was variable for about a week in culture, increasing in some cases or decreasing in others by a factor of up to 1.6 after day seven, but was relatively constant during the rest of the study in all cultures. However, the intracellular Na content presented more daily variations than K, in some cases changing from a value of 86.7 $\pm$ 3.7 nmoles/mg protein, n = 3, at day 11, to 203 $\pm$ 15, n = 28, at day 12. These changes in Na content were accompanied by a constant K content, and in some cases returned to the initial level on the next day, eliminating the possibility of cell death. So far, no steady state values for Na or K content have been obtained in any of the experiments described above. The mechanism underlying the changes in cation composition of cultured endothelial cells is under continued investigation. (Supported by grants AHA MV-86-01, AHA MV-87-01 and WSU Research Incentive Award.

2. THE INTERACTION OF DITHIOLS AND TWO DIFFERENT BINDING SITES FOR 5HT-REUPTAKE INHIBITORS WITHIN AND 5HT-REUPTAKE SYSTEM

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Chemical modification procedures have been used to study the interaction of tricyclic and non-tricyclic 5HT-reuptake inhibitors with the [$^{-3}$H] imipramine binding site (IBS). N-ethoxycarbonyl-3-ethoxy-1,2-dihydroquinoline (EEDQ) induced a pronounced loss in [$^{-3}$H] imipramine binding due to a reduction in Bmax. Preincubation with reuptake inhibitors and subsequent inactivation by EEDQ revealed that imipramine and 5HT prevented the EEDQ-induced-crosslinking, but citalopram and fluoxetine did not. Thiol modification studies demonstrated that DTT-reduction enhances the binding of [$^{-3}$H] imipramine by increasing the Bmax. 1,1-diazobis-(N,N-dimethylforamide) (diamide), phenyl-arsineoxide (PAO) and N-ethylmaleimide (NEM) attenuated the binding capacity by lowering the Bmax. Reversible treatment with PAO prior to NEM-induced alkylation prevented this alkylation, indicating that dithiols, indeed, are involved in NEM induced inactivation. Binding of tricyclics or nontricyclics prior to PAO inactivation revealed that tricyclics provide complete protection against thiol modification,

while the non-tricyclics do not. The results support the hypothesis of the 5HT-reuptake system being a complex aggregation of closely interacting binding sites.

3. ROLE OF THE 53 KILODALTON GLYCOPROTEIN OF THE SARCOPLASMIC RETICULUM

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When rabbit skeletal muscle sarcoplasmic reticulum (SR), at 2.5 mg/ml, was extracted with cholate (2.5 mg/ml) at low [KCl], the resulting preparation accumulated Ca^{++} and hydrolyzed ATP at levels similar to untreated SR. SR extracted with cholate at high [KCl] hydrolyzed ATP at normal rates, but was deficient in accumulating Ca^{++}. The preparations that accumulated Ca^{++} poorly had diminished content of the 53-kilodalton glycoprotein (GP-53) of the SR membrane. Preincubation of SR with an antiserum (AS) against GP-53 resulted in decreased ATP-driven Ca^{++} transport by the SR, but had no effect on the rate of Ca-stimulated hydrolysis of ATP. The effect of AS on Ca^{++} uptake was time and concentration dependent. Half-maximal effect of AS of Ca^{++} uptake required 30 min preincubation at 37 °C. Two-fold dilution of AS resulted in significant reduction of its potency. Preincubation with AS did not increase the passive permeability of SR to Ca^{++} and did not cause proteolysis of the Ca-ATPase. Under our conditions the estimated coupling ratio in control SR and in SR preincubated with preimmune serum was about 1.5 mole Ca^{++}/mole ATP, while the coupling ratio in SR preincubated with AS was 0.186. The transient state kinetics of cholate-extracted SR was studied with chemical quenching methods. Cholate-extracted SR that contained GP-53 at undiminished levels had steady-state levels of EP and rates of EP formation and ADP-induced EP decomposition that are similar to those of SR, and transported Ca^{++} with an initial burst followed by a steady-state rate of transport. Cholate-extracted SR with diminished content of GP-53 formed normal steady-state levels of EP, had normal kinetics of EP decomposition, formed EP more slowly than control preparations, and transported Ca^{++} with an initial burst, but with a greatly diminished steady-state rate. Our results are consistent with the interpretation that GP-53 may be involved in regulating coupling of Ca^{++} transport to ATP hydrolysis in the SR.

4. REACTIVITY OF THE LYSOSOMAL H^+ PUMP TO ANTIBODY ELICITED AGAINST THE CHROMAFFIN GRANULE H^+ PUMP

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The H^+ ATPases of eukaryotic cell organelles, including the rat liver lysosomal (tritosomal) H^+ ATPase and the bovine chromaffin granule H^+ ATPase, exhibit similarities in function, in substrate requirements, and inhibitor responses. We have explored the possibility that these pumps also exhibit immunological determinants which are similar. Toward this end, antibodies were raised in rabbits against a purified preparation of the chromaffin granule H^+ pump (Percy, *et al.* (1985) Biochem J. 231: 556–564), and this antibody was reacted with lysosomal proton pump preparations. Tritosomes were purified from livers of rats injected with WR1339. Membranes were prepared from these by osmotic lysis in 1 mM EDTA, 200 mM NaCl, 0.5 mM PMSF, 5 μg/ml leupeptin and 2 μg/ml pepstatin, followed by purification on sucrose gradients. The membranes were then incubated in 25% glycerol, 1% $C_{12}E_9$, 10 mM Pipes-Tris buffered sucrose, 1 mM EDTA, 2 μg/ml pepstatin and 5 μg/ml leupeptin, pH 7.4 on ice, and the soluble and insoluble fractions separated following centrifugation for 1 hour at 200 k × g at 0 °C. The antibody raised

against the chromaffin granule pump was reacted with each of these lysosomal membrane protein preparations on immunoblots. Immunoreactivity was detected by autoradiography following incubation with ^{125}I-Protein A. We have previously shown that the lysosomal H^+ pump and ATP hydrolysis activity can be inhibited by photolysis with 250 μM 8-azido ATP. Under similar conditions, a 70–80 kD lysosomal polypeptide, amongst others, was labelled by photolysis with 4 μM 8-azido-α[^{32}P]ATP (BBA 899 (1987) 276–284). A polypeptide of similar molecular weight, immunoreactive with the chromaffin granule antibody, is found in lysosomes, lysosomal membranes, and in detergent extracts of lysosomal membranes. This data raises the possibility that the 70–80 kD integral membrane proteins of the lysosome and chromaffin granule membranes play similar roles in H^+ pump function. Supported by the VA and NIH DK38808 (JC), and the American Heart Association Grant 87-1025 (GED).

5. MECHANISM OF URIDINE DIPHOSPHOGALACTOSE (UDPGAL) UPTAKE IN GOLGI VESICLES

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The Golgi apparatus contains a number of glycosyltransferases involved in the terminal glycosylation of N-asparagine linked glycoproteins. Galactosyltransferase is a membrane bound enzyme of the Golgi whose active site is luminally oriented. It utilizes UDPGal (which is synthesized in the cytoplasm) as galactosyl donor and one of the products, uridine diphosphate (UDP), is highly inhibitory to the enzyme and must be removed from the lumen. Previous studies have shown that UDPGal is taken up by isolated Golgi vesicles. In addition, we found that during galactosylation in these vesicles, UDP, formed by galactosyltransferase in the lumen of the Golgi, is rapidly degraded to uridine monophosphate (UMP) by nucleoside diphosphatase also present in the lumen. This UDPase activity is also membrane bound with its active center luminally oriented. UMP appears to readily diffuse out of the vesicles. To study whether uptake of UDPGal involves an antiport of UMP out of the Golgi, we studied the kinetics of efflux of ^{3}H-UMP from vesicles preloaded with various concentrations of UMP. We find that Golgi vesicles have a high binding capacity for UMP which is abolished by disruption of the membrane either with filipin of N_2 decompression. The Kd is about 30 μM. Measurable efflux occurs when the internal concentration of UMP exceeds 1 mM. Efflux is saturable with a V_{max} of 1 nmole $\times$ min^{-1} $\times$ mg^{-1}, an apparent K_m of 2 mM at 22 °C, and appears cooperative with a Hill coefficient of 4. The uptake of UMP by Golgi vesicles shows similar kinetics. At both low and high levels of UMP, efflux rates are not affected by the presence of UDPGal on the outside of the vesicles. These results indicate that this type of antiport mechanism for the uptake of UDPGal is unlikely. In addition, we find that uptake of UDPGal by Golgi vesicles is not stimulated by an imposed ΔpH or $\Delta\Psi$ in either direction but is stimulated four-fold by preloading the vesicles with N-acetylglucosamine, a galactosyltransferase acceptor. We conclude from these studies that UDPGal uptake in Golgi is a facilitated diffusion driven by the concentration gradient of the nucleotide sugar.
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6. MEMBRANE REGULATION OF MUSCLE CONTRACTION AND RELAXATION IN FAST TWITCH SKELETAL MUSCLE

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The sarcoplasmic reticulum (SR) serves a central role in Ca^{2+} uptake, storage and release. Highly purified sarcoplasmic reticulum (SR), subfractionated into light and heavy SR, derive from longitudinal (LC) and terminal cisternae (TC), respectively. The light SR fraction consists of one type of membrane, the Ca^{2+} pump containing membrane (CPM) with the calcium pump protein (CPP) constituting ~90% of the protein. The CPM is involved in energized Ca^{2+} uptake enabling muscle to relax. The CPM has been

extensively characterized in terms of structure, function and motional characteristics [1].

The molecular basis for Ca^{2+} release which triggers muscle contraction in excitation-contraction coupling is one of the important unsolved problems in cell biology and muscle physiology. In order to study the Ca^{2+} release process *in vitro*, distinct new membrane fractions have been prepared: 1) plasma membrane vesicles, capable of generating a Na^+ gradient, comparable to that *in situ*; 2) a purified triad fraction to study the influence of transverse tubule (TT) on Ca^{2+} release process; and 3) a junctional terminal cisternae (JTC) fraction of SR with intact feet structures which, *in situ*, together with TT forms the triad junction [1].

JTC consists of two morphologically distinct types of membranes, i.e., the well-characterized CPM (~80–85%) and the junctional face membrane (JFM) (~15–20%) containing the junctional feet structures [2]. The compartmental contents (C), mainly calcium binding protein, are anchored to the JFM, apparently via the junctional feet structures. The JTC has been disassembled by selective extraction of CPM to yield a JFM-C complex. In turn, C can be removed to yield purified JFM [3].

JTC vesicles, devoid of TT, are leaky to Ca^{2+}. The leak is closed by ruthenium red, whose action is blocked by ryanodine (Ki ~50 nM). Thus, ryanodine appears to lock the calcium release channels (CRC) in the 'open state'. Both ryanodine and ruthenium red react with CRC which we have localized to the JTC by pharmacological action and by ryanodine binding (Kd ~50 nM; Bmax ~20 pmol/mg protein) [4]. Smith, Coronado and Meissner have incorporated SR vesicles by fusion into phospholipid bilayers and described two types of calcium conducting channels [5]. We find that their high conductance channel (HCC) (~100 pS) is modulated by ryanodine, equating HCC to the CRC in excitation-contraction coupling [6].

The ryanodine receptor from skeletal muscle has been isolated. It consists mainly of a high molecular weight polypeptide, Mr ~360 KD, which has been identified by electron microscopy to be equivalent to the feet structures of the JTC. These studies: 1) pin point the calcium release process to the JFM and JTC; and 2) provide an approach to the molecular definition of the CRC [7].

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The purified ryanodine receptor has been reconstituted into bilayers. Channel gating activity is observed which is modulated (activated or inhibited) by ligands in a manner reflecting permeability changes observed in junctional terminal cisternae of SR. We conclude that the identity of the Ca^{2+}-release channel of SR is the foot structure, which consists of an oligomer of the high molecular weight polypeptide [8].

References

1. S Fleischer. In: S. Fleischer and Y Tonomura (eds) Structure and Function of Sarcoplasmic Reticulum. Academic Press, 1985, pp 155–162
2. A Saito, S Seiler, A Chu, S Fleischer: J Cell Biol 99:875–885, 1984
3. B Costello, C Chadwick, A Saito, A Chu, A Maurer, S Fleischer: J Cell Biol 103:741–754, 1986
4. S Fleischer, EM Ogunbunmi, MC Dixon, EAM Fleer: Proc Natl Acad Sci USA 82:7256–7259, 1985
5. JS Smith, R Coronado, G Meissner: Nature (London) 316:446–449, 1985
6. K Nagasaki, S Fleischer: Cell Calcium 9:1–7, 1988
7. M Inui, A Saito, S Fleischer: J Biol Chem 262:15637–15642, 1987
8. L Hymel, M Inui, S Fleischer, HG Schindler: Proc Natl Acad Sci USA 85:441–445, 1988

7. MODIFICATION BY FITC OF THE CAATPASE AND CA^{2+} TRANSPORT REACTIONS IN SKELETAL MUSCLE SARCOPLASMIC RETICULUM

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The fluorescent probe, fluorescein isothiocyanate (FITC), prevents ATP binding to the CaATPase of sarcoplasmic reticulum by covalent modification of a single reactive lysine residue at the catalytic site. The time courses of phosphoenzyme formation by ATP and phosphoenzyme decomposition produced by the addition of ADP were investigated by rapid

acid quenching following exposure of the membrane-bound CaATPase to various concentrations of FITC. Inhibition of 30–40% of the CaATPase activity by FITC had no effect on the pattern of ADP-induced dephosphorylation measured 116 msec after the start of phosphorylation. However, increasing the inhibition to 80–90% reduced the proportion of ADP-sensitive (E_1P) phosphoenzyme and increased the fraction of ADP-insensitive (E_2P) phosphoenzyme compared to the control enzyme. Moreover, the initial burst of P_i production associated with rapid decomposition of the phosphorylated enzyme was accelerated by FITC treatment. In addition to the changes produced in the CaATPase partial reactions, FITC-induced alterations in the pattern of $^{45}Ca^{2+}$ accumulation as determined by ADP and EGTA quenching were also seen. At levels of FITC producing modification of the enzymatic reactions, the initial burst of $^{45}Ca^{2+}$ accumulation was accelerated, consistant with the FITC-induced increase in the initial rate of P_i production. In addition, the linear phase of $^{45}Ca^{2+}$ uptake following the burst was strongly inhibited indicating that FITC was preventing recycling of the Ca^{2+} pump. Since FITC inhibits the enzyme by preventing ATP from binding to the catalytic site, the shift in the $E_1P \not\in E_2P$ equilibrium in the FITC-treated preparation may have resulted from an altered conformational interaction between active protein subunits and inactive adjacent subunits. These results suggest that subunit-subunit interactions are necessary for stabilizing the formation of E_1P and that loss of the stabilizing interactions through FITC modification increases the initial rate of Ca^{2+} uptake but prevents continuous operation (recycling) of the Ca^{2+} pump.

8. REGULATION OF MAMMALIAN PHOSPHOLIPASES: PEROXIDATION, PHOSPHOLIPID ALTERATIONS AND SARCOPLASMIC RETICULUM DYSFUNCTION

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The susceptibility of partially peroxidized liposomes of 2-[1-^{14}C]-linoleoyl-phosphatidylethanolamine (^{14}C-PE) to hydrolysis by cellular phospholipases was examined. ^{14}C-PE was peroxidized by exposure to air at 37 °C resulting in the formation of more polar derivatives as determined by thin-layer chromatographic analysis. Hydrolysis of these partially peroxidized liposomes by lysosomal phospholipase C associated with cardiac sarcoplasmic reticulum, and by rat liver lysosomal phospholipase C, was greater than hydrolysis of non-peroxidized liposomes. By contrast, hydrolysis of liposomes by purified human synovial fluid phospholipase A_2, or bacterial phospholipase C was also completely inhibited by partial peroxidation of PE. Lysosomal phospholipase C preferentially hydrolyzed the peroxidized component of the lipid substrate which had accumulated during autoxidation. The major product recovered under these conditions was 2-monoglyceride, indicating sequential degradation by phospholipase C and diglyceride lipase. Liposomes peroxidized at pH 7.0 were more susceptible to hydrolysis by lysosomal phospholipases C than liposomes peroxidized at pH 5.0, in spite of greater production of polar lipid after peroxidation at pH 5.0. Sodium bisulfite, an antioxidant and a reported inhibitor of lysosomal phospholipases, prevented: (1) lipid autoxidation, (2) hydrolysis of both non-peroxidized and peroxidized liposomes by sarcoplasmic reticulum and (3) loss of lipid phosphorus from endogenous lipids when sarcoplasmic reticulum was incubated at pH 5.0. These studies show that lipid peroxidation modulates the susceptibility of phospholipid to attack by specific phospholipases and may therefore be an important determinant in membrane dysfunction during injury.

9. ISOLATION OF THE RYANODINE RECEPTOR FROM CARDIAC SARCOPLASMIC RETICULUM AND IDENTITY WITH THE FEET STRUCTURES OF JUNCTIONAL TERMINAL CISTERNAE

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The ryanodine receptor from cardiac sarcoplasmic

reticulum (SR) has been purified, characterized, and compared with that from skeletal muscle SR. Ryanodine, a highly toxic alkaloid, reacts specifically with the Ca^{2+} release channels which in both cardiac and skeletal muscle SR are localized to the terminal cisternae (TC). Recently, we isolated the ryanodine receptor from skeletal muscle SR and showed it to be equivalent to the feet structures of TC (Inui, Saito and Fleischer (1987) J. Biol. Chem. 265, 1740–1747). We now describe the purification of receptor from heart. The cardiac ryanodine receptor was solubilized with CHAPS in the presnece of phospholipid. Purification was performed by sequential affinity chromatography followed by gel permeation chromatography in the presence of CHAPS and phospholipid. The enrichment of the receptor from cardiac microsomes was about 100-fold. The purified receptor contained a major polypeptide band of Mr 340 KD with a minor band of Mr 300 KD (less than 10%), on SDS-polyacrylamide gel electrophoresis. Electron microscopy of the purified receptor from heart showed square structures of 222 ± 21/side, which are the unique characteristics of feet structures of the junctional face membrane of TC. The ryanodine receptors from heart and skeletal muscle have similar characteristics in terms of protein composition, morphology, chromatographic behaviour, Ca^{2+}-, salt- and phospholipid-dependency of ryanodine binding. However, there are distinct differences: 1) the receptor from heart is slightly smaller than that for skeletal muscle (Mr 340 vs 360 KD); 2) the affinity of the purified cardiac receptor for ryanodine was 4 to 5-fold tighter than that of skeletal muscle, measured under identical conditions. The greater sensitivity to ryanodine in intact heart can be directly explained by the tighter binding of the ryanodine receptor from heart. The present study defines in molecular terms and characterizes at least a portion of the machinery involved in the calcium release process in heart and skeletal muscle. It indicates that basically similar but not identical machinery (the ryanodine receptor and foot structure) is involved in modulating Ca^{2+} release from cardiac and skeletal muscle SR [1].

Recently, the purified cardiac muscle ryanodine receptor has been reconstituted into planar bilayers and found to have channel gating activity. The channels are strongly activated by Ca^{2+} (10 nM) in the presence of ATP (1 nM) or ryanodine and inactivated by Mg^{2+} (3 mM) or ruthenium red (30 μM). These characteristics are diagnostic of calcium release from heart SR. Hence, the cardiac receptor, which has previously been equated to the foot structure, is now identified as the calcium release channel. The characteristics of the calcium release channel from skeletal muscle and heart are similar in that they: 1) consist of a single high molecular weight polypeptide (Mr 360000 in skeletal muscle and 340000 for heart); 2) exist morphologically as the foot structure; 3) are activated (ATP, Ca^{2+}, ryanodine) and inhibited (ruthenium red and Mg^{2+}) by a number of the same ligands. Important differences include: 1) Ca^{2+} activation at lower concentration of Ca^{2+} for the heart; 2) more dramatic stabilization by ryanodine of the open state for the skeletal muscle channel; and 3) different relative permeabilities (P_{Ca}/P_K) [Hymel, Schindler, Inui, Fleischer: Biochem Biophys Res Communs 152:308–314, 1988].

Reference

Inui, Saito, Fleischer: J Biol Chem 262:15637–15642, 1987

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10. PHOTOAFFINITY LABELLING OF THE SARCOPLASMIC RETICULUM JUNCTIONAL FEET-RYANODINE RECEPTOR COMPLEX BY 8-AZIDO-[α-^{32}P]ATP

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Vesicle ion flux studies and single channel recordings have indicated that the Ca^{2+} release channel of skeletal muscle sarcoplasmic reticulum (SR) is

stimulated by μM Ca^{2+} and mM adenine nucleotide, and inhibited by mM Mg^{2+} and μM calmodulin (Meissner 1986, Biochemistry 25, 244; Smith *et al.*, 1985, Nature 316, 446). Recently, the neutral plant alkaloid, ryanodine, has been shown to interact specifically and with nM affinity to the SR Ca^{2+} release channel, using vesicle ion flux, [^{3}H] ryanodine binding and single channel measurements (Pessah *et al.*, 1985, BBRC 128, 449; Fleischer *et al.* 1985, PNAS82, 7256; Meissner, 1986, J. Biol. Chem. 261, 6300; Rousseau *et al.*, 1987, Am. J. Physiol., 253, (364). The morphology of the isolated ryanodine receptor (Inui *et al.*, 1987, J. Biol. Chem. 262, 1740; Lai *et al.*, 1987, BBRC 143, 704) has revealed that it is identical to the junctional feet structures, which, in intact muscle, have been found to connect the SR to the transverse tubules (T-system). It is across this T-SR junction that the triggering signal for Ca^{2+} release is believed to occur upon depolarization of the T-system.

We have purified the rabbit muscle ryanodine receptor to homogeneity and found that it comprises a single polypeptide of M_r ~400000 upon SDS-PAGE. We also now show, that the ryanodine receptor can be affinity photolabelled by 8-azido-[α^{32}]ATP in the membrane bound, solubilized and purified forms. Specific labelling of the M_r ~400000 protein was obtained using 'heavy' SR vesicles (0.5 mg protein/ml) with 2 μM 8-azido-[α-^{32}P]ATP in 50 mM Na-PIPES pH 7, 1 M NaCl, 1 mM EDTA, 0·2 mM EGTA, 50 mM mannitol. The presence of mM concentrations of either AMP-PCP, ATP, azido-ADP, ADP or AMP inhibited specific labelling of the M_r ~400000 protein band. Similar results were obtained using vesicles solubilized in CHAPS and with the purified ryanodine receptor. No labelling was found in the absence of UV irradiation. These results, combined with observations that the M_r ~400000 protein present in 'heavy' SR is 1) the sole constituent of the purified ryanodine receptor (this abstract), 2) a Ca^{2+}-binding protein (Zorzato and Volpe, 1987, Biophys. J. 51, 353a; Seiler *et al.*, 1984, J. Biol. Chem. 259, 8550), 3) photoaffinity labelled by calmodulin and doxorubicin, both modulators of SR Ca^{2+} release (Zorzato *et al.*, 1986, J. Biol. Chem. 261, 13252; Seiler *et al.*, 1984, J. Biol. Chem. 259, 8550), indicates that the junctional feet-ryanodine receptor complex contains all the regulatory sites of the SR Ca^{2+} release channel which mediates excitation-contraction coupling.

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11. DIFFERENTIAL ALTERATION IN MYOCARDIAL EXCITATION-CONTRACTION COUPLING BY ALIPHATIC ALCOHOLS OF VARYING SIZE

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Depending on the phenomenon or tissue studied, the aliphatic alcohols have pharmacologic effects similar to both the local and general (volatile) anesthetics, which are well known to alter or depress myocardial contractility. Therefore, effects of the alcohols upon isometric contractions of guinea pig papillary muscles were examined. Contractions were studied following rest and at frequencies of 0.1, 0.25, 0.5, 1, 2, & 3 Hz, with the increase in stimulation frequency causing enhanced contractility ('positive staircase'). The effects of the alcohols were also examined in contractions which had been enhanced by: 1) low Na (40 mM) Tyrode, which caused rapid early tension development after rest; or 2) 26 mM K Tyrode with 0.1 μM isoproterenol, which permitted propagated slow action potentials. The latter inotropic enhancement results in a distinctive late peaking contraction after rest and at 0.1–0.5 Hz, with rapid tension development at 2–3 Hz. The following alcohol concentrations in Tyrode solution were employed: 100–300 mM ethanol, 10 mM n-butanol, 3 mM n-pentanol, 1–3 mM n-hexanol, 0.5–1 mM n-heptanol, 0.1–.2 mM n-octanol, and 1 mM benzyl alcohol. The concentration range of each alcohol in Tyrode is that which: 1) is of similar anesthetic potency; and 2) results in a similar estimated concentration of each alcohol in lipid, based upon the individual lipid-water partition coefficient.

Ethanol, butanol, pentanol and hexanol caused depression of the rapid tension development observed at 2–3-Hz, or when induced by low Na Tyrode. In contrast, octanol consistently increased the contractions at 2–3 Hz and in low Na, but markedly

and reversibly depressed the late peaking tension. The ability of the alcohols to reversibly depress the late-peaking tension decreased with chain length. The effects of benzyl alcohol were similar to those of hexanol, causing relatively selective depression of the late-peaking tension, with only modest depression of early rapid tension development. Previous experiments have demonstrated the sensitivity of the late-peaking tension to local anesthetics, which caused little or no depression of the early, rapid tension development. In contrast, ryanodine irreversibly blocked the early rapid tension development. None of the alcohols depressed the slow AP rate of depolarization, which could have been interpreted as depression of slow inward (Ca) current. Likewise, the effects demonstrated were unlike those of the Ca entry blockers nifedipine, verapamil, or diltiazem.

The different aliphatic alcohols cause dissimilar changes in tension development of ventricular muscle. These changes can be attributed to differential alteration in functions of the sarcoplasmic reticulum, depresing a ryanodine-sensitive and/or a local anesthetic sensitive component. Such effects suggest that certain alcohols interact with the membrane proteins and/or lipids which control intracellular Ca fluxes with some specificity, and that effects are not simply related to the alcohol's lipid solubility.

12. SH GROUPS AND WATER TRANSPORT IN RED CELLS

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Evidence for the existence of water channels in red cells is based on comparisons of its water permeability properties with corresponding measurements in lipid bilayers. (In red cells the magnitude of the permeability as well as the ratio, P_{osm}/P_d, of osmotic to diffusional permeabilities is mich higher, while the activation energy is much lower). After treatment with PCMBS these discrepancies vanish; apparently PCMBS closes the water channels. Measurements of the ratio of osmotic to diffusional flow show that channel closure is all or none. The assumption of single file diffusion indicates that there are 11 water molecules per channel. Studies of the equilibrium of PCMBS and the putative SH receptor indicate that the channel reaction is entropically driven. Contrary to previous reports (Naccache, P., Sha'afi, R., J. Cell. Physiol. 83, 449, 1974), our studies on pH dependence suggest only a single case of sites (presumably SH sites) involved in water transport, that are acted on by PCMBS. Further, results with anion inhibitors indicate that these sites are fully accessible from the external medium. The PCMBS receptor groups involved in water and various solute transport systems can be distinguished by kinetic means. Results on the inhibition of water, urea, glucose, monocarboxylic acid, and Na^+-K (leakage acceleration) will be compared.

These results require accurate measurements of both P_{osm} and P_d. Although P_d has been measured by independent techniques, the only measurements of P_{osm} have relied exclusively on empirical optical techniques for measurements of cell volume. We describe results obained with a new EPR method for estimation of P_{osm}, which is based on the virtually instantaneous equilibration of the spin label tempone between intra and extracellular spaces. In the presence of an extracellular paramagnetic quenching agent, the measured signal is proportional to the cell water. Results obtained with this method and with light scattering compare very favorably. Supported by NIH GM-19981.

13. COMPARISON OF THE RYANODINE RECEPTOR OF CARDIAC AND SKELETAL MUSCLE SARCOPLASMIC RETICULUM BY TARGET INACTIVATION ANALYSIS

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In excitation-contraction coupling, excitation at the sarcolemma causes an increase in intracellular calci-

um concentration which triggers muscle contraction. In cardiac muscle this is achieved in two stages: (1) Ca^{2+} influx through voltage gated sarcolemmal Ca^{2+} channels; and (2) subsequent Ca^{2+} release from sarcoplasmic reticulum (SR). In skeletal muscle, all of the Ca^{2+} for contraction is stored within the SR. In both types of muscle, Ca^{2+} release from SR occurs via specific release channels localized in the terminal cisternae (TC). Ryanodine specifically modulates the Ca^{2+} release channel locking it in the open state. For cardiac SR the K_d is 7.1 ± 1.7 nM, whereas for skeletal SR the K_d is 39.1 ± 21.8 nM. The ryanodine receptor from both cardiac and skeletal muscle has been isolated and consists of a single high molecular weight (HMW) polypeptide of 340 and 360 kD, respectively. The ryanodine receptor has been identified with the foot structure which spans the dyad or triad junction between TC and transverse tubules.

Radiation inactivation analysis studies indicate a target size for ryanodine binding of 139 ± 1 kD and 138 ± 21 kD for cardiac and skeletal SR, respectively. This is significantly lower than the size of the HMW polypeptide from these sources, suggesting that only a portion of the HMW polypeptide is involved in ryanodine binding. Analysis of the amount of HMW polypeptide remaining after irradiation, as assessed by densitometry of SDS PAGE gels, indicates a target molecular weight of 1 063 ± 78 kD and 1069 ± 243 kD for cardiac and skeletal SR, respectively. Given a monomer molecular weight of ~350 kD, this indicates a structual unit of a timer. The estimated molecular weight of the foot structure calculated from its dimensions and the density of protein is 4.4–4.8 million, suggesting that ~12 HMW polypeptides are present in one foot structure. The foot structure approximates a square rectangle with four-fold symmetry. Therefore, an association of four trimeric subunits forming the square is a reasonable model for the arrangement of the subunits of the foot structure.

We conclude that the ryanodine receptor of cardiac and skeletal muscle are similar with regard to the nature of their ryanodine binding domains and oligomeric structure. (Supported by NIH DK 14632 and HL 32711 and the Muscular Dystrophy Association (MDA) to SF and Fellowships from American Heart Assoc., Middle Tenn. Chapter (SM) and MDA (to CC). MI is Investigator of AHA Tenn. Affiliate).

14. RYANODINE SENSITIVITY OF THE CALCIUM RELEASE CHANNEL OF SARCOPLASMIC RETICULUM

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Ryanodine modulates Ca^{2+} permeability in isolated junctional terminal cisternae (JTC) of sarcoplasmic reticulum (SR), suggesting it to be a specific ligand for the calcium release channel. Our laboratory has recently purified the ryanodine receptor and found it to be equivalent to the feet structures, which are involved in the junctional association of the transverse tubule with the terminal cisternae [Inui, Saito and Fleischer (1987) J. Biol Chem. 262, 1740–1747]. Smith, Coronado and Meissner [(1985) Nature (London) 316, 446–449] have incorporated sarcoplasmic reticulum into bilayers and found a high conductivity channel (~100 pS) which has a number of characteristics expected of the Ca^{2+} release channel in SR. We now find that the high conductivity channel in the bilayer is sensitive to ryanodine. Low concentrations of ryanodine in the cis side (sub μM) 1) lock the channels in the open state; and 2) prevent the action of ruthenium red (μM) to completely close the channel. 3) Much higher concentrations of ryanodine (300 μM) close the channel. Ryanodine up to 10 μM added to the trans side has no effect. In these three respects, ryanodine acts similarly on the channel in the bilayer as in JTC vesicles. Further, the bilayer studies provide new insights into the action of ryanodine on the channel in that: 1) ryanodine locks the channel in the open state, but the conductivity is reduced to about 40%; 2) ryanodine prevents ruthenium red from closing the channel, although there is a further 50% decrease in the open current. These studies provide support that the high conductivity calcium channel in sarcoplasmic reticulum is involved in excitation-contraction coupling. By same token the pharmacological action of ryanodine is pinpointed to the calcium release channel (Nagasaki and Fleischer Cell Calcium 9:1–7, 1988).

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15. THE ESCHERICHIA COLI PHOSPHOENOLPYRUVATE-DEPENDENT PHOSPHOTRANSFERASE SYSTEM: THE MANNITOL CARRIER CONTAINS TWO PHOSPHORYLATIONSITES PER MONOMER AND ONE HIGH-AFFINITY MANNITOL BINDING SITE PER DIMER

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The membrane bound mannitol-specific Enzyme II (EII^{Mtl}) of the *Escherichia coli* phosphoenolpyruvate-dependent phosphotransferase system (PTS) transports mannitol over the cytoplasmic membrane. The driving force for transport is phosphoenolpyruvate, which, via two cytoplasmic PTS proteins, EI and HPr, phosphorylates the carrier. During transport this phosphoryl group is transferred to mannitol which enters the cell as mannitol phosphate. The stoichiometry of phosphoryl group incorporation was determined by phosphorylation of Enzyme II from (^{14}C) phosphoenolpyruvate. The native reduced enzyme incorporated two phosphoryl groups per monomer. Both phosphoryl groups were shown to be transferred to mannitol. Oxidation or N-Ethylmaleimide (NEM) labelling of Cys-384 resulted in incorporation of only one phosphoryl group per monomer, which was unable to be transferred to mannitol. The number of mannitol binding sites on Enzyme II was determined using an Amicon Centricon centrifugation method. The reduced unphosphorylated enzyme contained one high-affinity binding site (K_d = 0.1 μM) per dimer, implying that the purified unphosphorylated enzyme is at least present as a dimer. Oxidation or NEM labelling did not change the number of binding sites.

16. ENZYME II^{MTL} OF THE ESCHERICHIA COLI PHOSPHOENOLPYRUVATE-DEPENDENT PHOSPHOTRANSFERASE SYSTEM; IDENTIFICATION OF THE CYSTEINE ALKYLATION SITES ON THE MANNITOL CARRIER

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The membrane bound mannitol-specific Enzyme II (EII^{Mtl}) of the *Escherichia coli* phosphoenolpyruvate-dependent phosphotransferase system (PTS) transports mannitol over the cytoplasmic membrane. The driving force for transport is phosphoenolpyruvate, which, via two cytoplasmic PTS proteins, EI and HPr, phosphorylates the carrier. During transport this phosphoryl group is transferred to mannitol which enters the cell as mannitol phosphate. Treatment of the reduced unphosphorylated enzyme with the cysteine specific reagent N-Ethylmaleimide (NEM) results in incorporation of one NEM label per monomer and loss of enzymatic activity. NEM treatment of the phosphorylated enzyme also results in incorporation of one NEM label per molecule, but without loss of catalytic activity. The cysteines of Enzyme II were labelled with 4-vinylpyridine. After proteolytic breakdown and reversed-phase HPLC, the peptides containing cysteines 110, 384 and 571 could be identified. NEM treatment and inactivation prevented 4-vinylpyridine incorporation into the Cys-384 containing peptide. Both oxidation and phosphorylation of the native enzyme protected the protein against NEM labelling of Cys-384. Under phosphorylated conditions Cys-320 was labelled by NEM.

17. CATECHOLAMINE-INDUCED DOWN-REGULATION OF B-ADRENERGIC RECEPTOR FUNCTION

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Exposure of tissues or cells in culture to catechola-

mines results not only in activation of adenylate cyclase but also initiates a set of reactions leading to down-regulation of B-adrenergic receptor (BAR) function. Current evidence supports the hypothesis that such down-regulation involves a series of modifications of the BAR *per se*. An initial 'uncoupling' reaction, which may result from BAR phosphorylation, leads to a 40–60% loss of catecholamine-stimulated adenylate cyclase activity. The uncoupling reaction proceeds with a t 1/2 ot 1–2 min and precedes by 45–90 sec the appearance of BAR in cytoplasmic vesicles. Such BAR exhibit ligand binding properties consistant with their existance on the inside of vesicles; thus, they appear to be formed by a process of agonist-induced BAR internalization (endocytosis). During the early phase of agonist-induced BAR transformation receptors cycle from the surface to endosomes and back to the surface with a mean transit time of about 6 min. A third process results in the actual loss of BAR, at least in part due to receptor protein degradation. In general, catecholamine-induced uncoupling, endocytosis and recycling, and degradation do not require protein systhesis, whereas, recovery from the down-regulated state (degraded receptors) requires new receptor synthesis. Our recent experiments indicate that BAR internatlization can be reversibly blocked by phenylarsine oxide (J. Biol. Chem. 260: 12457, 1985) or by a reduction in cellular ATP content (J. Biol. Chem. 261: 5974, 1986). Such conditions do not prevent agonist-induced uncoupling of the response of adenylate cyclase to catecholamines. These results, coupled with the results of kinetic studies indicate that 1) receptor internalization is not required for functional desensitization, and 2) if a linear sequence of events is involved in desensitization, BAR internalization must follow an earlier uncoupling reaction. Other studies have addressed a related question; namely, is BAR internalization required for BAR loss?

18. A FLUORESCENCE QUENCHING ASSAY SHOWING PERMEABILITY OF BOVINE LENS MEMBRANE RECONSTITUTED VESICLES

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Gap junctions in the lens fiber cell membrane are thought to mediate transport of metabolites and waste products in this avascular organ. Additionally, gap junctions are thought to be compromised in the events leading to cataractogenesis, which is a leading cause of blindness in this country. In order to understand the molecular details of the function of this intrinsic membrane protein, we have developed a new, more sensitive assay for gap junction-mediated permeability. Lens fiber cell membranes are prepared by standard techniques, and gap junction proteins are solubilized in octylglucoside. These solubilized proteins are then incorporated into liposomes which contain 1 mol% of the fluorescent phospholipid analogue, NBD-PE. Quenching of the NBD-PE fluorescence by cobalt ion is used as an assay for liposome permeability. In liposomes without gap junction protein, approximately 50% of the fluorescence is quenched after addition of cobalt. In liposomes containing gap junction proteins, cobalt ion quenches up to 90% of the fluorescence, indicating that NBD-PE on both the inner and outer membrane monolayers is accessible to the quenching agent. We have also recently shown that lens membrane preparations which have been stripped of extrinsic proteins by the urea/NaOH treatment described by Russell, *et al.* (Exp. Eye Res. (1981) 32:511) are more active in this permeability assay than less purified preparations.

19. SINGLE Cl^- CHANNEL FROM CARDIAC SARCOPLASMIC RETICULUM

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The behavior of single Cl^- channel from canine cardiac sarcoplasmic reticulum was examined by

fusing isolated vesicle fractions into planar lipid bilayer with an asymmetric choline chloride gradient. Electrical measurements were performed with a patch clamp amplifier and data were stored on a VCR and analyzed by a computer program. The channel exhibited a steady state activation and a full open state with a unit conductance of 55 pS (in 260 mM Cl^-), as compared to 95 pS for the rabbit skeletal SR Cl^- channel. Several subconductance states were observed. The reversal potentials and unit conductance values were dependent on the Cl^- gradient across the bilayer. The open probability was dependent on holding potential (−60 to +60 mV) and displayed a bell shape relationship, with P_o values ranging from 0.2 to 0.8 with a maximum at −10 mV. Within the resolution of our system (>2 ms), time analysis has revealed the presence of one time constant for the full open state and two time constants for the closed state. The open and long closed time constants were found to be voltage dependent. Different time constant values were also obtained by varying Cl^- concentrations. The kinetic behavior was not affected by changing Ca^{2+} concentrations in both chambers, by adding millimolar ATP cis or by changing the pH from 7.4 to 6.8. In the presence of SO_4^{2-}, the unit current-amplitude was decreased but the P_o was unaffected. Channel activity was irreversibly inhibited by DIDS, a stilbene derivative. Using heavy or light SR fractions, multichannel recordings were consistently obtained with an average of three active Cl^- channels per vesicle fusion. This argues in favor of a high density and an homogeneous distribution of the anionic selective channel at the surface of the cardiac SR membrane. Our data suggests that the Cl^- selective pathway is capable of compensating electrogenic Ca^{2+} fluxes during rapid Ca^{2+} release and uptake.

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20. INVESTIGATION OF MONO AND DITHIOLS INVOLVED IN GABA TRANSPORT IN MEMBRANE VESICLES OF RAT BRAIN SYNAPTOSOMES

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The properties of γ-aminobutyric acid (GABA) transport in membrane vesicles derived from synaptomoses of rat brain have been studied using membrane permeable and impermeable sulfhydryl reagents, dithiol specific reagents and oxidizing reagents. GABA transport is inhibited, reversibly, by very low concentrations of the membrane permeable trivlent arsenical, phenylarsine oxide. Preincubation with this reagent partially protects GABA transport from inactivation by N-ethylmaleimide (NEM). Thorin, a negatively charged trivalent arsenical, has no influence on GABA transport at concentrations 100-fold higher than the inhibitory phenylarsine oxide concentration. The impermeant oxidizing agent, potassium ferricyanide, did not inhibit transport whereas the permeant reagent, diamide, was inhibitory. These data indicate that the GABA transporter possesses an activity-linked dithiol in a hydrophobic region of the carrier not accessible to charge, polar reagents. PCMBS also inhibits but does not protect against NEM inactivation suggesting that there is an activity linked mono-thiol in a polar region of the carrier.

21. SKELETAL MUSCLE SARCOPLASMIC RETICULUM CA^{2+} CHANNELS RECORDED IN NATIVE MEMBRANE

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Recent advances in the study of excitation-contraction (E-C) coupling have made it clear that Ca release by the sarcoplasmic reticulum (SR) is mediated by Ca^{2+} conducting channels in the SR membrane. The initial studies demonstrating the

presence and properties of these SR Ca^{2+} channels were carried out using biochemically isolated membrane vesicles inserted into artificial lipid bilayers (Smith *et al.* Nature 316: 446–9, 1985; Suarez-Isla *et al.* PNAS 83: 7741–5, 1986). More recently, concern over the degree to which the isolation procedures and artificial membranes influence the channel properties has served as an impetus for exploration of the intriguing possibility that more direct methods may be applied to the study of these channels. In this context, we have found the frog semitendinosus muscle fibers, mechanically skinned in Ca^{2+}-containing Ringers solution, exhibit small evaginations of internal membranes seconds after removal of the sarcolemmae. The evaginations coalesce in minutes to form large (10 to 100 μm) hemispheres extending from the surface of the skinned fibers. The membranes are clean enough to allow the formation of high resistance (1–50 GΩ) seals against conventional patch-pipette glass such that low noise current recordings can be made. Using this preparation, we have found the membrane to contain Ca^{2+}, K^+ and Cl^- conductances. To date, our studies have focused on the largest Ca conductance present because of its similarity to the putative SR Ca^{2+}-release channel described by others (Smith *et al.* 1985). This current record, filtered at 2 kHz, was made with 100 mM K gluconate in the pipette and 50 mM Ca gluconate in the bath. Arrows indicate the closed current levels. The I/V plot represents maximal unitary conductance at various potentials for the same patch. As illustrated, this channel has several distinguishing characteristics including: 1) relatively high (100–110 pS) slope conductance for Ca^{2+}, 2) the presence of a 65 pS subconductance state 3) relatively poor selectivity for Ca^{2+} over K^+ (P_{Ca}:$P_K \cong 2.0$), 4) pronounced sensitivity to voltage, becoming inactivated at high positive (over +70 mV) holding potentials (V_h) and low negative V_h (below −10 mV) and exhibiting high open time probabilities between these extremes. 5) sensitivity to caffeine (1–2 mM), manifest as an increase in P_o at negative V_h. The simplicity of this technique suggests that it will be generally useful in the study of skeletal muscle SR channels. Supported by PHS 1 POL HL37044.

22. REGULATION OF CALCIUM PUMP ATPASE BY PHOSPHOLAMBAN IN CARDIAC SARCOPLASMIC RETICULUM

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Phospholamban, an intrinsic component of cardiac sarcoplasmic reticulum (SR), was found to mediate intracellular effect of cAMP by serving as a substrate for cAMP-dependent protein kinase (PK) as well as by functioning as an activator of Ca pump ATPase. Transient state kinetic studies were indicative of the possibility that phosphorylation of phospholamban augments ATP hydrolysis by enhancing the cation-mediated conformational change of ATPase through a protein-protein interaction. Phospholamban was thus assumed to serve as a regulatory cofactor of Ca pump, that operate to mediate altered intracellular Ca signaling when β-adrenergic agonists increase intracellular levels of cAMP. In order to understand the molecular mechanism by which phospholamban exerts its stimulatory action on Ca pump ATPase in cardiac SR, we purified phospholamban from canine cardiac SR to near homogeneity and performed the primary structural analysis. The partial amino acid sequence of phospholamban, determined by chemical sequence analysis, indicated that the peptide is initiated with N^{α}-acetylated Met followed by 44 amino acids intervening two unidentified residues. This enabled us to isolate and sequence the complementary DNA (cDNA) specific for phospholamban isolated from a canine cardiac cDNA library. The amino acid sequence, deduced from sequencing the cDNA clones, indicated that phospholamban consists of 52 amino acid residues and lacks an amino-terminal signal sequence. The protein has an inferred MW 6080 that is in agreement with its apparent monomeric MW 6000 estimated by SDS-PAGE. Phospholamban monomer contains two distinct domains, a hydrophilic region at the amino terminus (domain I) and a hydrophobic region at the carboxy terminus (domain II); the former is probably located at the cytoplasmic surface, offering phosphorylation sites,

whereas the latter is anchored into the SR membrane. Most of domains I and II is assumed to form α-helix. Ser 16 and Thr 17 in domain I are the specific sties at which phosphorylation by cAMP- and calmodulin-PKs, respectively, occurs. Domain I $^{\alpha}$-helix probably breaks into two portions (domain IA and IB) at Pro 21, which would give a kink and allow side chains of IA and IB to express hydrophobic interactions. We propose an oligomeric model of phospholamban, in which four or five identical protomers of phospholamban assemble into oligomeric array with each of phosphorylation site facing the cytoplasmic milieu and Pro 21 serving as a hinge. The hydrophobic pocket formed by the oligomer may operate to exert a direct interaction with a key domain of Ca pump ATPase. Macromolecular properties of phospholamban oligomer and its molecular association with Ca pump ATPase are being investigated employing low-angle laser light scattering photometry.