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Changes in the surface charge properties of isolated cardiac sarcolemmal vesicles measured by light scattering. I. Characteristics of rat and canine preparations

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The cation-binding characteristics of isolated sarcolemmal vesicles from rat and canine cardiac muscle cells were investigated. To help elucidate the molecular properties involved in these interactions the cation-induced aggregation behavior of rat and canine cardiac sarcolemmal vesicles, sonicated unilamellar vesicles (SUVs) made from sarcolemmal lipid extracts, and SUVs generated from combinations of synthetic lipids similar to those found in the sarcolemmal membrane, as well as mitochondrial and sarcoplasmic reticulum enriched membrane fractions were examined. Our results indicate that cations, such as Ca^{2+}, do indeed bind to the sarcolemmal membrane surface. They also suggest that two (or more) interacting sites are involved in the Ca^{2+}-induced aggregation of the isolated sarcolemmal vesicles, and that sarcolemmal lipid components could be the primary binding sites. The modulating (secondary) sites on the other hand may be protein or carbohydrate in nature, or require specific lipid organizational properties. Finally, the results indicate that the interactions of cations, such as Ca^{2+}, with the sarcolemmal surface are species specific, with the sarcolemmal membranes of both rat and canine preparations having different physico-chemical properties.

Introduction

Numerous physiological studies suggest that cation–sarcolemmal interactions play an essential role in the excitation/contraction/relaxation cycle of cardiac muscle cells [1–11]. However, while some of these studies have demonstrated a close correlation between sarcolemmal associated Ca^{2+} and cardiac cell contractility, they have not been able to ascertain if Ca^{2+} was actually binding (adsorbing) to the sarcolemmal surface under these conditions or only present in the diffuse double layer [5,11–27]. (The difficulties are mainly due to the limitations inherent in the use of $^{45}Ca^{2+}$ as a probe for determining Ca^{2+}–sarcolemmal interactions involving low-affinity sites, which make it impossible to ascertain the spatial localization of Ca^{2+} within the system and thus the molecular mechanisms involved. These problems are compounded in the isolated vesicle studies by the sensitivity of the results obtained to the exact

Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Ches, 2(*N*-cyclohexylamino)ethanesulfonic acid; DTT, DL-dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; eggPC, egg phosphatidylcholine; eggPE, egg phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SUVs, sonicated unilamellar vesicles; TLC, thin-layer chromatography; LUVs, large unilamellar vesicles; PG, phosphatidylglycerol; MLVs, multilamellar vesicles; PA, phosphatidic acid.

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experimental protocol being employed. In the Millipore Filtration assay commonly used very small procedural changes can alter the amount of $^{45}Ca^{2+}$ 'bound', in nmol/mg protein, by up to 400% [21,22]. It is therefore important to point out that the term Ca^{2+} 'binding' employed in the studies cited above is used to describe the 'sum' of all the $^{45}Ca^{2+}$ which may be associated with the sarcolemma (and may also include internally trapped $^{45}Ca^{2+}$ in some cases). This associated $^{45}Ca^{+}$ is actually composed of two components: (1) the accumulation of cations, such as Ca^{2+}, in the aqueous region directly adjacent to a negatively charged membrane surface (the diffuse double layer), and (2) the actual binding, or adsorption, of cations to specific membrane components. As noted by McLaughlin [64], Ohki and Sauve [65], and McLaughlin et al. [61] these two types of Ca^{2+}–membrane interactions are very difficult to distinguish using radioactive agents such as $^{45}Ca^{2+}$, although their physiological consequences may be completely different.)

At the same time, other studies have demonstrated that the membrane proteins responsible for transsarcolemmal ion movements (i.e., ion channels, cation pumps, the Na^{+}/Ca^{2+} exchanger, etc.) are quite sensitive to their membrane environment [28–35]. The physico-chemical properties of the sarcolemmal membranes involved in regulating these processes, however, remain completely unknown. Consequently, it has not been possible to determine whether the effects of such cation–sarcolemmal interactions are due to a direct effect on channel or exchanger activity, an indirect effect of altered lipid–protein interactions, or a direct function of the amount of Ca^{2+} 'bound' to the sarcolemmal phospholipids, as proposed by Langer [5,11]. These uncertainties have led to intense controversy concerning the potential role(s) of cation–sarcolemmal interactions in these processes.

In order to help resolve these issues it is first necessary to ascertain the basic cation binding properties of the cardiac myocytes' sarcolemma and to determine the components participating in these interactions. With this end in mind we have begun a systematic examination of the interactions of cations with the cardiac sarcolemmal surface. This examination involves an analysis of the cation-induced aggregation behavior of the isolated cardiac sarcolemmal vesicles, and represents the first step towards a quantitative understanding of cation–cardiac sarcolemmal interactions. A major advantage of this experimental approach is that the interactions we are observing (aggregation) are directly related to the specific adsorption (binding) of cations to the sarcolemmal surface and not diffuse double layer effects [36,37]. We are therefore able to clearly distinguish between these two types of membrane associations and ascertain the contributions of ion binding (adsorption) to cation–sarcolemmal interactions. In addition, the presence or absence of internally trapped Ca^{2+} has no effect on vesicle aggregation behavior. We have previously employed this method to analyze mono- and divalent cation binding in model membrane systems [38,39] and to examine the roles of lipids and proteins in the (Ca^{2+}-PO_4^{3-})-induced aggregation of erythrocyte derived vesicles [40,41].

In the present study, we have examined the cation-induced aggregation behavior of isolated rat and canine cardiac sarcolemmal vesicles, sonicated unilamellar vesicles (SUVs) made from the sarcolemmal lipid extracts, and SUVs generated from combinations of synthetic lipids similar to those found in the sarcolemmal membrane, as well as mitochondrial and sarcoplasmic reticulum enriched membrane fractions. The purpose of this study is three-fold: (1) to ascertain whether cations do indeed bind (adsorb) to the sarcolemmal surface, rather than being present only in the diffuse double layer, (2) to characterize these interactions in order to help define a physico-chemical baseline for future quantitative analysis, and (3) to compare the cation binding properties of cardiac sarcolemmal membranes from two different species (rat and canine). The third aim is important for two reasons: (a) although it is known that the relative importance of sarcolemmal vs. sarcoplasmic reticulum Ca^{2+} to muscle contraction is different in these two species, most investigators assume that the physico-chemical properties of their sarcolemmal membranes are the same, as evidenced by the fact that many researchers combine the results obtained from different species (i.e., isolated sarcolemmal vesicles from canine, septal preparations from rabbits, single-cell studies from rats) in the same investigation to draw a

common conclusion concerning Ca^{2+} cardiac cell interactions, and (b) by comparing the results obtained for both species we will be better able to determine the basic molecular mechanisms involved (shared by the two species) and to identify the particular characteristics which may be species specific.

Portions of the reuslts obtained in this study were presented previously [42].

Materials and Methods

Materials. Phospho*enol*pyruvate (trisodium salt), pyruvate kinase (type III), L-lactic acid dehydrogenase (type XI), Na_2-ATP (vanadium free), β-NADH (from yeast grade III, disodium salt), *p*-nitrophenyl phopshate (diTris salt), Mes, Hepes, Ches, Tris-maleate, Mops, EDTA, DL-dithiothreitol, PMSF, sodium pyrophosphate, EGTA were obtained from Sigma Chemical Co. Chloride salts (vesicle isolation buffers, enzyme assays) were from Mallinckrodt Chemical Co. Ultrapure mono- and divalent cation salts for vesicle aggregation experiments (Gold Label 99.999%) and fluorescamine were from Aldrich Chemical Co. DNAase I (bovine pancreas, lyophilized) was from Worthington Biochemical. Egg phosphatidylcholine (PC) (> 99%), egg phosphatidylethanolamine (PE) (made by transphosphatidylation of egg PC (> 99%), and bovine phosphatidylserine (PS) (disodium salt, > 99%) were obtained from Avanti Polar Lipids, Inc. All three lipids gave single spots on silica gel G or H thin-layer chromatography plates. Distilled, de-ionized water was further purified (18 MΩ) with a Water-I apparatus (Gelman Sciences). Organic solvents were redistilled before use.

Isolation and characterization of cardiac sarcolemmal vesicles. Sarcolemmal vesicles were isolated from rat (male, Sprague-Dawley) or canine hearts according to the method of Bers and Langer [16] as modified by Frank et al. [43] with the further change that the protease inhibitor PMSF (200 μM) was added to the isolation buffers. Enzymatic activities of isolated canine and rat sarcolemmal vesicles were measured spectrophotometrically using a Hewlett/Packard model 8450A Photodiode Array Spectrophotometer equipped with a thermoelectric temperature controller: K^+-stimulated *p*-nitrophenylphosphatase activity (K^+-*p*-NPPase) was measured according to Bers and Langer [16]; K^+-stimulated ($Na^+ + K^+$)-ATPase with either the coupled enzyme assay according to Forgac [44] and Luciani [45] or using the more traditional end point method of Philipson and Nishimoto [46,47]; Ca^{2+}-stimulated ($Ca^{2+} + Mg^{2+}$)-ATPase using the coupled enzyme assay as described by Madden et al. [48]. Protein concentrations were determined according to the method of Lowry et al. [49], as modified for membrane proteins by Wang and Smith [50], using bovine serum albumin as standard. Phosphate was measured according to Bartlett [51]. Lipids were extracted from the isolated sarcolemmal vesicles using $CHCl_3/CH_3OH$ (2:1, v/v) according to the method of Bligh and Dyer [52]. The extracted lipids were stored in $CHCl_3/CH_3OH$ (2:1, v/v) under a N_2 atmosphere at $-20\,^\circ C$. Sonicated unilamellar vesicles (SUVs) were prepared from synthetic phospholipids or isolated sarcolemmal vesicle lipid extracts using standard methods [38]. The resultant vesicles were centrifuged at 178000 $\times g$ for 35 min at room temperature (Beckman Airfuge) to remove any multilamellar vesicles, and the supernatant used for aggregation experiments.

Vesicle aggregation. The aggregation behavior of the vesicle suspensions was measured turbidometrically over the wavelength range 200–800 nm as a function of cation concentration or at 300 nm as a function of time at constant cation concentration using the Hewlett/Packard 8450A Photodiode Array Spectrophotometer. Although the spectrophotometer recorded the entire spectrum (200–800 nm) of the vesicle suspension simultaneously, 300 nm was used to generate the absorbance (A) vs. [cation] and absorbance (A) vs. time curves because of its sensitivity to small changes in aggregation behavior. Control plots using a number of other wavelengths greater than 300 nm gave qualitatively identical curves, but with reduced absorbance values. For most experiments the sarcolemmal vesicles were suspended at a concentration of 60 or 120 μg protein/ml by injecting an aliquot of the concentrated vesicle preparation into 3 ml of the aggregation buffer, vortexing for a few seconds, and then sonicating the sample two times for 15 s (1 minute between bursts) using a bath sonicator to generate a homogeneous suspension. Experiments employing SUVs

made from extracted or synthetic lipids were conducted using lipid concentrations of 0.1–2.5 μmol phospholipid/ml depending on composition. Control experiments demonstrated that the sonication step improved the reproducibility of the absorbance values obtained, but had no effect on the shapes of the A vs. [cation] curves. Unless indicated otherwise, the aggregation buffers employed ('standard buffers') consisted of 100 mM monovalent cation (usually NaCl), 5.0 mM buffer (Mes, Hepes, or Ches depending on pH) and 0.05 mM Na_2-EDTA to remove any residual divalent cations. All aggregation assays were conducted at 25°C. Ultrapure (18 MΩ) water was used in all cases.

The A vs. [cation] experiments were conducted as described by Ohki et al. [38]. Control experiments confirmed that both curve shape and the [cation] corresponding to the A vs. [cation] curve maximum value were independent of vesicle concentration over the range employed, although the absolute value of the absorbance at 300 nm (A_{300}) maximum might be significantly increased or decreased.

The kinetics of cation-induced vesicle aggregation were determined for each cation by injecting an aliquot of the concentrated salt solution (final concentration 3 or 10 mM) into the vesicle suspension, mixing, and measuring the change in A_{300} vs. time. To insure the internal consistency of the results obtained numerous sarcolemmal vesicle samples were prepared as described for the A_{300} vs. [cation] experiments and then combined to form a common sample. Aliquots of this pool were then removed for the individual aggregation kinetics experiments. Previous studies [36,37,53] have shown that for vesicles having negatively charged surfaces the initial increase in turbidity is essentially governed by the aggregation rate constant C_{11}, which is a direct function of cation binding (i.e., the dissociation rate constant, D_{11}, equals zero). Consequently, under identical experimental conditions (kinetic energy constant) the rate of vesicle aggregation, measured turbidometrically, for a specific cation concentration increases as cation binding increases. Although this kinetic method cannot be used to quantitatively analyze cation binding, it can be employed to determine the selectivity series for the binding of cations, as well as to indicate alterations in the vesicle surface which change cation binding.

Fluorescamine labelling. Isolated cardiac sarcolemmal vesicles were labelled with fluorescamine using the method of Hidalgo and Ikemoto [54], and Hidalgo et al. [55]. Vesicles were suspended in buffer (100 mM NaCl/5.0 mM Hepes/0.05 mM Na_2-EDTA (pH 8.0)) to a final concentration of 2 mg protein/ml at room temperature. An aliquot of the fluorescamine stock solution (50 mM in dimethylsulfoxide (DMSO)) was then injected into the sample (rapidly stirred) to give a final fluorescamine concentration of 0.16 mM (final [DMSO] less than 0.4%). The suspension was further stirred for about 2 minutes and then centrifuged (178 000 × g) for 10 min at room temperature in a Beckman airfuge. The supernatant was removed and the pellet resuspended in fresh buffer (above) at a final concentration of 3–5 mg protein/ml. This preparation was then used as the starting material for the vesicle aggregation experiments. Control experiments were conducted using vesicle preparations treated in the same way, but without fluorescamine in the DMSO.

SUVs made from synthetic phospholipids or the total lipid extract of the isolated sarcolemmal vesicles were labelled with fluorescamine using a slightly modified procedure because the SUVs would not be separated from unreacted fluorescamine by high speed centrifugation. An aliquot of fluorescamine (50 mM in DMSO) was added to a concentrated suspension of lipid vesicles (10.0 μmol PL/ml) in buffer (above) under rapid stirring to give a final fluorescamine concentration of 1 mM (DMSO concentration = 1%). The sample was stirred for approx. 2 minutes and then used directly for experiments. (After the vesicle preparation was diluted to the concentrations employed in the aggregation experiments the final fluorescamine concentration was reduced to 0.03 mM and the DMSO concentration to less than 0.07%).

Fluorescamine labelling of both the synthetic and extracted lipids was monitored by TLC with Silica gel G and H plates (Supelco, Inc.) using $CHCl_3$/ CH_3OH/ H_2O (65 : 25 : 4, v/v) and $CHCl_3$/ CH_3OH/ NH_4OH/H_2O (90 :54 : 5.5 : 5.5, v/v) as solvents. Fluorescamine labelling of the lipid components of the labelled, isolated

sarcolemmal vesicles was examined by extracting the lipids from the vesicles with $CHCl_3/CH_3OH$ (2:1, v/v) as previously described, followed by TLC as above.

Results

Biochemical assays

The enzymatic activities of the isolated canine sarcolemmal vesicle preparations were: K^+-*p*-nitrophenylphosphatase (K^+-*p*-NPPase), 24.9–29.0 μmol *p*-nitrophenol/mg protein per h; K^+-stimulated ($Na^+ + K^+$)-ATPase (Philipson method), 12.8–20.2 μmol inorganic phosphate (P_i)/mg protein per h; K^+-stimulated ($Na^+ + K^+$)-ATPase after stimulation by alamethicin (12.5 μg/ml), 97.3–127 μmol P_i/mg protein per h. The enzymatic activities of the isolated rat sarcolemmal vesicle preparations were: K^+-*p*-nitrophenylphosphatase (K^+-*p*-NPPase), 7.0–11.9 μmol *p*-nitrophenol/mg protein per h; K^+-stimulated ($Na^+ + K^+$)-ATPase (Philipson method) 40.7 μmol P_i/mg protein per h; K^+-stimulated ($Na^+ + K^+$)-ATPase after stimulation by alamethicin (12.5 μg/ml) 74.8 μmol P_i/mg protein per h; K^+-stimulated ($Na^+ + K^+$)-ATPase activity (coupled enzyme assay) * 64.8–94.5 μmol P_i/mg protein per h; Ca^{2+}-stimulated ($Ca^{2+} + Mg^{2+}$)-ATPase, 0–0.84 μmol P_i/mg protein per h. No azide (NaN_3) sensitive ATPase activity was observed in the isolated rat sarcolemmal vesicle preparations using the coupled enzyme assay methods.

Effects of surface charge density on vesicle aggregation: model system studies

In order to provide a reference point for this study the effect of surface charge density on the Ca^{2+}-induced aggregation behavior of synthetic PC/PE/PS SUVs was examined as a function of $[Ca^{2+}]$. PC, PE, and PS were chosen because they are: (1) the major zwitterionic and anionic phospholipids present in the cardiac sarcolemmal membrane [21,22,56–58] and (2) they are also the lipids of choice employed in most model membrane studies. The surface charge densities of the SUVs were altered by changing the mol% of PS present in the vesicles, while keeping the mole% PC equal to the mol% PE. The results are shown in Fig. 1. They indicate that the aggregation curves are shifted to progressively lower $[Ca^{2+}]$ as the mol% PS of the SUVs increases. The results also demonstrate that it requires progressively larger concentrations of phospholipid vesicles to give the same A_{300} change as the mol% PS is decreased (0.1 μmol PL/ml for PC/PE/PS (0:0:100) to 2.5 μmol PL/ml for PC/PE/PS (50:50:0)). Thus at a constant phospholipid (PL) concentration, one experimentally observes an increase in the degree of aggregation (A_{300} max ↑) and a shift in the aggregation curves to lower $[Ca^{2+}]$ as the surface charge density increases. Similar results have been obtained with SUVs made from various PC/PS combinations [38].

Ca^{2+}-induced vesicle aggregation: Cardiac sarcolemmal, sarcoplasmic reticulum and mitochondrial fractions

Given this reference point, the aggregation behavior of isolated canine and rat cardiac sarcolemmal vesicles could be evaluated. In the first series of experiments the relative aggregation behavior of isolated rat cardiac sarcolemmal vesicles, and mitochondrial or sarcoplasmic reticulum enriched fraction vesicles (from the sucrose gradient-isolation procedure) were investigated as a function of $[Ca^{2+}]$. This was done in order to determine the possible effects of mitochondrial or sarcoplasmic reticulum membrane contamination on sarcolemmal vesicle aggregation. All experiments were conducted using 'standard buffers', at constant temperature (25°C) and protein concentration (60 μg/ml) *. In each case the initital

* The enzymatic activities of the various ATPases using the coupled enzyme assay methods were always proportionally higher than the activities measured for the same preparation using the more traditional end-point method. A detailed examination of these two approaches to measuring enzyme activities in cardiac sarcolemmal vesicle preparations will be presented separately (Leonards and Dhers, unpublished data).

* All samples were normalized to 60 μg protein per ml for these experiments. In terms of nmol PL/ml the values were: (a) rat: sarcolemmal vesicles, 120 nmol PL/ml; sarcoplasmic reticulum (SR) enriched fraction, 90 nmol PL/ml; mitochondrial enriched fraction, 48 nmol PL/ml; (b) canine: sarcolemmal vesicles, approx. 110 nmol PL/ml; mitochondrial enriched fraction, approx. 50 nmol PL/ml.

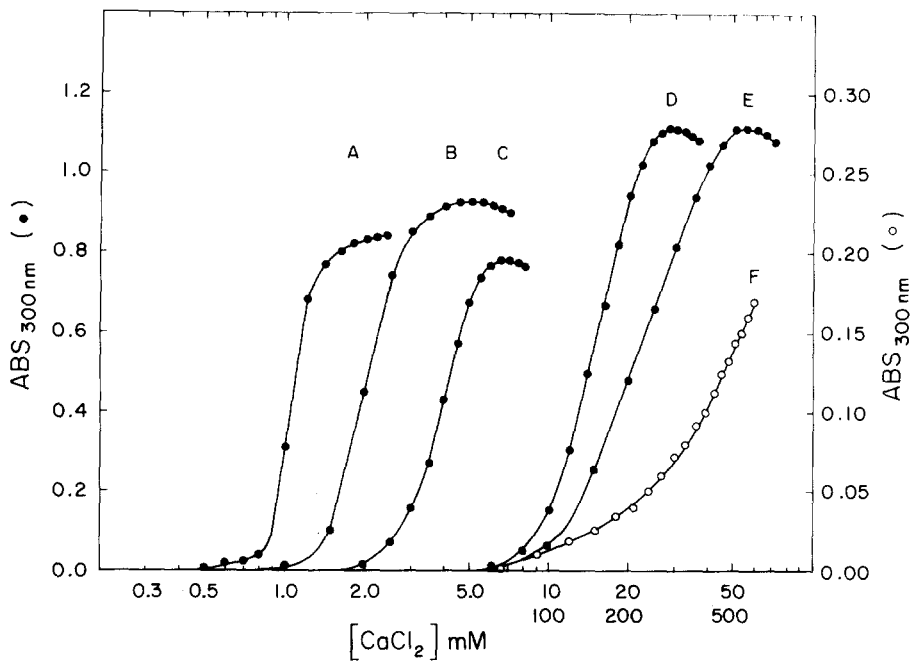

Fig. 1. Effects of surface charge density on the Ca^{2+}-induced aggregation behavior (A_{300}) of synthetic PC/PE/PS SUVs. Lipid composition and concentration: (A) PC/PE/PS (0:0:100 mol%), 0.1 μmol PL/ml; (B) PC/PE/PS (10:10:80 mol%), 0.2 μmol PL/ml; (C) PC/PE/PS (20:20:60 mol%), 0.2 μmol PL/ml; (D) PC/PE/PS (30:30:40 mol%), 0.4 μmol PL/ml; (E) PC/PE/PS (40:40:20 mol%), 0.4 μmol PL/ml; (F) PC/PE/PS (50:50:0 mol%), 2.5 μmol PL/ml. (Note: the *X*-axis values for (F) is the lower range (100–500 mM) Ca^{2+} concentrations.) Buffer: 100 mM NaCl/5.0 mM Hepes/0.05 mM Na_2-EDTA (pH 7.0). Temperature 25 ° C. Curves are averages of $n \geq 3$ experiments each curve.

turbidity of the sample was analyzed over the entire wavelength range (200–800 nm) to confirm that the size distribution of the vesicles from the various preparations was equivalent. The results are shown in Fig. 2A. They demonstrate that sarcolemmal vesicles, isolated from rat cardiac myocytes, were readily induced to aggregate with Ca^{2+}, indicating that Ca^{2+} is binding (adsorbing) to the sarcolemmal membrane surface. In contrast, the mitochondrial or sarcoplasmic reticulum enriched fraction vesicles (also at 60 μg protein/ml) displayed little or no aggregation in the presence of Ca^{2+} under these experimental conditions. Similar results were obtained with canine preparations (not shown). Thus any potential contamination by these organelles should not adversely affect the results obtained for the isolated sarcolemmal vesicles.

Effects of pH on Ca^{2+}-induced sarcolemmal vesicle aggregation

In the second series of experiments the effects of pH on the Ca^{2+}-induced aggregation behavior of isolated rat and canine sarcolemmal vesicles were examined as a function of [Ca^{2+}]. Theoretically, if the surface of the sarcolemmal vesicles contains ionizable sites which dissociate over a given pH range, there should be an increase in the surface charge density of the vesicle surface as the pH of the buffer is increased over this range. This is analogous to increasing the mole% PS present in the synthetic lipid SUVs (Fig. 1) and, assuming that Ca^{2+} can bind to these sites, should therefore result in an increase in the degree of aggregation (A_{300} maximum value, ↑) and a shift of the aggregation curves to lower [Ca^{2+}] (as in Fig. 1). Vesicle aggregation experiments were conducted under standard conditions using a constant protein concentration (60 μg/ml) at four different pH values (6.0, Mes; 7.0, Hepes; 8.0, Hepes; 9.0 Ches). Figs. 2A and B illustrate the results obtained. (The pH 6.0 results for rat sarcolemmal vesicles were deleted because of complications due to the direct effects of pH on sarcolemmal vesicle

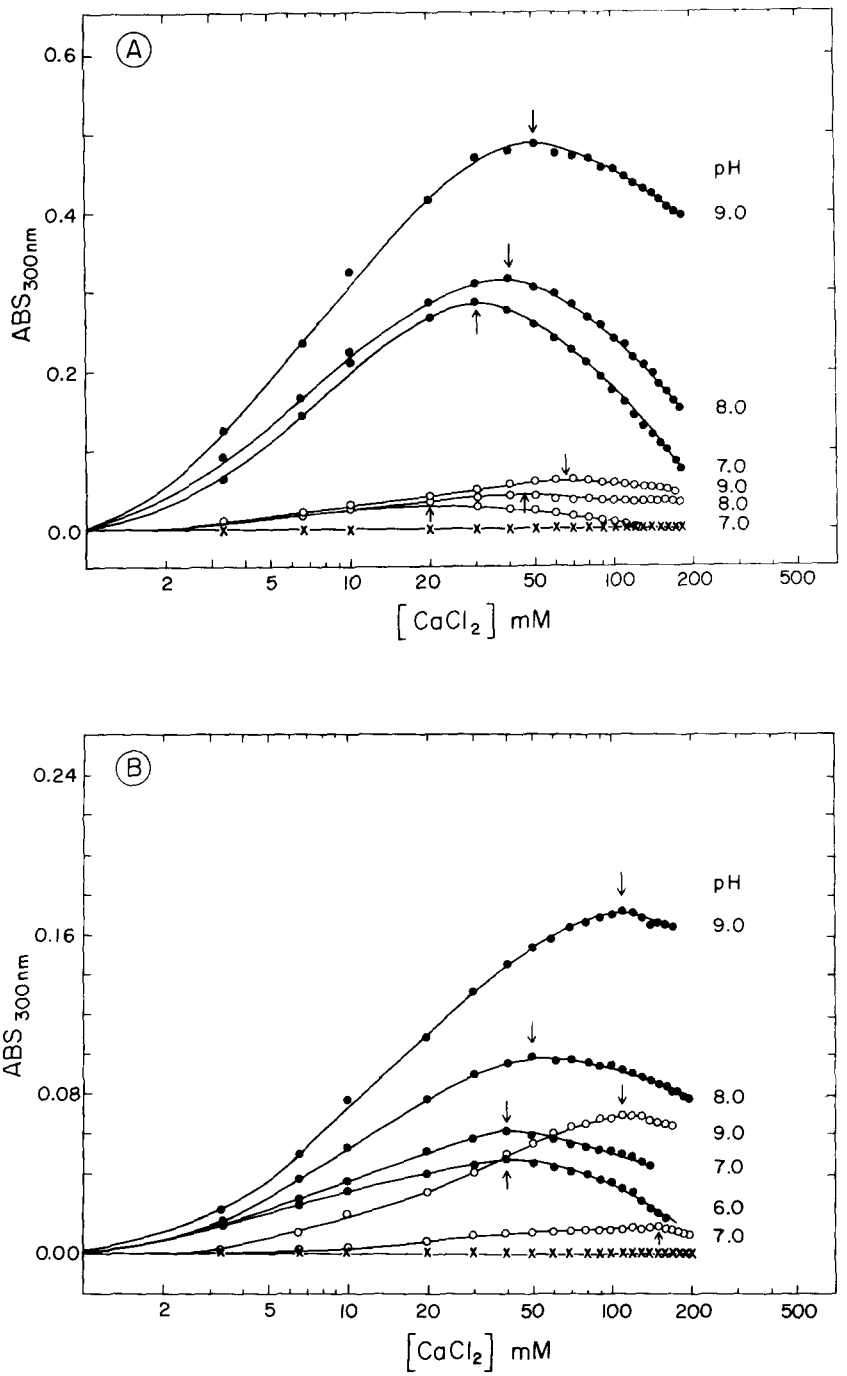

Fig. 2. Aggregation behavior (A_{300}) of isolated sarcolemmal, mitochondrial, and sarcoplasmic reticulum vesicles from (A) rat and (B) canine cardiac muscle at different pH values as a function of divalent (Ca^{2+} or Mg^{2+}) cation concentration. (A) Rat preparations: Upper set of curves (●), isolated sarcolemmal vesicles; middle set of curves (○), sarcoplasmic reticulum enriched fraction vesicles; lowest set of curves (×), mitochondrial enriched fraction vesicles (zero at all pH values). (B) Canine preparations: Upper set of curves (●), isolated sarcolemmal vesicles + Ca^{2+}; middle set of curves (○), isolated sarcolemmal vesicles + Mg^{2+}; lowest set of curves (×), mitochondrial-enriched fraction vesicles (zero at all pH values). Note: sarcoplasmic reticulum enriched fraction vesicles deleted for clarity. Buffers: 100 mM NaCl/5.0 mM (Mes pH 6.0–6.5; Hepes pH 7.0, 8.0; Ches, pH 9.0)/0.05 mM Na_2-EDTA. Temperature 25 °C. Protein concentration all samples, 60 μg/ml. Arrows indicate A_{300} maximum values. Curves are averages of $n > 3$ experiments each curve.

aggregation, discussed below.) The results indicate that the degree of aggregation (A_{300} maximum value) increased; as expected, in both the canine and rat (sarcolemmal vesicle preparations as the suspension pH was raised. However, in both cases, the aggregation curves were shifted to higher, not lower, [Ca^{2+}] with increasing pH (even as the magnitude of aggregation was increasing). This is the opposite result of that expected given the PC/PE/PS SUV results shown in Fig. 1. Control experiments with canine sarcolemmal vesicles at twice the protein concentration (120 μg/ml) gave identical results, except that the absolute A_{300} values were larger. Other control experiments (not shown) using mixed populations of PC/PE/PS SUVs having different mole percentages of PS indicate that these results could not be explained on the basis of a mixed population of right-side out and inside out sarcolemmal vesicles having different surface charge densities.

The results shown in Figs. 2A and B also indicate that the extent of these shifts in A_{300} maximum values were species dependent, with canine sarcolemmal vesicles being much more sensitive to pH changes than the rat sarcolemmal vesicles. For canine sarcolemmal vesicles, the [cation] corresponding to the the A_{300} maximum values obtained shifted from 40 mM Ca^{2+} (pH 6.0 and pH 7.0), to 50 mM Ca^{2+} (pH 8.0), to 110–120 mM Ca^{2+} (pH 9.0). In contrast the rat sarcolemmal vesicle values were: 30 mM Ca^{2+} (pH 7.0), 40 mM Ca^{2+} (pH 8.0), and 50 mM Ca^{2+} (pH 9.0).

Effects of pH on Mg^{2+}-induced sarcolemmal vesicle aggregation

The results described above indicate that Ca^{2+} binds to the sarcolemmal surface. To help evaluate the specificity of this interaction, the aggregation behavior of canine sarcolemmal vesicles was examined as a function of [Mg^{2+}] for comparison. The aggregation experiments were conducted at pH 7.0 (Hepes) and pH 9.0 (Ches) under conditions identical to those employed in the Ca^{2+} studies. The results are also included in Fig. 2B.

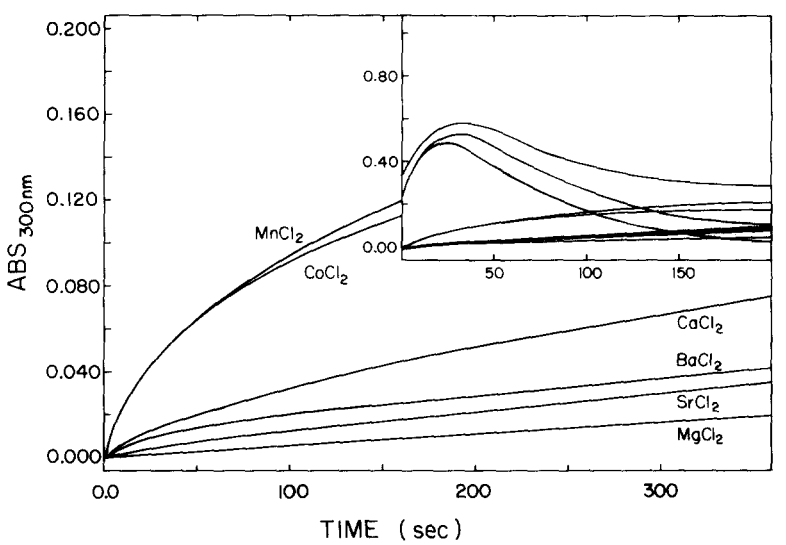

Fig. 3. Aggregation kinetics of isolated rat sarcolemmal vesicles for various di- and trivalent cations under identical experimental conditions. Buffer: 100 mM NaCl/5.0 mM Hepes/0.05 mM Na_2-EDTA (pH 7.0). Temp. 25 °C. Protein concentration all samples, 60 μg/ml. An aliquot of the respective di- or trivalent cation salt was injected into the vesicle suspension to give a final test cation concentration of 10 mM. Changes in turbidity (A_{300}) were then monitored as a function of time. Since these experiments are conducted under identical conditions at a constant protein concentration the differences observed for these curves correspond to differences in cation binding to the sarcolemmal vesicle surface. The main portion of the figure (expanded y scale): the aggregation curves obtained for Mn^{2+}, Co^{2+}, Ca^{2+}, Ba^{2+}, Sr^{2+} and Mg^{2+}. Inset (full scale): aggregation curves obtained for all cations, including (from top) La^{3+}, Cd^{2+}, and Zn^{2+}. The overall selectivity series for cation binding is: $La^{3+} \geq Cd^{2+} = Zn^{2+} \ggg Mn^{2+} \geq Co^{2+} \gg Ca^{2+} > Ba^{2+} = Sr^{2+} > Mg^{2+}$. Curves are averages of three separate runs. The selectivity series is based on four such triplicate runs.

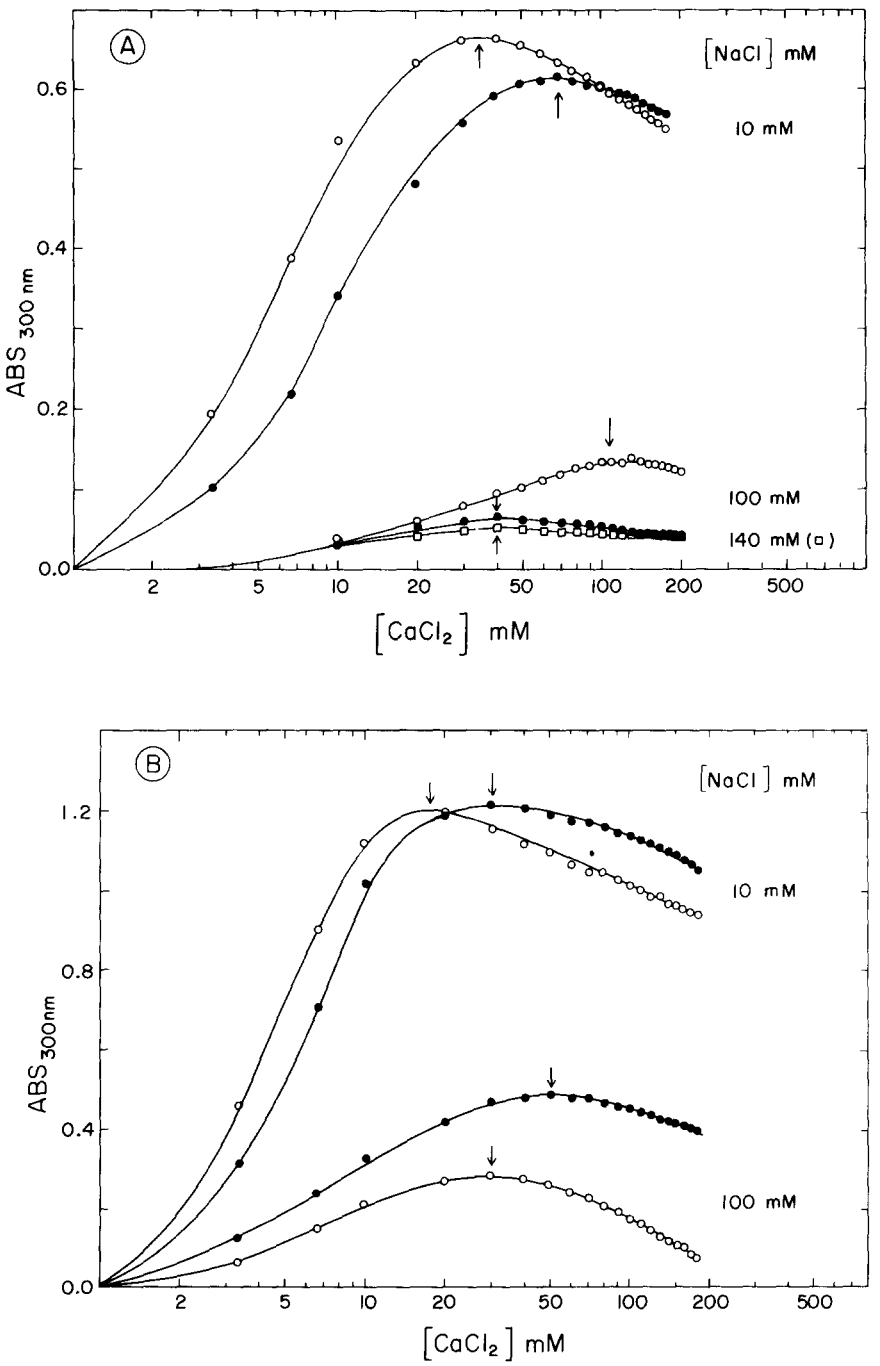

Fig. 4. Effects of ionic strength on the Ca^{2+}-induced aggregation behavior of (A) canine and (B) rat cardiac sarcolemmal vesicles. (A) Canine sarcolemmal vesicles: Upper set of curves: ○, aggregation in pH 7.0 buffer (10 mM NaCl); ●, aggregation in pH 9.0 buffer (10 mM NaCl). Lower set of curves: ●, aggregation in pH 7.0 buffer (100 mM NaCl); ○, aggregation in pH 9.0 buffer (100 mM NaCl); □, aggregation in pH 7.0 buffer (140 mM NaCl). (B) Rat sarcolemmal vesicles: Upper set of curves: ○, aggregation in pH 7.0 buffer (10 mM NaCl); ●, aggregation in pH 9.0 buffer (10 mM NaCl). Lower set of curves: ○, aggregation in pH 7.0 buffer (100 mM NaCl); ●, aggregation in pH 9.0 buffer (100 mM NaCl). Buffers: 10, 100, or 140 mM NaCl; 5.0 mM Hepes (pH 7.0) or Ches (pH 9.0); 0.05 mM Na_2-EDTA; Temp. 25 °C. Protein concentration all samples, 60 μg/ml. Arrows indicate A_{300} maximum values. Curves are averages of $n \geq 3$ experiments per curve.

They indicate that, as was the case for Ca^{2+}, aggregation increased (A_{300} maximum value, ↑) as the suspension pH was raised from 7.0 to 9.0. The A_{300} values obtained for both pH values, however, were much smaller for Mg^{2+} at all concentrations, indicating that the ability of Mg^{2+} to induce sarcolemmal vesicle aggregation was significantly less than that for Ca^{2+} under the same conditions. Thus, in terms of cation binding, the sarcolemmal vesicle surface can be considered to be highly selective for Ca^{2+} over Mg^{2+} under these conditions. This selectivity is also apparent from the concentration of Mg^{2+} giving the A_{300} maximum values. (approx. 120 mM Mg^{2+} (pH 9.0) and 130–150 mM Mg^{2+} (pH 7.0)). Although the data suggests that the shifts in the aggregation curves as a function of pH may be less than those found for Ca^{2+}, the shallowness of the pH 7.0 aggregation curve makes it difficult to ascertain the A_{300} maximum value with any certainty.

Selectivity series for cation binding to sarcolemmal vesicles

To further expand upon the cation binding behavior of the sarcolemmal vesicle surface the kinetics of cation-induced sarcolemmal vesicle aggregation were measured for a series of di- and trivalent cations under the same experimental conditions (final concentration injected test cation = 3 or 10 mM). Fig. 3 illustrates the results obtained for rat sarcolemmal vesicle preparations. They indicate that the selectivity series for cation binding to the rat sarcolemmal vesicle surface is $La^{3+} \geq Cd^{2+} = Zn^{2+} \ggg Mn^{2+} \geq Co^{2+} \gg Ca^{2+} > Ba^{2+} = Sr^{2+} > Mg^{2+}$. Under the same conditions the selectivity series for cation binding to canine sarcolemmal vesicles is $Mn^{2+} > Ca^{2+} > Sr^{2+} \geq Mg^{2+}$ (not shown).

Effect of ionic strength on vesicle aggregation

In the fourth series of experiments the effects of ionic strength on the Ca^{2+}-induced aggregation behavior of canine and rat sarcolemmal vesicles were investigated by varying the monovalent cation concentration, [NaCl], of the vesicle suspension. In principle decreasing the monovalent cation concentration of the sarcolemmal vesicle suspension buffer should result in increased Ca^{2+} binding to the sarcolemmal vesicle surface. Experimentally the observed changes should be very similar to those obtained for the PC/PE/PS SUV surface charge density experiments above (i.e., the degree of aggregation (A_{300} max) should increase and the aggregation curves should be shifted to lower [Ca^{2+}] as the ionic strength is decreased) although in this case Ca^{2+} binding is being altered without concominant changes in surface charge density (assuming no Na^+ binding). These experiments therefore provide an alternative method for altering Ca^{2+} binding to the sarcolemmal vesicle surface. Figs. 4A and B illustrate the results obtained for canine and rat sarcolemmal vesicles, respectively, using 10, 100, and 140 mM NaCl containing buffers. With the exception of [NaCl] all conditions were identical to those used in the preceding Ca^{2+}/pH experiments. The results obtained with both the canine and rat sarcolemmal vesicle preparations indicate that reducing the monovalent cation concentration from 100 mM to 10 mM NaCl resulted in a marked increase in the extent of vesicle aggregation (A_{300} maximum, ↑) and a shift of the aggregation curves to lower [Ca^{2+}]. This was true whether the experiments were conducted at pH 7.0 or 9.0. With canine sarcolemmal vesicles the shifts in the [Ca^{2+}] corresponding to the A_{300} maximum values were: 40 mM Ca^{2+} (pH 7.0, 100 mM NaCl) → 35 mM Ca^{2+} (pH 7.0, 10 mM NaCl); and 110–120 mM Ca^{2+} (pH 9.0, 100 mM NaCl) → 70 mM Ca^{2+} (pH 9.0, 10 mM NaCl). For rat sarcolemmal vesicles the values were: 30 mM Ca^{2+} (pH 7.0, 100 mM NaCl) → 17 mM Ca^{2+} (pH 7.0, 10 mM NaCl); and 50 mM Ca^{2+} (pH 9.0, 100 mM NaCl) → 30 mM Ca^{2+} (pH 9.0, 10 mM NaCl). On the other hand increasing the monovalent cation concentration from 100 to 140 mM NaCl had no significant effect on either of these parameters (canine sarcolemmal vesicles at pH 7.0 shown). In control experiments (not shown) the results obtained using 10 mM NaCl → isoosmotic sucrose buffer were essentially the same as those obtained in 10 mM NaCl buffer, indicating that the observed changes were not due to variations in osmotic strength. These results match those predicted above from Fig. 1, and indicate that, in marked contrast to the previous Ca^{2+}/pH experiments, Ca^{2+}/ sarcolemmal vesicle binding increases as expected as a function of ionic strength changes at constant surface charge density.

The results shown in Figs. 4 A and B also allow one to further evaluate the effects of pH on the Ca^{2+}-induced aggregation behavior of canine and rat sarcolemmal vesicles. The results demonstrate that the species specific differences in aggregation behavior, observed as a function of pH in the 100 mM NaCl containing buffers, are retained in the 10 mM NaCl containing buffers, although they are not as pronounced. The results also indicate that the shifts in the aggregation curves to lower $[Ca^{2+}]$, observed as the ionic strength of the buffer was reduced, were smaller at pH 7.0 than 9.0. However, even though the A_{300} maximum values were quite similar in the low ionic strength case, the aggregation curves were still shifted to higher, not lower $[Ca^{2+}]$ as the pH was raised, indicating that increasing Ca^{2+} binding via reduced ionic strength can only partially overcome the effects of pH on the surface charge density of the sarcolemmal vesicles.

Effects of fluorescamine labelling of NH_2 groups on vesicle aggregation

In both the canine and rat sarcolemmal vesicle studies the aggregation curves were shifted to higher $[Ca^{2+}]$ with increasing pH, even as the extent of aggregation was increasing (Fig. 2). This was contrary to the results expected, based on model membrane systems having a single type of charged molecule [38] (Fig. 1), and studies of vesicles derived from human and rabbit erythrocytes [40]. However, these results are consistent with the proposal by Langer [5,11] that two types of charged groups are involved in the regulation of Ca^{2+} binding to the sarcolemmal vesicle surface, one of which binds Ca^{2+} and the second which modifies this Ca^{2+} binding as a function of pH, but does not itself bind Ca^{2+}. In this model the Ca^{2+} binding sites are acidic phospholipids in the sarcolemmal membrane. Access to these binding sites is controlled by zwitterionic phospholipid NH_2 groups (i.e. PE) which go from $-NH_3^+$ to $NH_2 + H^+$ as the pH is increased.

If this hypothesis is correct, then removing the NH_2 groups from the sarcolemmal vesicle surface should eliminate the pH dependency of this influence (i.e., the pH related shift in the sarcolemmal vesicles aggregation curves to higher $[Ca^{2+}]$ should be essentially removed). To examine this possibility isolated canine and rat sarcolemmal vesicles were labelled with fluorescamine as described in Methods. Fluorescamine treatment functionally removed the NH_2 groups by incorporating them into a ringed (and now fluorescent) structure. Control preparations were carried through the same labelling procedure, but in the absence of fluorescamine. The Ca^{2+}-induced aggregation behavior of the labelled and control sarcolemmal vesicle preparations were then examined at pH 7.0 and pH 9.0 under standard conditions. Fig. 5 illustrates the results obtained for canine sarcolemmal vesicle preparations under these conditions. At pH 9.0 there was no observed difference in the aggregation behavior of fluorescamine labelled and control sarcolemmal vesicles. In contrast, labelling the sarcolemmal vesicles with fluorescamine resulted in a dramatic shift in the aggregation curves at pH 7.0 (A_{300} max control = 40 mM $Ca^{2+} \rightarrow A_{300}$ max + fluorescamine = 100 mM Ca^{2+}). In addition the $[Ca^{2+}]$ giving the A_{300} max values obtained for the fluorescamine labelled sarcolemmal vesicles at pH 7.0 were the same as those obtained for labelled sarcolemmal vesicles at pH 9.0, indicating that removing sarcolemmal surface NH_2 groups did indeed eliminate the pH dependence of the sarcolemmal vesicle aggregation curves. Similar results were obtained for the fluorescamine labelled rat sarcolemmal vesicle preparations (not shown). The observation that fluorescamine labelling shifted all of the sarcolemmal vesicle aggregation curves to the higher $[Ca^{2+}]$ also suggests that the molecules labelled with fluorescamine could still be affecting the access of Ca^{2+} to its primary binding site (assuming Langer's proposal [5,11]). This influence however would no longer be pH dependent.

Effects of monovalent cations on vesicle aggregation

To determine if monovalent cation binding to the sarcolemmal vesicle surface was significant the aggregation behavior of canine and rat sarcolemmal vesicles were evaluated as a function of [NaCl] or [KCl]. The aggregation experiments were conducted using the same initial conditions as the divalent cation experiments. Aliquots of a concentrated monovalent cation solution (NaCl or

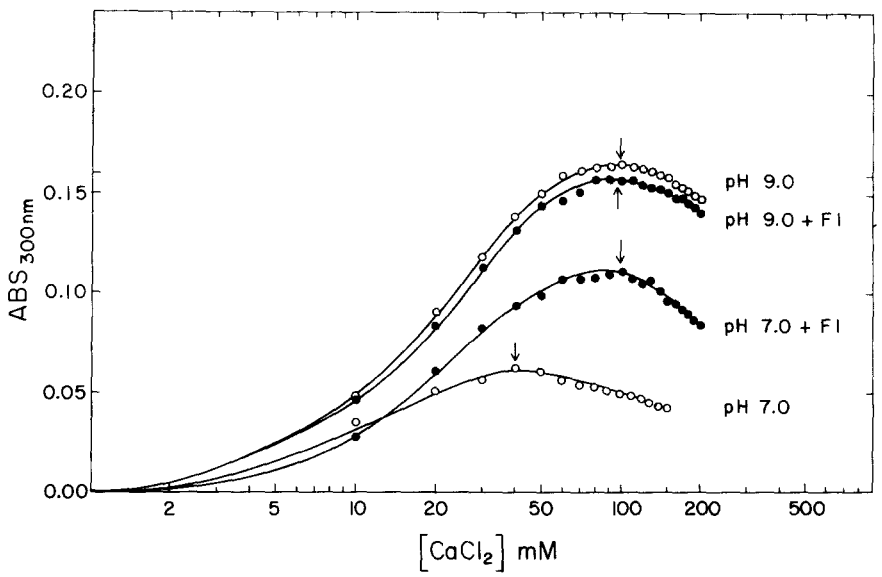

Fig. 5. Effect of fluorescamine labelling of surface NH_2 groups on the pH dependence of Ca^{2+}-induced vesicle aggregation (A_{300}) of isolated canine cardiac sarcolemmal vesicles. ○, control curve – pH 9.0 buffer, no fluorescamine; ●, test curve – pH 9.0 buffer, sarcolemmal vesicles labelled with fluorescamine; ○, control curve – pH 7.0 buffer, no fluorescamine; ●, test curve – pH 7.0 buffer, sarcolemmal vesicles labelled with fluorescamine. Fluorescamine labelling conducted at pH 8.0, 2 mg/ml sarcolemmal vesicles protein, room temperature. Preparations then centrifuged to remove unreacted fluorescamine. Buffer: 100 mM NaCl; 5.0 mM Hepes (pH 7.0) or Ches (pH 9.0); 0.05 mM Na_2-EDTA. Temp. 25° C. Protein concentration only approximate (about 60 μg/ml). Arrows indicate A_{300} maximum values. (Equivalent results were obtained from rat cardiac sarcolemmal vesicles preparations (not shown).) Curves are averages of $n \geq 3$ experiments per curve.

KCl) were then injected using the same protocol as that employed in the A_{300} vs. $[Ca^{2+}]$ experiments. Na^+ and K^+ were chosen for these experiments because: (1) they are the major physiologically important monovalent cations present in cardiac tissue, and (2) they are known to induce the aggregation of synthetic PS or phosphatidic acid (PA) containing liposomes (SUVs and LUVs) [39]. The results obtained from such experiments (not shown) demonstrated that neither Na^+ nor K^+ could induce the aggregation of either the canine or rat sarcolemmal vesicles at any of the pH values tested, even at a 1.0 M final monovalent cation concentration. Thus monovalent cation binding to the sarcolemmal vesicle surface is not significant, relative to that for divalent cations.

Effects of protons (H^+) on vesicle aggregation

In a related series of experiments the effects of protons (H^+) on canine and rat sarcolemmal vesicle aggregation were also investigated. The experiments were conducted by suspending the sarcolemmal vesicles in either pH 8.0 (Hepes) or pH 9.0 (Ches) 'standard buffers' under identical conditions and then increasing the $[H^+]$ in a stepwise fashion using HCl. In contrast to the results obtained in the Na^+ and K^+ experiments, protons (H^+) were found to have a pronounced effect on both canine and rat sarcolemmal vesicle aggregation. For rat sarcolemmal vesicles the pH values corresponding to the midpoints of the sarcolemmal vesicle aggregation curves were found to be essentially the same in both buffers (pH 6.0 in Ches buffer and 6.2 in Hepes buffer), indicating that the sarcolemmal vesicles were aggregating in direct response to the pH of the suspension, and not charged forms of the buffer molecules themselves. Under identical conditions canine sarcolemmal vesicles displayed the same massive aggregation behavior, but at pH 4.8 in Ches buffer and 5.0 in Hepes buffer, indicating significant differences in the sarcolemmal vesicle surface characteristics of these two species. Moreover, labelling the sarcolemmal vesicle NH_2 groups with

fluorescamine had no effect on the pH induced aggregation behavior of the sarcolemmal vesicles, indicating that NH_2 groups were not involved.

Potential roles of sarcolemmal vesicles lipids in vesicle aggregation

To ascertain the potential roles of the sarcolemmal lipids in the interactions, the aggregation behavior of SUVs made from the total lipid extracts of both canine and rat sarcolemmal vesicles were examined, and compared to the results obtained for synthetic PC/PE/PS SUVs under the same conditions.

To provide a reference point for the extracted sarcolemmal vesicle lipid studies the effects of pH on the Ca^{2+}-induced aggregation behavior of synthetic PC/PE/PS SUVs were evaluated. The experiments were conducted using PC/PE/PS (0 : 0 : 100 mole%) and PC/PE/PS (40 : 40 : 20 mol%) SUVs, and carried out under conditions identical to those employed in the Ca^{2+}/pH studies of the sarcolemmal vesicles (Fig. 2). The results indicate that buffer pH had no effect on the Ca^{2+}-induced aggregation of either 100% PS SUVs (pH values 6.0, 7.0, 8.0) or PC/PE/PS (40 : 40 : 20) SUVs (pH values 6.0, 7.0, 8.0, 9.0) under these conditions. Reducing the vesicle concentration (PC/PE/PS (40 : 40 : 20) pH 7.0 samples) decreased the absolute magnitude of the A_{300} values obtained, but had no other effect on vesicle aggregation, indicating that the results obtained were not a coincidence of the vesicle concentrations used.

Given this reference point the effects of pH on the Ca^{2+}-induced aggregation behavior of the SUVs generated from the canine and rat sarcolemmal vesicle lipid extracts were examined. All experiments were conducted using 'standard buffer' conditions and at phospholipid concentrations similar to those employed in the synthetic

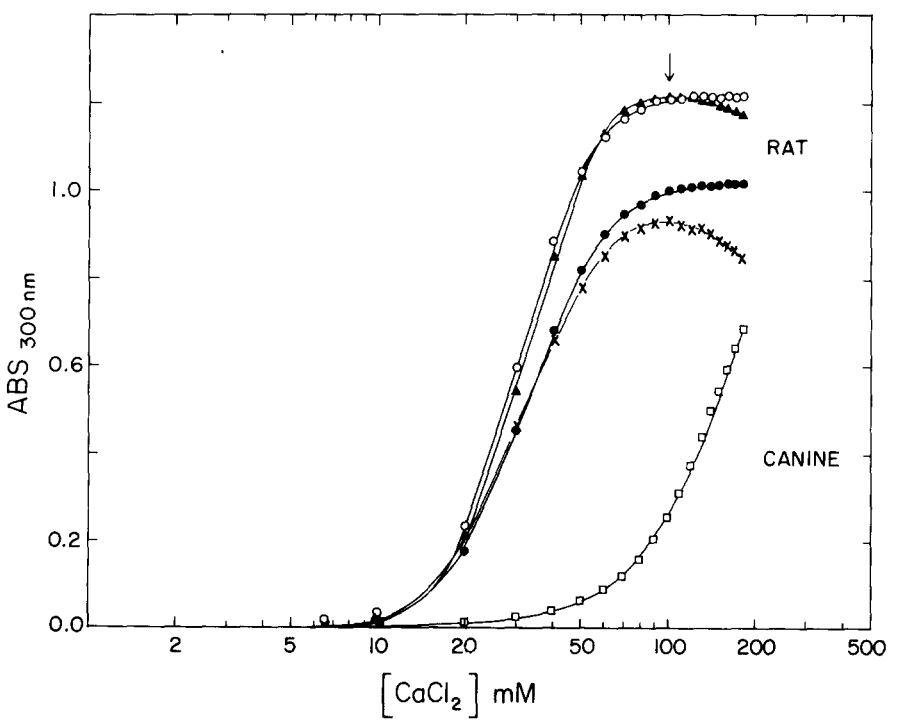

Fig. 6. Effects of pH on the Ca^{2+}-induced aggregation behavior of SUVs generated from canine and rat sarcolemmal vesicle lipid extracts. Upper set of curves: ▲, SUVs (rat) in pH 7.0 buffer (0.18 μmol PL/ml): ○, SUVs (rat) in pH 9.0 buffer (0.18 μmol PL/ml). Middle set of curves: ●, SUVs (rat) in pH 8.0 buffer (0.09 μmol PL/ml): ×, SUVs (rat) labelled with fluorescamine in pH 8.0 buffer (0.09 μmol PL/ml). Lower curve: □, SUVs (canine) in pH values 7.0, 8.0, and 9.0 buffer (0.07 μmol PL/ml). Buffers: 100 mM NaCl; 5.0 mM Mes (pH 6.0), Hepes (pH 7.0, 8.0), or Ches (pH 9.0); 0.05 mM Na_2-EDTA. Temp. 25 °C. Arrows indicate A_{300} maximum values (rat). Curves are averages of $n \geq 3$ experiments per curve.

PC/PE/PS SUVs experiments. Fig. 6 illustrates the results obtained for both canine and rat sarcolemmal vesicle derived lipid SUVs. The data show that Ca^{2+} induced the aggregation of the SUVs generated from both rat and canine sarcolemmal vesicle lipid extracts, indicating a significant level of Ca^{2+} binding to both membranes. In addition the aggregation behavior of the rat sarcolemmal vesicle lipid SUVs are quite different from that of the canine sarcolemmal vesicle lipid SUVs, even though both types of sarcolemmal vesicles were extracted using the same procedure. Thus, the species specific differences in Ca^{2+}–sarcolemmal interactions observed in the isolated canine and rat sarcolemmal vesicles seem to be retained in their lipid extracts. However, in contrast to the isolated sarcolemmal vesicle studies, neither the rat nor canine sarcolemmal vesicle lipid extract SUVs displayed any effect of pH on Ca^{2+}-induced vesicle aggregation. Instead, their behavior was the same as that found for the synthetic PC/PE/PS SUVs above. As in the synthetic PC/PE/PS SUV experiments, reducing the vesicle concentration did not alter these results (Fig. 6).

To determine if phospholipid NH_2 groups played any role in the Ca^{2+}-induced aggregation of the lipid extract vesicles, rat sarcolemmal vesicles lipid SUVs were labelled with fluorescamine as described in Methods, and then examined as above. The results, also shown in Fig. 6, indicate that fluorescamine labelling of the SUV PE groups (confirmed by TLC) had no effect on Ca^{2+}-induced vesicle aggregation.

The results obtained in the isolated sarcolemmal vesicle studies also indicated that H^+ could induce a massive aggregation of both canine and rat sarcolemmal vesicles, and that this aggregation behavior was species dependent. To ascertain if the sarcolemmal vesicle lipids played a role in this process rat and canine sarcolemmal vesicle lipid extract SUVs were examined using the same protocol employed in the A_{300} vs. [HCl] experiments. The results indicate that it required much larger [HCl] to induce the aggregation of the lipid extract SUVs than the corresponding isolated sarcolemmal vesicles. Although an A_{300} maximum value was not found over the [HCl] range tested, if one takes the 50% value of the largest A_{300} value obtained as the mid-point for determining the highest pH at which the lipid SUVs aggregate, one obtains a pH value of 2.7 for both the rat and canine preparations. This is markedly different from the values obtained for the rat sarcolemmal vesicles (pH 6.0–6.2) and canine sarcolemmal vesicles (pH 4.8–5.0), but very similar to those previously reported for the H^+-induced aggregation of PS SUVs (pH 2.6) [38]. Together these results suggest that the species dependent H^+-induced aggregation of the isolated canine and rat sarcolemmal vesicles is not due to the membrane lipids.

Discussion

While previous physiological studies have demonstrated a close correlation between sarcolemmal associated Ca^{2+} and cardiac cell contractility [5,11–27] they have not been able to determine if Ca^{2+} was actually binding (adsorbing) to the sarcolemmal surface under these conditions. Thus the first aim of this study was to ascertain whether cations do bind to the sarcolemmal surface, or are present only in the diffuse double layer. As indicated by the results obtained (Figs. 2–4) there is a significant degree of binding by a number of cations, including Ca^{2+}, to the sarcolemmal surface. Moreover, the results of this study also show that cation binding to the sarcolemmal vesicle surface is quite selective among the different divalent cations (and La^{3+}) examined. In contrast, sarcolemmal vesicles could not be induced to aggregate at all with monovalent cations (except H^+) over the concentration range tested (up to 1.0 M), indicating very weak, if any, monovalent cation binding to the sarcolemmal vesicle surface. The sarcolemmal vesicle aggregation kinetics experiments indicate that for the di- and trivalent cations evaluated the selectivity series for cation binding to the sarcolemmal vesicle surface is the same for both rat and canine sarcolemmal vesicles under the conditions employed ($La^{3+} \geq Cd^{2+} = Zn^{2+} \ggg Mn^{2+} \geq Co^{2+} \gg Ca^{2+} > Ba^{2+} = Sr^{2+} > Mg^{2+}$). The relative relationships between the various curves were, however, different for the two species, with Ca^{2+} and Mn^{2+} being more similar to each other in the canine sarcolemmal vesicle preparations. The A vs. [cation] experiments for

Ca^{2+} and Mg^{2+} also display this cation binding selectivity (Fig. 2) indicating that the selectivity is maintained over the pH range of 7.0 to 9.0 and that a significant difference exists in the interactions of these two cations with the sarcolemmal membrane.

The selectivity series obtained in these experiments is different from those previously obtained in vesicle aggregation experiments for SUVs made from PS or various PC/PS combinations [38], or in microelectrophoresis experiments for multilamellar vesicles (MLVs) generated from PS, PC, PG or PC/PS combinations [59–61]. However, this selectivity series is very similar to that obtained for the displacement of $^{45}Ca^{2+}$ in canine cardiac sarcolemmal vesicle experiments ($La^{3+} \geq Mn^{2+} > Sr^{2+} \geq Ba^{2+} > Mg^{2+}$) [20] and those obtained for $^{45}Ca^{2+}$ displacement from 'gas-dissected' sarcolemmal membranes of neonatal rat heart cells ($La^{3+} > Cd^{2+} > Mn^{2+} > Mg^{2+}$) [25]. Moreover this series is the same as that found to progressively uncouple excitation from contraction in neonatal rat papillary muscle ($La^{3+} > Cd^{2+} > Mn^{2+} > Mg^{2+}$) [12,25].

Having established that cations bind to the sarcolemmal vesicle surface the second section of this study was concerned with describing some of the general characteristics of this binding in both rat and canine sarcolemmal vesicle preparations. From the results obtained with the isolated sarcolemmal vesicles under various environmental conditions three properties of the sarcolemmal vesicles stand out. The first, apparent from the observed shifts in the A vs. $[Ca^{2+}]$ aggregation curves obtained at different pH values, is that Ca^{2+} binding to the sarcolemmal vesicle surface cannot be explained by a model involving a simple pH induced increase in the surface charge density of a membrane containing a single type of charged molecule (i.e., phospholipid like PS). Instead the results suggest that two (or more) interacting sites are involved in the Ca^{2+}-induced aggregation of isolated sarcolemmal vesicles, one of which binds Ca^{2+} and the second which modifies this Ca^{2+} binding as a function of pH, but does not itself bind Ca^{2+}. This 'working hypothesis' is essentially the same as that proposed by Langer [5,11] to explain the relationship between $^{45}Ca^{2+}$ 'binding' and contractility observed with cultured myocytes, and is also similar in many respects to the surface charge regulation model discussed by Sculley [62]. In such a model Ca^{2+} binding to the primary (binding) sites would increase as the pH was raised (A_{300} ↑), but not as quickly as the net surface charge density of sites inaccessible to Ca^{2+} (secondary regulatory sites) on the sarcolemmal vesicle surface. This inaccessibility would in turn increase the electrostatic repulsion between vesicle surfaces and shift the sarcolemmal vesicle aggregation curves to higher $[Ca^{2+}]$. In a similar fashion reducing the ionic strength of the vesicle suspension medium at constant pH would result in increased Ca^{2+} binding to the primary sites, but without a concomitant change in the secondary site component. Under these conditions the A vs. $[Ca^{2+}]$ aggregation curves would increase in height and be shifted to lower $[Ca^{2+}]$. These are the results actually obtained (Figs. 4A, B). This working hypothesis is also supported by the results obtained from the fluorescamine experiments which show that the pH dependence (but not Ca^{2+} binding) is eliminated by covalently labelling the sarcolemmal amino (NH_2) groups with fluorescamine. However, we cannot ascertain at this time whether the results of such labelling indicates that particular sarcolemmal membrane components containing NH_2 groups are themselves the secondary (regulatory) sites and are exerting their influence directly on the degree of accessibility of the primary Ca^{2+} binding sites, or are acting indirectly by blocking the regulatory affect of a second independent site which is inaccessible to Ca^{2+}.

The second major feature of the sarcolemmal vesicles is that SUVs made from the lipid extracts of rat of canine sarcolemmal vesicle preparations demonstrate a significant degree of Ca^{2+} binding to the membrane surface. If one assumes, for illustrative purposes, that these SUVs were equivalent to the PC/PE/PS mixtures used to generate the SUVs examined in Fig. 1, the rat lipid extract SUVs would contain approx. 15 mole% PS and the canine lipid extract SUVs approx. 10 mol% PS. These values are similar to those reported for the (PS + PI) contents of isolated cardiac sarcolemmal vesicle preparations (6–13%) [57,58,63]. Given the working hypothesis above, these results thus suggest that specific lipid components (acidic phos-

pholipids in Langer's model [5,11]) could function as the primary Ca^{2+} binding sites on the sarcolemmal membrane surface.

In contrast to these results, the pH dependence of Ca^{2+}-induced vesicle aggregation has disappeared in the SUVs as have the effects of fluorescamine labelling, and H^+-induced vesicle aggregation. Instead the properties of the lipid extract SUVs are very similar to those obtained for the PC/PE/PS SUV model membrane systems. Thus, the physiologically important modulating effects of pH on cation binding, contractility, $^{45}Ca^{2+}$-sarcolemmal association, etc. correlate much better with the results obtained in this study for the 'complete' isolated sarcolemmal vesicles (i.e., sarcolemmal vesicles containing protein, lipid, and carbohydrate components) than the lipid extract SUVs. This suggests that the molecular interactions which give rise to these modifications of Ca^{2+} binding to the sarcolemmal vesicle surface (involving the second regulatory sites) may either be a property of a protein or carbohydrate component of the membrane, or due to a specific two-dimensional organization of the sarcolemmal membrane lipids which is lost during the extraction and SUV formation procedure. In either case our results argue against a simple role for the NH_2 groups on PE as the regulatory (secondary) sites on the sarcolemmal vesicle surface, as proposed by Langer [5,11].

The third significant property of the sarcolemmal vesicles is that the aggregation behavior of the canine derived preparations is different from those obtained for the rat derived preparations under all conditions tested. These differences were first noted in the effects of pH in the Ca^{2+}-induced sarcolemmal vesicle aggregation experiments, and were maintained in both the H^+-induced sarcolemmal vesicle aggregation and lipid extract SUV experiments. Thus, while this study shows that both species share many general features in the interactions of cations with their cardiac sarcolemmal surfaces, it also indicates the presence of consistent species specific differences in the physico-chemical properties of their sarcolemmal membranes. Consequently, both the differences in the physico-chemical properties of the sarcolemmal membrane, as well as the relative roles of the sarcolemmal vs. sarcoplasmic reticulum, must be included in evaluating Ca^{2+} cardiac cell interactions in different species.

In closing, this study represents a first step in the systematic examination of the interactions of cations with the cardiac sarcolemmal membrane. Given the reuslts obtained we can now begin to selectively dismantle the membrane in order to identify the components involved, and quantitatively analyze their interactions.

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References

1 Tsien, R.W. (1983) Annu. Rev. Physiol. 45, 341–358.
2 Lee, K.S. and Tsien, R.W. (1984) J. Physiol. 354, 253–272.
3 Schilling, W.P. and Lindenmeyer, G.E. (1984) J. Membr. Biol. 79, 163–173.
4 Bean, B.P. (1985) J. Gen. Physiol. 86, 1–30.
5 Langer, G.A. (1985) Circ. Res. 57, 374–382.
6 Philipson, K.D. (1985) Annu. Rev. Physiol. 47, 561–571.
7 Hilgemann, D.W. (1986) J. Gen. Physiol. 87, 675–706.
8 Hilgemann, D.W. (1986) J. Gen. Physiol. 87, 707–735.
9 Hess, P., Lansman, J.B. and Tsien, R.W. (1986) J. Gen. Physiol. 88, 293–319.
10 Lansman, J.B., Hess, P. and Tsien, R.W. (1986) J. Gen. Physiol. 88, 321–347.
11 Langer, G.A. (1987) Can. J. Physiol. Pharmacol. 65, 627–631.
12 Langer, G.A., Serena, S.D. and Nudd, L.M. (1974) J. Mol. Cell. Cardiol. 6, 149–161.
13 Tillisch, J.H. and Langer, G.A. (1974) Circ. Res. 34, 40–50.
14 Rich, T.L. and Langer, G.A. (1975) J. Mol. Cell. Cardiol. 7, 747–765.
15 Limas, C.J. (1977) Arch. Biochem. Biophys. 179, 302–309.
16 Bers, D.M. and Langer, G.A. (1979) Am. J. Physiol. 237, H332–H341.
17 Horackova, M. and Vassort, G. (1979) J. Gen. Physiol. 73, 403–424.
18 Philipson, K.D. and Langer, G.A. (1979) J. Mol. Cell. Cardiol. 11, 857–875.

19 Tillisch, J.H., Fung, L.K., Hom, P.M. and Langer, G.A. (1979) J. Mol. Cell. Cardiol. 11, 137–148.
20 Pang, D.C. (1980) Biochim. Biophys. Acta 598, 528–542.
21 Philipson, K.D., Bers, D.M. and Nishimoto, A.Y. (1980) Am. J. Physiol. 238, H373–H378.
22 Philipson, K.D., Bers, D.M. and Nishimoto, A.Y. (1980) J. Mol. Cell. Cardiol. 12, 1159–1173.
23 Bers, D.M., Philipson, K.D. and Langer, G.A. (1981) Am. J. Physiol. 240, H576–H583.
24 Burt, J.M., Duenas, C.J. and Langer, G.A. (1983) Circ. Res. 53, 679–687.
25 Langer, G.A. and Nudd, L.M. (1983) Circ. Res. 56, 146–149.
26 Frankis, M.B. and Lindenmeyer, G.E. (1984) Circ. Res. 55, 676–688.
27 Philipson, K.D., Langer, G.A. and Rich, T.L. (1985) Am. J. Physiol. 248, H147–H150.
28 Langer, G.A., Frank, J.S. and Philipson, K.D. (1982) Pharmac. Ther. 16, 331–376.
29 Philipson, K.D., Frank, J.S. and Nishimoto, A. (1983) J. Biol. Chem. 258, 5905–5910.
30 Philipson, K.D. (1984) J. Biol. Chem. 259, 13999–14002.
31 Philipson, K.D. and Nishimoto, A. (1984) J. Biol. Chem. 259, 16–19.
32 Philipson, K.D. and Ward, R. (1985) J. Biol. Chem. 260, 9666–9671.
33 Soldati, L., Longoni, S. and Carafoli, E. (1985) J. Biol. Chem. 260, 13321–13327.
34 Coronado, R. and Affolter, H. (1986) J. Gen. Physiol. 87, 933–953.
35 Ehrlich, B.E., Schen, C.R., Garcia, M.L. and Kaczorowski, G.J. (1986) Proc. Natl. Acad. Sci. USA 83, 193–197.
36 Verwey, E.J.W. and Overbeek, J.T. (1948) Theory of Stability of Hydrophobic Colloids. Elsevier/North Holland, Amsterdam.
37 Bentz, J. and Nir, S. (1981) Proc. Natl. Acad. Sci. USA 78, 1634–1637.
38 Ohki, S., Düzgünes, N. and Leonards, K.S. (1982) Biochemistry 21, 2127–2133.
39 Ohki, S., Roy, S., Ohshima, H. and Leonards, K.S. (1984) Biochemistry 23, 6126–6132.
40 Leonards, K.S. and Ohki, S. (1984) Biochemistry 23, 2718–2725.
41 Ohki, S. and Leonards, K.S. (1984) Biochemistry 23, 5578–5581.
42 Leonards, K.S. (1986) Biophys. J. 49, 510a.
43 Frank, J.S., Philipson, K.D. and Beydler, S. (1984) Circ. Res. 54, 414–423.
44 Forgac, M.D. (1980) J. Biol. Chem. 255, 1547–1553.
45 Luciani, S. (1984) Biochim. Biophys. Acta 772, 127–134.
46 Philipson, K.D. and Nishimoto, A.Y. (1982) J. Biol. Chem. 257, 5111–5117.
47 Philipson, K.D. and Nishimoto, A.Y. (1982) Am. J. Physiol. 243, C191–C195.
48 Madden, T.D., Chapman, D. and Quinn, P.J. (1979) Nature (London) 279, 538–541.
49 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
50 Wang, C.S. and Smith, R.L. (1975) Analyt. Biochem. 63, 414–417.
51 Bartlett, G.R. (1959) J. Biol. Chem. 234, 446–468.
52 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
53 Bentz, J., Düğünes, N. and Nir, S. (1985) Biochemistry 24, 1064–1072.
54 Hidalgo, C. and Ikemoto, N. (1977) J. Biol. Chem. 252, 8446–8454.
55 Hidalgo, C., Petrucci, D.A. and Vergara, C. (1982) J. Biol. Chem. 257, 208–216.
56 Weglecki, W.B., Owens, K., Kennett, F.F., Kessner, A., Harris, L., Wise, R.M. and Vahouny, G.V. (1980) J. Biol. Chem. 255, 3605–3609.
57 Tibbits, G.F., Sasaki, M., Ikeda, M., Shimada, K., Tsuruhara, T. and Nagatomo, T. (1981) J. Mol. Cell. Cardiol. 13, 1051–1061.
58 Gross, R.W. (1984) Biochemistry 23, 158–165.
59 McLaughlin, A., Grathwohl, C. and McLaughlin, S. (1978) Biochim. Biophys. Acta 513, 338–357.
60 Lau, A., McLaughlin, A. and McLaughlin, S. (1981) Biochim. Biophys. Acta 645, 279–292.
61 McLaughlin, S., Malrine, N., Gresalf, T. Vaio, G. and McLaughlin, A. (1981) J. Gen. Physiol. 77, 445–473.
62 Sculley, M.J. (1986) Biophys. Chem. 24, 33–39.
63 Dhalla, N.S. and Pierce, G.N. (1984) in Methods in Studying Cardiac Membranes (Dhalla, N.S., ed.), Vol. I, pp. 3–18, CRC Press, Boca Raton, FL.
64 McLaughlin, S. (1977) Curr. Top. Membr. Transp. 8, 71–114.
65 Ohki, S. and Sauve, R. (1978) Biochim. Biophys. Acta 511, 377–387.