Contribution of myocardial diffuse double-layer calcium to contractile function

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FINTEL, MARION, GLENN A. LANGER, JOHN C. ROHLOFF, AND MICHAEL E. JUNG. *Contribution of myocardial diffuse double-layer calcium to contractile function.* Am. J. Physiol. 249 (Heart Circ. Physiol. 18): H989–H994, 1985.—The role of diffuse double-layer calcium in cardiac excitation-contraction coupling was examined using rabbit interventricular septa, cultured neonatal rat myocardial cells, and gas-dissected sarcolemmal membranes. The diffuse double layer refers to the space directly adjacent to the sarcolemma where the ionic composition of the media is a direct function of the membrane surface potential. The divalent cation dimethonium was used as a specific probe for the diffuse double layer. According to Gouy-Chapman theory, replacement of sodium with sucrose should increase the amount of calcium located in this compartment. Dimethonium (10 mM) was found to decrease calcium uptake and contractility during low-sodium (33 mM) perfusion when the perfusate contained sucrose but not LiCl. Dimethonium did not decrease calcium uptake or contractility during control perfusion. The results suggest that calcium present in the myocardial diffuse double layer can be augmented or reduced in accordance with Gouy-Chapman theory. Changes in diffuse double-layer calcium are accompanied by small (7.8%) but significant changes in contractility.

surface charge; Gouy-Chapman theory; electrostatic potentials; rabbit; neonatal rat

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EXTRACELLULAR CALCIUM plays a crucial role in excitation-contraction coupling in cardiac muscle. However, the concentration of calcium in the extracellular space is not entirely homogeneous. According to Gouy-Chapman theory, the aqueous space adjacent to the sarcolemma contains an excess of cations compared with the bulk aqueous phase. McLaughlin (14) has written an in depth review of this compartment, which is termed the diffuse double layer. The existence of the diffuse double layer is attributed to the negative surface potential of the sarcolemma, produced in part by the negatively charged phospholipids of the bilayer. The distance (Debye length) over which the diffuse double layer extends from the bilayer depends not only on the surface charge density but also on the ionic composition of the bulk aqueous phase. Replacement of sodium ions with nonionic sucrose should increase the surface potential of the sarcolemma as well as the area encompassed by the diffuse double layer. Therefore, the concentration of calcium located in the diffuse double layer would be expected to increase.

McLaughlin et al. (13) recently proposed that the divalent cation dimethonium would be a useful tool in the study of the diffuse double layer. The usefulness of dimethonium as a probe for diffuse double-layer calcium depends on its ability to screen negative charges of the sarcolemma without displacing bound calcium. McLaughlin et al. (13) investigated dimethonium's effects on the surface potential of multilamellar phospholipid vesicles. The effects of dimethonium on the ζ-potential, the potential within the membrane (nonactin-induced conductance), and the potential at the phosphodiester groups were determined. These investigators (13) found that dimethonium significantly reduced the ζ-potential of phosphatidylserine vesicles that were prepared in the presence of 100, 10, and 1 mM NaCl. Dimethonium was more effective with respect to reducing the ζ-potential as the NaCl concentration was lowered. The changes in ζ-potential that occurred in response to dimethonium matched the changes predicted using the Gouy-Chapman theory, provided the assumption was made that dimethonium did not adsorb to the vesicles. The effect of dimethonium on conductance of planar phosphatidylserine membranes and the potential at the phosphodiester group of phosphatidylserine vesicles also conformed to the predictions made using the Gouy-Chapman theory. McLaughlin et al. (13) concluded that dimethonium effectively screened negative membrane charges but did not adsorb to the bilayer.

In the present investigation, the effects of dimethonium on myocardial calcium uptake and contractile function were measured during low-sodium and control perfusion. Dimethonium was found to reduce calcium uptake and tension development during perfusion with low-sodium media containing sucrose but not during perfusion with low-sodium media containing LiCl or with control media. The results suggest that the calcium located in the diffuse double layer plays a role, but of relatively minor degree, in excitation-contraction coupling when the amount of calcium located in this compartment has been augmented. Diffuse double-layer calcium does not, however, represent an important compartment under control conditions.

METHODS

Cultured cells. Calcium exchange was measured using cultured neonatal rat myocardial cells (4). Whole hearts were excised from neonatal rats and digested with 0.1% trypsin. By means of a process of centrifugation and washing, most of the fibroblastic elements of the heart were removed. The suspended myoblasts were pipetted onto disks composed of polystyrene combined with scintillator material (Bicron, Newbury, OH 44065). The cells (80–90% myoblasts) were grown as a thin monolayer culture for 3–4 days before being used for experiments.

Gas-dissected sarcolemmal membranes were used to specifically measure calcium binding to the sarcolemma (8). Membranes were prepared from cultured rat myocardial cells grown on scintillator disks. The cultured cells were exposed to the shearing force of a high-velocity stream of nitrogen gas such that the cells were disrupted. As a result of this procedure, only the sarcolemmal membranes were left attached to the scintillator disks. This procedure resulted in a membrane preparation having a high degree of purity (less than 1% intracellular content) and structural integrity. The membrane markers Mg^{2+}-ATPase, Na^+-K^+-ATPase, and 5′-AMPase were increased 7- to 15-fold, whereas the mitochondrial marker succinic dehydrogenase was not detectable.

A modified Beta II spectrometer (Beckman) was used to measure calcium exchange. Scintillation disks containing cultured cells or gas-dissected membranes were mounted in a flow cell with the cells or membranes directed inward. The flow cell was then inserted into the spectrometer. During each experiment, the cells and membranes were perfused with HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered perfusate that contained ^{45}Ca (1 μCi/ml) at a flow rate of 10 ml/min. ^{45}Ca associated with the cells or membranes excited the scintillator material embedded in the disks, producing a light signal. The light signal was continuously monitored by the photomultiplier tubes of the spectrometer. ^{45}Ca associated with the cells and membranes was counted with an efficiency of 35–51%, whereas the perfusate was counted with an efficiency of less than 5%. Experiments were performed on blank scintillation disks to determine if lithium, sucrose, or dimethonium affected the signal measured by the spectrometer. Lithium was found to have no effect, dimethonium had a slight but nonsignificant effect, whereas sucrose produced a marked quench effect. Because of the quench effect of sucrose, the data obtained for calcium uptake in response to perfusion with sucrose underestimated the actual amount of calcium taken up. After an experiment, the cells and membranes were scraped from the disks. The cells were dried in a 100°C oven for 24 h and the dry cell weight measured. Membranes were assayed for protein content by use of the Lowry method (12).

Septum. The mechanical response of the heart to various interventions was investigated using the isolated, arterially perfused rabbit interventricular septum (6). Male New Zealand rabbits (1.8–2.3 kg) were injected intravenously with an overdose of pentobarbital sodium (200 mg) and 1,000 U of heparin. The heart was quickly excised and a cannula placed within the septal artery, restoring septal perfusion. The septum was then isolated from the rest of the heart. The septum was paced at 60 beats/min, and tension development was monitored continuously. The temperature of the septum was maintained at 28°C. Initially the septum was perfused with oxygenated (95% O_2-5% CO_2) Krebs-Ringer-bicarbonate (KRB) perfusate. Thereafter the septum was perfused with oxygenated (100% O_2) HEPES-buffered solution. Perfusion flow rate was kept constant at 3 ml/min (approx 2.2 $ml \cdot g^{-1} \cdot min^{-1}$).

Solutions. KRB perfusate contained the following (in mM): NaCl 120, $CaCl_2$ 1.5, KCl 6, $MgCl_2$ 1, NaH_2PO_4 0.435, dextrose 5.5, and $NaHCO_3$ 25. HEPES-buffered perfusate used in experiments on rabbit septa contained the following (in mM): NaCl 139, KCl 5.0, $MgCl_2$ 1.0, $CaCl_2$ 1.0, dextrose 6, and HEPES 6.0. Either 178 mM sucrose or 89 mM LiCl was used as the sodium substitute when the concentration of sodium in the HEPES perfusate was reduced to 50 mM. The pH of the perfusate was adjusted to 7.40. The HEPES perfusate used in the experiments on cultured cells and gas-dissected membranes contained the following (in mM): NaCl 133, $CaCl_2$ 1.0, KCl 3.6, $MgCl_2$ 0.3, dextrose 16, and HEPES 3.0. Either 200 mM sucrose or 100 mM LiCl was used as the sodium substitute when the concentration of sodium was reduced to 33 mM. The pH of the perfusate was adjusted to 7.15.

Synthesis of dimethonium. Ethane-bis-trimethylammonium bromide (dimethonium) was synthesized by reacting *N,N,N′,N′*-tetramethylethylenediamine with methyl bromide in acetonitrile (20). The resulting precipitate was then recrystallized from isobutanol-methanol. Appropriate safety precautions were taken due to the toxic nature of methyl bromide. NMR and melting point analyses of the compound did not disclose the presence of any major contaminants.

Statistical analysis. Data were analyzed by use of the Student's *t* test and, when appropriate, the paired *t* test.

RESULTS

The effect of 10 mM dimethonium on calcium uptake was examined in cultured neonatal rat myocardial cells during low-sodium and control perfusion. The concentration of 10 mM was selected because, according to Gouy-Chapman theory, divalent cations such as dimethonium are 10 times more effective at changing membrane surface potential than monovalent cations, given the surface charge and surface potential of most biological membranes (14). Cells were first perfused for approximately 35 min with HEPES-buffered control (133 mM NaCl) perfusate that contained ^{45}Ca. The ^{45}Ca taken up by the cells was continuously monitored as described in METHODS. In control experiments, once the level of radioactivity reached asymptote, cells were perfused for approximately 35 min with an aliquot of the control perfusate to which dimethonium had been added. No effect of dimethonium on control calcium uptake was noted (Table 1, Fig. 1).

TABLE 1. *Effect of 10 mM dimethonium on calcium uptake in cultured myocardial cells and gas-dissected sarcolemmal membranes*

NaCl, mM	Na Substitute	ΔCa Uptake of Cells, mmol/kg dry wt		ΔCa Uptake of Membranes in Response to Dimethonium	
		Low Na	Dimethonium	nmol/mg protein	mmol/kg dry wt*
133			+0.13±0.06	−7.3±1.1	−0.14±0.02
33	Sucrose	+1.43±0.17	−0.88±0.11†	−25.4±2.7†	−0.49±0.05
33	LiCl	+0.45±0.10	−0.08±0.05	−6.8±1.3	−0.13±0.02

Data are means ± SE. * These values were obtained using the factor of 19.1 μg membrane protein/mg dry cell wt (9) to compare the Ca uptake of membranes with the Ca uptake of whole cells. † Decrease in Ca uptake in response to dimethonium was significantly greater ($P < 0.001$) when perfusate Na was replaced with sucrose compared with control.

Low-sodium experiments were performed on cultured cells in which perfusion with control perfusate was followed by perfusion with low-sodium perfusate containing sucrose or LiCl. Dimethonium was then added to an aliquot of the low-sodium perfusate, and the cells were perfused for an additional 35 min. The results of these experiments are illustrated in Fig. 1 and summarized in Table 1. Low-sodium perfusion (33 mM NaCl) resulted in a significant increase in calcium uptake compared with control (133 mM NaCl) perfusion (Table 1). The increment in calcium uptake was significantly greater ($P < 0.001$) when sucrose was used as the sodium substitute, compared with when LiCl was used (Table 1). Burt and Langer (3) found that exchangeable calcium of cultured neonatal rat myocardial cells was 3.57 ± 0.28 mmol/kg dry wt under control conditions. The increment in calcium uptake induced by low-sodium perfusion with sucrose was found to be 1.43 ± 0.17 mmol calcium/kg dry wt (Table 1). This represents an increase in calcium uptake of approximately 40%. Administration of 10 mM dimethonium substantially reduced the calcium uptake that occurred in response to a decrease in extracellular sodium when sucrose was used (Table 1, Fig. 1). In contrast, dimethonium did not decrease calcium uptake when LiCl was used as the sodium substitute (Table 1, Fig. 1).

The effect of 10 mM dimethonium on calcium uptake in gas-dissected sarcolemmal membranes was measured during low-sodium and control perfusion. Perfusion with low-sodium perfusate containing sucrose resulted in an increase in sarcolemmal calcium uptake, although perfusion with low-sodium media containing LiCl did not. Dimethonium caused the same reduction in calcium uptake during low-sodium perfusion with LiCl as it did during control perfusion (Table 1). The reduction in calcium uptake in response to dimethonium was significantly greater when sucrose was used as a sodium substitute compared with control or compared with when LiCl was used as a sodium substitute (Table 1).

The effect of dimethonium on mechanical function was investigated using rabbit interventricular septa. Septa were perfused for 20 min with either low-sodium (50 mM NaCl) or control (139 mM NaCl) HEPES-buffered perfusate. Low-sodium perfusion induced a rapid ($t_{½}$ = 10 s) increase in myocardial contractility. The septa were then perfused for 10 min with an aliquot of the perfusate to which dimethonium had been added. The effect of dimethonium on peak tension is shown in Fig. 2 and summarized in Fig. 3. Dimethonium induced a small but significant decrease in peak tension during perfusion with low-sodium (50 mM) perfusate when sucrose was used as the sodium substitute (Figs. 2 and 3). Dimethonium had no effect during perfusion with low-sodium media containing LiCl (Figs. 2 and 3).

Dimethonium caused a small but significant increase in peak tension when administered in control perfusate. This increase in tension could be attributed to the increase in perfusate osmolarity of 30 mosM that occurred when 10 mM dimethonium bromide was added to solution. An increase in perfusate osmolarity has been shown to result in an increase in myocardial contractility (5). Perfusion of septa (n = 4) with hyperosmolar perfusate that contained 30 mM sucrose was found in the present investigation to result in an increase in peak tension of 2.3 ± 0.4 g at 10 min. This increase in tension was comparable to the increase in tension of 1.2 ± 0.3 g that occurred in response to dimethonium. Tension development during administration of dimethonium did not change appreciably after the initial 5 min, either during control perfusion or during low-sodium perfusion.

DISCUSSION

The plasma membranes of most cells, including cardiac cells, are negatively charged because of the presence of anionic phospholipids and other negatively charged components. Extracellular cations such as sodium and calcium affect the electrostatic potential at the membrane in two ways, by binding directly to the negatively charged sites and by accumulating in the diffuse double layer. The diffuse double layer, also known as the screening layer, refers to the aqueous region adjacent to the charged membrane. In cardiac cells, the calcium associated with the sarcolemma has been shown to be important in contractile function (1, 17, 18). In particular, recent studies using various probes and phospholipases to alter the surface charge density of the sarcolemma have demonstrated a striking correlation between calcium associated with the sarcolemma and contractility (2, 11, 19). However, until now it has not been possible to assess the importance of calcium located in the diffuse double layer but not specifically bound to the membrane. The present investigation demonstrates that extracellular calcium located in the diffuse double layer does, under certain conditions, play a small but significant role in cardiac excitation-contraction coupling. Calcium located in this

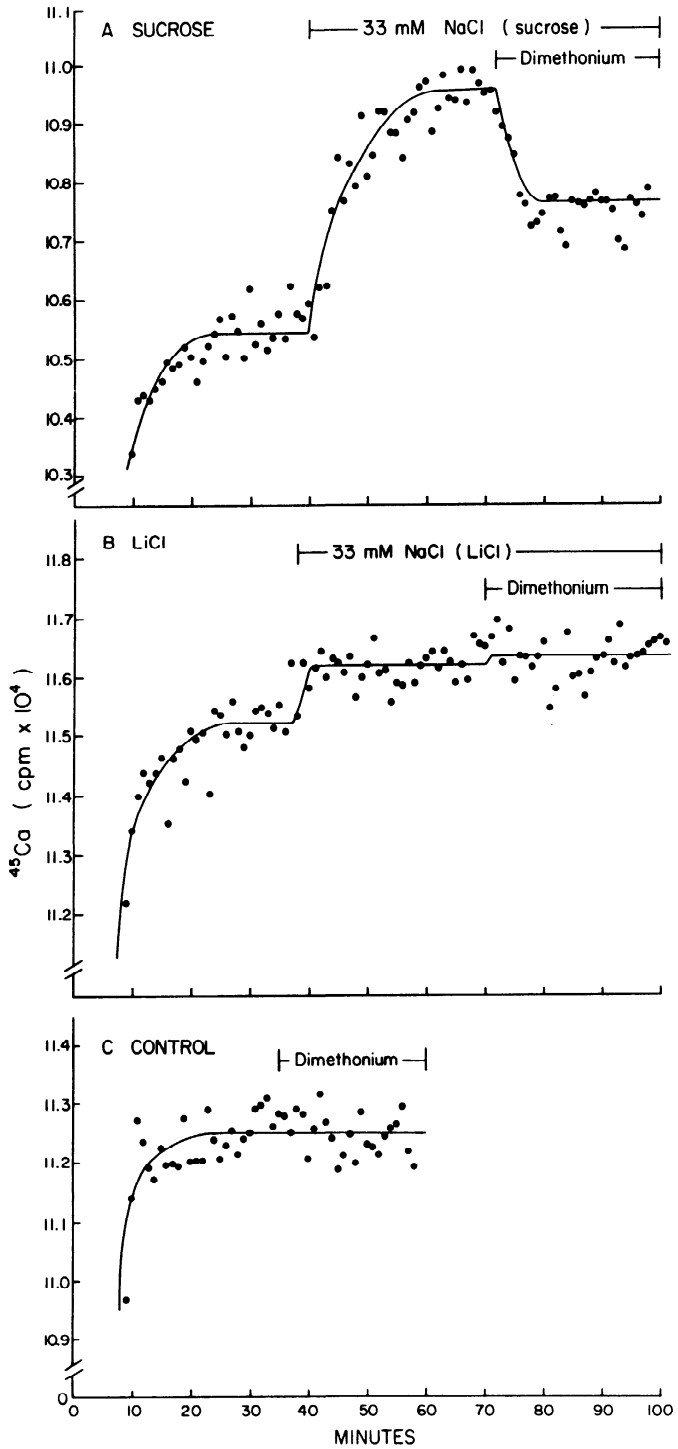

FIG. 1. Effect of 10 mM dimethonium on Ca uptake in cultured neonatal rat myoblasts. Cells were perfused with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered control media for approximately 35 min before any interventions were performed. Dimethonium induced a significant decrease in Ca uptake during perfusion with low-Na media containing sucrose (*A*). Significant decrease in response to dimethonium was not observed during perfusion with low-Na perfusate containing LiCl (*B*) or control perfusate (*C*). It should be noted that rate of cellular Ca uptake is perfusion limited at flow rate used (7).

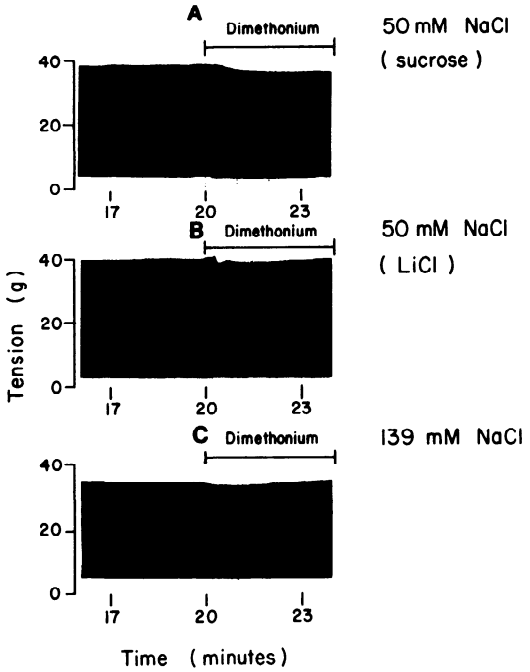

FIG. 2. Representative tracings showing effect of dimethonium on tension development of rabbit interventricular septa. Septa were perfused for 20 min with either low-Na perfusate or control (139 mM NaCl) perfusate. Septa were then perfused for an additional 10 min with an aliquot of perfusate to which 10 mM dimethonium was added. Dimethonium was administered during control perfusion, low-Na perfusion with sucrose, and low-Na perfusion with LiCl in each septum. Dimethonium caused a small but significant decrease during perfusion with low-Na perfusate containing sucrose (*A*) but not during low-Na perfusion with LiCl (*B*) or during control perfusion (*C*).

compartment does not, however, play a significant role under control conditions.

The results of this study are consistent with the earlier work of McLaughlin et al. (13), in which dimethonium was put forth as a specific probe for membrane surface potential. The decrease in calcium uptake by cells and isolated membranes in response to dimethonium when sucrose was used as a sodium substitute suggests that dimethonium is effective at screening negative membrane charges. McLaughlin and co-workers (13) reported that dimethonium, unlike calcium, did not adsorb to phospholipid vesicles. In the present investigation, dimethonium did not appear to compete with calcium for phospholipid binding sites because calcium uptake by cultured cells was not affected by dimethonium under control conditions.

It would be expected that at a perfusate sodium concentration of 133 mM the predominant cation in the diffuse double layer would be sodium. However, on decrease of sodium by 100 mM the surface potential would be expected to increase by almost twofold if sodium were replaced by a neutral compound such as sucrose. This is because surface potential varies inversely with the square root of the salt concentration (14). The increase in surface potential and the reduction in monovalent cations would favor the movement of divalent calcium into the diffuse double layer. In addition, if sodium were at sites that were competitive for calcium, additional calcium binding to the membrane would take place on removal of sodium. The increment in calcium uptake induced by low-sodium perfusion can be separated, through the use of dimethonium, into that fraction which is affected by

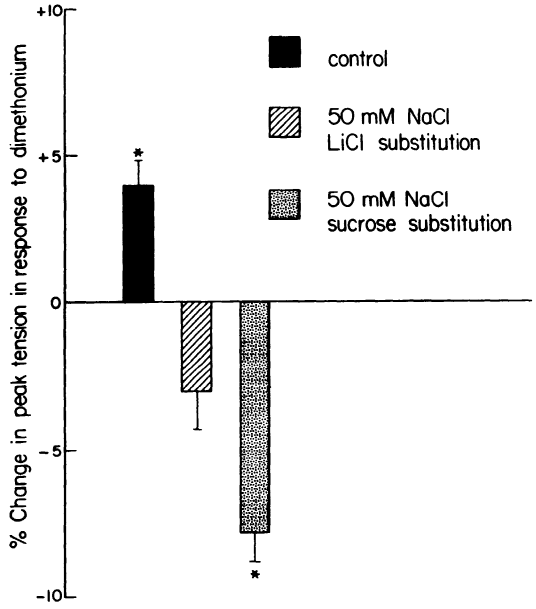

FIG. 3. Change in peak tension of rabbit septa in response to 10 mM dimethonium. Septa ($n = 6$) were perfused with control or low-Na perfusate for 20 min followed by 10 min with an aliquot of perfusate which contained dimethonium. Peak tension at end of 10 min of dimethonium perfusion was compared with peak tension just prior to addition of dimethonium. Data were analyzed using a paired t test. Each septum was perfused with dimethonium during control perfusion, low-Na perfusion with sucrose, and low-Na perfusion with LiCl. Dimethonium induced a small but significant decrease in peak tension during perfusion with low-Na perfusate containing sucrose ($^*P < 0.001$). In contrast, dimethonium did not affect tension during low-Na perfusion with LiCl and actually increased tension slightly ($^*P < 0.001$) during control perfusion.

the sarcolemmal surface potential and that fraction which is not.

Low-sodium perfusion has been shown repeatedly to increase myocardial contractility and calcium uptake (10, 15, 16, 21). As can be seen from Fig. 1 and Table 1, there is a significantly larger increase in calcium uptake with sucrose substitution than with LiCl substitution. Dimethonium (10 mM) displaced a significant fraction of the calcium increment in sucrose and had virtually no effect on the increment in LiCl. It should be noted that the scintillator disk technique cannot distinguish between ^{45}Ca within the cell, ^{45}Ca bound to the sarcolemma, or ^{45}Ca that has moved into the diffuse double layer; all locations would count with an efficiency typical of the cell layer (~41%) as opposed to the ^{45}Ca in the bulk solution (<5% efficiency). However, the marked "displacement" produced by 10 mM dimethonium in sucrose indicates that a substantial part of the uptake is related to movement of calcium into the diffuse double layer. The lack of effect of dimethonium when LiCl is used indicates that little of the increment in calcium is attributable to the diffuse double layer. These results are those predicted for a neutral (sucrose) substitution for sodium as opposed to a cationic (lithium) substitution. For the same reason, dimethonium had negligible effect on calcium uptake in the presence of 133 mM NaCl (Fig. 1*C* and Table 1).

The use of gas-dissected membranes allows sarcolemmal calcium to be measured separately from intracellular calcium. Partial replacement of sodium with sucrose resulted in an increase in calcium uptake of gas-dissected membranes, as well as whole cells (Table 1). The results with dimethonium indicate that part of this increase in calcium can be localized to the diffuse double layer when sucrose is used as the sodium substitute. The response of the membranes to dimethonium was not different from control when LiCl was used; this is consistent with results in whole cells (Table 1). Calcium uptake of sarcolemmal membranes was not increased, however, when lithium was used as a sodium substitute. In contrast to the results obtained when sucrose was used, the increase in calcium uptake of whole cells that occurred when LiCl was used as a sodium substitute does not appear to involve a measurable increase in calcium associated with the sarcolemma.

It was predicted that force development of the rabbit heart would not be decreased by dimethonium under control conditions or in the LiCl-substituted condition, since no significant calcium displacement was produced in the cultured cells. This was found to be the case. On the other hand, dimethonium produced a significant, albeit small, decrease of force development during sucrose-substituted perfusion in the interventricular septum. This indicates that specific elevation of the calcium concentration in the diffuse double layer in whole ventricular muscle can contribute to force development, but the contribution is relatively minor. Contractile function appears to be supported, in all cases tested, mainly by calcium that is independent of the diffuse double layer.

It is clear from previous studies that calcium associated with the sarcolemma is of considerable importance in the support of contraction (1, 2, 11, 17–19). The results of the present investigation suggest that the calcium that is significant is the calcium that is actually bound to membrane constituents.

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