*Biochimica et Biophysica Acta*, 774 (1984) 127–137
Elsevier



BBA 72155

# THE EFFECT OF LIPID INTERMEDIATES ON $Ca^{2+}$ AND $Na^+$ PERMEABILITY AND $(Na^+ + K^+)$-ATPase OF CARDIAC SARCOLEMMA

## A POSSIBLE ROLE IN MYOCARDIAL ISCHEMIA

JOS M.J. LAMERS [a,*], HANNY T. STINIS [a], AD MONTFOORT [b] and WILLEM C. HÜLSMANN [a]

*Departments of [a] Biochemistry I and [b] Pathological Anatomy, Medical Faculty, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam (The Netherlands)*

(Received January 25th, 1984)

*Key words: Ion transport; $Ca^{2+}$ transport; $(Na^+ + K^+)$-ATPase; Fatty acid; Acylcarnitine; Ischemia; (Pig heart sarcolemma)*

**The effect of fatty acid and acylcarnitine on $Ca^{2+}$ and $Na^+$ transporting enzymes and carriers was studied in sealed cardiac sarcolemma vesicles of mixed polarity. Palmitoylcarnitine markedly reduced the $Na^+$ gradient-induced $Ca^{2+}$ uptake. Half-maximal reduction was obtained at 15 μM of the carnitine derivative. In a same concentration range palmitoylcarnitine caused a rapid release of accumulated $Ca^{2+}$ when added to $Ca^{2+}$-filled vesicles, which suggests that palmitoylcarnitine increases the permeability of the sarcolemma vesicles to $Ca^{2+}$. A rapid release of $Ca^{2+}$ was also observed if $Ca^{2+}$ was taken up by action of the $Ca^{2+}$ pump. The $(Ca^{2+} + Mg^{2+})$-ATPase, which most likely drives this active $Ca^{2+}$ uptake, was 90% increased by 50 μM palmitoylcarnitine and evidence was presented that the acylcarnitine effect again was linked to an alteration of $Ca^{2+}$ permeability of the vesicles. At the same concentration acylcarnitine was not able to unmask the latent protein kinase, so that probably the sarcolemma ATP permeability was not affected. Palmitoylcarnitine at 25 μM did not affect the ouabain-sensitive $(Na^+ + K^+)$-ATPase in native sarcolemma vesicles, however, it inhibited markedly if the enzyme was measured in SDS-treated vesicles. The effect of increased free fatty acid concentration on some of the sarcolemma transporting properties was tested by adding oleate-albumin complexes with different molar ratios to the sarcolemma vesicles. In contrast to molar ratios 1 and 5, the ratio of 7 was able to induce a rapid $Ca^{2+}$ release and to inhibit $(Na^+ + K^+)$-ATPase in either native or SDS-treated vesicles markedly. $^{22}Na$ release from $^{22}Na$-preloaded sarcolemma vesicles was shown to be stimulated by either palmitoylcarnitine (50 μM) or oleate-albumin complex (with a molar ratio of 7). The possible significance of the observed effects of lipid intermediates on ion permeability and $(Na^+ + K^+)$-ATPase activity in isolated sarcolemma vesicles for the derangement of cardiac cell function in ischemia is discussed.**

## Introduction

In recent years, free fatty acids and in particular long chain fatty acylcarnitines have been thought to play an important role in the pathophysiology of the ischemic myocardium [1–6]. Large accumulation of long chain fatty acids, and their derivatives fatty acyl-CoA-thioesters and fatty acylcarnitine esters, have been demonstrated in ischemic myocardium and ascribed to oxygen deficiency of the mitochondria [7–9]. These compounds are active detergents and bind extensively to membranes [3,4,10]. A large body of evidence

* To whom correspondence should be addressed.

Abbreviations: Mops, 4-morpholinepropanesulfonic acid; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; SDS, sodium dodecylsulfate.

[2–4,11,12] now has indicated that these intermediates alter functional properties of myocardial membranes in vitro and that these changes may contribute in the decline of the myocardial contractility and the generation of arrhythmia. By this mechanism the lipid intermediates may also cause intracellular $Ca^{2+}$ overload, which may lead to cell death [13–15].

Long chain fatty acids and acylcarnitines have been found to inhibit $(Na^+ + K^+)$-ATPase in sarcolemma isolated from cardiac muscle [2,3,16]. In these studies $(Na^+ + K^+)$-ATPase-enriched membrane particles were prepared by using a surface-active agent (desoxycholate) to partially solubilize the membranes. After this treatment, however, $Ca^{2+}$ transport is no longer measurable because the membrane particles are not well sealed. There are no studies available on the influence of lipid intermediates on sarcolemma $Ca^{2+}$-transporting systems such as the electrogenic $Na^+/Ca^{2+}$-antiporter and the $Ca^{2+}$ pump [17,18]. Moreover, recently reported work suggested that $(Na^+ + K^+)$-ATPase activity, when measured in native sarcolemma vesicles, is resistant to pertubation by acylcarnitine [12]. Therefore in the present study the sarcolemma membranes were not treated with surface-active agents during isolation to attempt to maintain in vitro the native environment of the sarcolemma as much as possible. In a previous report it was shown that a highly purified sarcolemma preparation contained $Na^+/Ca^{2+}$-antiporter and $Ca^{2+}$-pumping (ATPase) activity [19,20]. It contained also a large amount of latent $(Na^+ + K^+)$-ATPase activity which could be attributed to the presence of tightly sealed vesicles of rightside-out orientation [19,21]. In the present work the effects of fatty acid and acylcarnitine on sarcolemma $Na^+/Ca^{2+}$ antiporter, $Ca^{2+}$-pumping ATPase and $(Na^+ + K^+)$-ATPase are investigated using this purified sarcolemma vesicle preparation.

## Materials and Methods

Sarcolemma was isolated from pig heart by the procedure described by Reeves and Sutko [22] except that a protease inhibitor phenylmethylsulfonyl fluoride was added to all media in 0.5 mM concentration. The preparation was highly enriched in sarcolemma as was revealed by the high specific activity of ouabain-sensitive $(Na^+ + K^+)$-ATPase: 50–75 μmol/mg per h if pre-incubated with 0.3 mg SDS or 0.5 mg alamethicin per mg membrane protein (compare Refs. 18, 19 and 21). Cross contamination with sarcoplasmic reticulum membranes is minimal as controlled by $Na^+$- and $Ca^{2+}$-dependent $^{32}P$ incorporation into 120 kDa proteins separated on polyacrylamide gel electrophoresis [19].

*Assay of the $Na^+$-$Ca^+$ exchange and passive $Ca^{2+}$ permeability.* $Na^+$-$Ca^{2+}$ exchange was measured in sarcolemma vesicles loaded with 160 mM NaCl (20 mM Mops (pH 7.4)) by preincubation in this salt medium at 37 °C for 20 min essentially according to the method previously described [17]. To estimate $Ca^{2+}$ uptake a 10 μl aliquot (12.5 μg protein) was added to 200 μl 160 mM KCl or NaCl medium containing 20 mM Mops (pH 7.4) and 50 μM $^{45}CaCl_2$ (0.1 Ci/mmol). The $^{45}Ca$ uptake reaction was terminated at 15, 30, 60 and 120 s by Millipore filtration (50 μl) and washed twice with ice-cold 160 mM KCl, 20 mM Mops (pH 7.4) containing 0.1 mM $LaCl_3$. $^{45}Ca$ uptake reactions in NaCl were used as blank values and subtracted from corresponding time values in dilution with KCl. The passive efflux from preloaded vesicles was estimated by dilution of 50 μl sample of a 2 min $Na^+$-$Ca^{2+}$ exchange reaction into 1.2 ml 160 mM KCl, 20 mM Mops (pH 7.4) containing 0.1 mM EGTA at 37 °C. Samples of 200 μl stopped by Millipore filtration after 15 s, 1, 3, 5 and 10 min were used for estimation of the release rate.

*Assay of the ATPases.* $(Na^+ + K^+)$-ATPase activity was determined by incubating 12.5 μg membrane protein (either preincubated in 0.25 mg/ml SDS, 20 mM imidazole. (pH 7.4), or not) in 200 μl medium containing 50 mM Tris-maleate (pH 7.4), 100 mM NaCl, KCl concentration as indicated, 2.5 mM $MgCl_2$, 1 mM EGTA, 2 mM [γ-$^{32}$P]ATP (0.25 mCi/mmol). Reactions were carried out in the absence or presence of 1 mM ouabain. $(Ca^{2+} + Mg^{2+})$-ATPase reactions were done in 200 μl medium containing 12.5 μg membrane protein, 0.1 mM [γ-$^{32}$P]ATP (0.01 Ci/mmol), 50 mM Tris-maleate (pH 6.8), 100 mM KCl and 5 mM $MgCl_2$ at 37 °C. Free $Ca^{2+}$ was controlled in the μM range by buffering with 100 μM EGTA [23]. Reactions were stopped by adding 20 μl 20% trichloro-

acetic acid containing 5 mM $P_i$ and the produced $^{32}P_i$ was complexed with molybdate for extraction into an isobutanol phase as previously described [23,24].

*Assay of cyclic AMP-dependent protein kinase.* Phosphorylations were carried out by adding 10 μM [γ-$^{32}$P]ATP (20 Ci/mmol) to 50 μl medium containing 5 μg membrane protein, 50 mM KCl, 10 mM Mops (pH 7.4), 20 mM potassium phosphate, 5 mM $MgCl_2$, 0.5 mM EGTA 5 μM cyclic AMP and 10 mM theophylline. Incubations were carried out for 2 min at 25 °C and terminated by adding a SDS/β-mercaptoethanol/glycerol mixture, further incubated at 95 °C for 10 min and analyzed for SDS-polyacrylamide gelelectrophoresis (15%) as described previously [23,24].

*Assay of $^{22}$Na release from $^{22}$Na-preloaded vesicles.* Membrane samples (500 μg protein) were preincubated for 20 h at 0–4 °C in 60 μl medium containing 160 mM $^{22}$NaCl (0.4 mCi/mmol), 20 mM Mops (pH 7.4). $^{22}$Na efflux was initiated by adding a 5 μl aliquot of this suspension into 200 μl medium containing 160 mM KCl, 20 mM Mops (pH 7.4). Samples of 50 μl were withdrawn for the estimation of vesicular $^{22}$Na content by Milipore filtration as described for the assay of $Na^+$-$Ca^{2+}$ exchange.

*Materials.* Radioactive [γ-$^{32}$P]ATP, $^{45}$Ca and $^{22}$Na were obtained from Amersham International PCL (Amersham, U.K.). A23187 was purchased from Boehringer (Mannheim, F.R.G.). Alamethicin was a kind gift from Dr. J.E. Grady (The Upjohn Company, Kalamazoo, Michigan). L-Palmitoylcarnitine was a gift from Sigma-Tau (Rome, Italy). Oleate-albumin complexes were prepared as described previously [2].

## Results

### *Effect of palmitoylcarnitine on the $Na^+$-$Ca^{2+}$ exchange*

Palmitoylcarnitine produced a concentration-dependent reducing effect on the sarcolemma $Na^+$-$Ca^{2+}$ exchange activity as illustrated in Fig. 1. Almost complete loss of $Na^+$ gradient-induced $Ca^{2+}$ uptake was observed at 100 μM and half-maximal decrease was seen at 15 μM palmitoylcarnitine. By others [32] was demonstrated that the extent of inhibition of the sarcoplasmic reticulum $Ca^{2+}$ pump by palmitoylcarnitine mostly depended on the ratio of the lipid intermediate to sarcolemma protein. It was also shown previously [12] that 90% of the palmitoylcarnitine added to a sarcolemma vesicle suspension was bound at a lipid intermediate concentration range of 0.2–1.5 μmol/mg sarcolemma protein. Therefore in the experiments that will be described in the present work not only the absolute concentrations but also the amounts/mg membrane protein should be taken into account in comparing stimulatory or inhibitory potency of the lipid intermediate. The ratio of fatty acyl derivative to sarcolemma amounted to 0.4 μmol/mg membrane protein at 25 μM palmitoylcarnitine in the experiments illustrated in Fig. 1. From the results presented in Fig. 1 can be inferred that the initial rate of the sarcolemma $Na^+$-$Ca^{2+}$ exchange has not been determined. However, from the first time point at 15 s and the last at 2 min can be inferred that presumably the initial rate and the maximum $Ca^{2+}$ uptake are both affected by the lipid intermediate. To test the possibility that palmitoylcarnitine

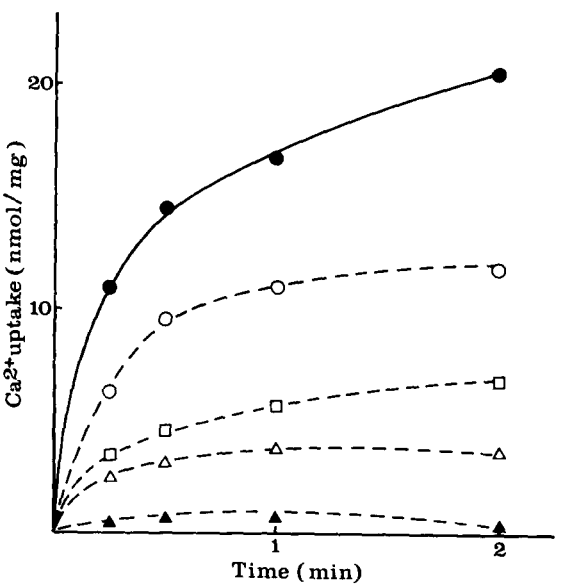


Fig. 1. Effect of palmitoylcarnitine on $Na^+$-$Ca^{2+}$ exchange in cardiac sarcolemma vesicles. Assays were performed as described under Methods. (●) Control; (○) 10 μM, (□) 25 μM; (△) 50 μM; (▲) 100 μM palmitoylcarnitine added at time zero. Blank $Ca^{2+}$ uptake values that were obtained by dilution of the sarcolemma vesicles in a medium containing 50 μM $^{45}CaCl_2$, 160 mM NaCl and 20 mM Mops (pH 7.4) were subtracted. Each point represents the mean of two experiments.

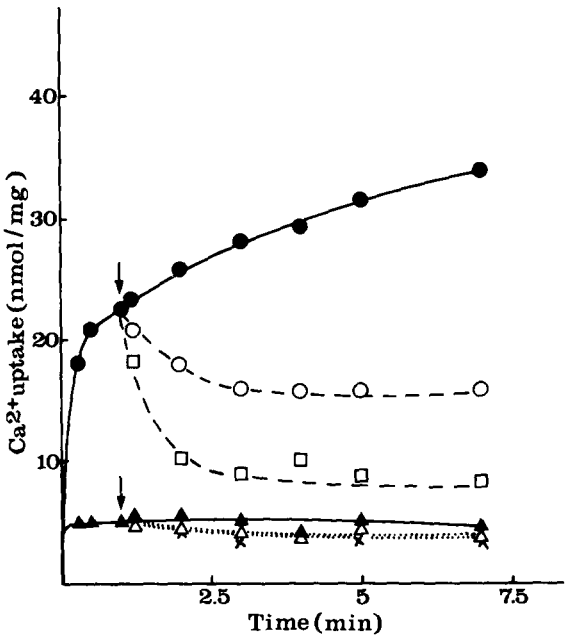


Fig. 2. Effect of palmitoylcarnitine on $Na^+$-$Ca^{2+}$ exchange and passive $Ca^{2+}$ uptake in cardiac sarcolemma vesicles if added after 1 min reaction. $Ca^{2+}$ uptake was determined in vesicles diluted in a medium containing 50 $\mu$M $^{45}CaCl_2$, 20 mM Mops (pH 7.4) and either 160 mM KCl (●, ○, □) or 160 mM NaCl (▲, △, ×) as described under Methods. The arrow indicates palmitoylcarnitine addition at a final concentration of 0 $\mu$M (●, ▲), 10 $\mu$M (○, △) and 25 $\mu$M (□, ×). Each point represents the mean of two experiments.

caused sarcolemma vesicles to become leaky, $Ca^{2+}$ uptake was allowed to proceed for 1 min before the addition of the acylcarnitine (Fig. 2). Accumulated $^{45}Ca$ was rapidly released by the carnitine derivative which result readily explains the observed effects on the initial rate and maximum uptake of the $Na^+/Ca^{2+}$ antiporter. To further differentiate the effects of palmitoylcarnitine on either the $Na^+/Ca^{2+}$ antiporter of the $Ca^{2+}$ and $Na^+$ permeability of the sarcolemma, the lipid intermediate was tested on the passive $Ca^{2+}$-release process. Sarcolemma vesicles were preloaded with $Ca^{2+}$ by the action of the $Na^+/Ca^{2+}$ antiporter. After 2 min incubation (compare Fig. 1), the sarcolemma vesicles were 24-fold diluted in 160 mM KCl containing 0.1 mM EGTA. This dilution step reduced the external $Na^+$ and $Ca^{2+}$ concentrations to extremely low values. In some unpublished experiments it was shown that the addition of 50 mM $Na^+$ released the $Ca^{2+}$ within 1 min, indicating that the vesicle $Ca^{2+}$ still was removable by reversed $Na^+$-$Ca^{2+}$ exchange. As can be seen from Fig. 3, palmitoylcarnitine addition instead of $Na^+$, resulted also in a rapid release of accumulated $Ca^{2+}$ within the first min of the measurements. It should be pointed out that these incubations do contain about 10-fold lower sarcolemma protein amounts, which means that at 2 $\mu$M concentration of the lipid intermediate it is present in 0.4 $\mu$mol/mg membrane protein. Thus the potency of palmitoylcarnitine to stimulate passive $Ca^{2+}$ release under this condition is somewhat lower than the potency to inhibit $Na^+$-$Ca^{2+}$ exchange. However, it should be noted that, as will be shown later, palmitoylcarnitine may also have affected the $Na^+$-$Ca^{2+}$ exchange activity by its effect on $Na^+$ permeability of the sarcolemma membrane.

*Effect of palmitoylcarnitine on the $Ca^{2+}$ pumping (ATPase)*

The effect of palmitoylcarnitine on the ATP-dependent $Ca^{2+}$ uptake was also studied and again a

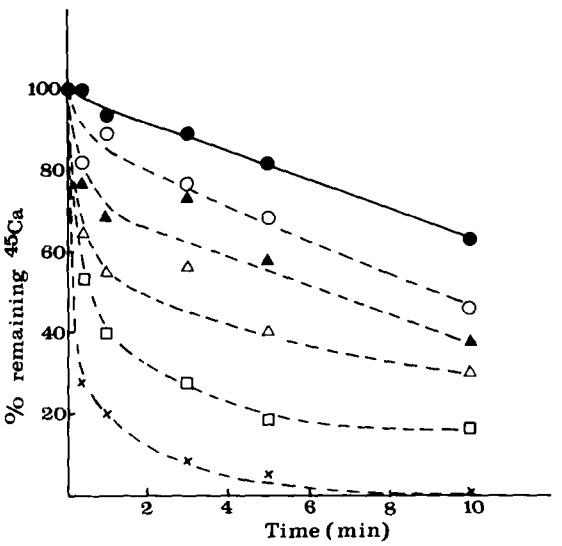


Fig. 3. Effect of palmitoylcarnitine on passive $Ca^{2+}$ release from cardiac sarcolemma vesicles. Passive $Ca^{2+}$ efflux from preloaded vesicles was estimated by dilution of a 50 $\mu$l sample (6.5 $\mu$g sarcolemma protein) of a 2 min $Na^+$-$Ca^{2+}$ exchange reaction into 2 ml medium containing 160 mM KCl, 20 mM Mops (pH 7.4), 0.1 mM EGTA and either 0 $\mu$M (●), 2 $\mu$M (○), 5 $\mu$M (▲), 10 $\mu$M (△), 25 $\mu$M (□) or 50 $\mu$M (×) palmitoylcarnitine as described under Methods. Each point represents the mean of two experiments.

rapid release of $^{45}Ca$ could be demonstrated if the lipid intermediate was added after 2 min $^{45}Ca$ uptake reaction (a concentration range of 25–50 μM palmitoylcarnitine was tested in these unpublished experiments). The $Ca^{2+}$-dependent ATP hydrolysis, driving this $Ca^{2+}$ accumulation, was subsequently investigated. It exhibited a maximum rate 19-fold higher than that of the $Ca^{2+}$ uptake process (6 nmol/min per mg, compared with the control rate of $(Ca^{2+}/Mg^{2+})$-ATPase which is presented in Table I). A low coupling ratio of the amount of the $Ca^{2+}$ taken up and of the ATP hydrolyzed has been found repeatedly in cardiac membranes [17,18,24,25]. It indicates that the inside-out oriented particles in the sarcolemma preparation contain a large amount of unsealed vesicles. Table I demonstrates that palmitoylcarnitine in a concentration range of 10–50 μM (0.2–0.9 μmol/mg membrane protein) was able to stimulate the $(Ca^{2+} + Mg^{2+})$-ATPase. The ATPase was estimated at saturating free $Ca^{2+}$ concentration of 12 μM after it first had been shown that the affinity of the enzyme for calcium ions ($K_a$ = 0.35 μM, compare also Ref. 23) was not affected by the acylcarnitine. The stimulatory effect of palmitoylcarnitine on the $(Ca^{2+} + Mg^{2+})$-ATPase most likely is unrelated to a calmodulin-like effect of the lipid intermediate, because from previous work it appeared that the $(Ca^{2+} + Mg^{2+})$-ATPase in native sarcolemma vesicles is saturated with calmodulin [18,20]. Moreover, in some unpublished experiments it was demonstrated that palmitoylcarnitine also had no effect on another sarcolemma bound calmodulin regulated enzyme: the intrinsic $Ca^{2+}$-calmodulin-dependent protein kinase [20,23,26]. The findings presented in Figs. 2, 3 and Table I suggest a possible relationship between the stimulation of the $(Ca^{2+} + Mg^{2+})$-ATPase and the increased leakiness of the sarcoplasma vesicles for calcium ions. Indeed the $Ca^{2+}$ ionophore A23187 similarly increased the $(Ca^{2+} + Mg^{2+})$-ATPase activity and palmitoylcarnitine had no additive effect (Table I). Therefore these data indicate that the electrochemical $Ca^{2+}$ gradient may inhibit the $Ca^{2+}$ pumping ATPase. Indeed time dependence of ATP-dependent $Ca^{2+}$ uptake deviates from linearity already after 1 min reaction (results not shown) and thus palmitoylcarnitine may have uncoupled the uptake process from ATP hydrolysis.

TABLE I

EFFECT OF PALMITOYLCARNITINE ON CONTROL AND A23187-STIMULATED SARCOLEMMAL $(Ca^{2+} + Mg^{2+})$-ATPase

$(Ca^{2+} + Mg^{2+})$-ATPase was determined by subtracting activities obtained at zero concentration of $Ca^{2+}$ (in the presence of EGTA) from those obtained at 12 μM free concentration of $Ca^{2+}$. The ionophore A23187 was present in the assay medium at 6 μM (0.1 μmol/mg membrane protein). Each column represents the mean of four experiments (± S.E.).

| Palmitoylcarnitine (μM) | $(Ca^{2+} + Mg^{2+})$-ATPase (nmol/min per mg protein) | |
|---|---|---|
| | Control | In the presence of A23187 |
| 0 | 96.5 ± 5.3 | 173.7 ± 14.8 |
| 10 | 161.3 ± 25.0 | 212.3 ± 26.3 |
| 25 | 177.5 ± 13.3 | 211.7 ± 30.7 |
| 50 | 184.3 ± 5.2 | 188.5 ± 30.0 |

*Influence of palmitoylcarnitine on $(Na^+ + K^+)$-ATPase*

Palmitoylcarnitine at 0.4 μmol/mg membrane protein (25 μM), a concentration that markedly increased $Ca^{2+}$ permeability, did not significantly affect the $(Na^+ + K^+)$-ATPase activity (Fig. 4). Previously we showed that the potency of free fatty acids to inhibit sarcolemma $(Na^+ + K^+)$-ATPase increased if suboptimal $K^+$ concentrations were used in the assay [2]. Therefore the acylcarnitine effect was also studied $K^+$ concentrations lower than 10 mM (Fig. 4), however, still no significant effect of the lipid intermediate was observed.

Previously it was reported by us and by others that $(Na^+ + K^+)$-ATPase, adenylate cyclase and cyclic AMP-dependent protein kinase showed considerably latent activities in cardiac sarcolemma, which could be unmasked by adding the peptidic ionophore alamethicin [19,21]. SDS could also be used for this purpose in the case of $(Na^+ + K^+)$-ATPase, however, not with the other latent enzymes because of their instability in the presence of this detergent. It can be seen from Fig. 4 that SDS treatment of the vesicles (1 μmol/mg membrane protein) typically increased $(Na^+ + K^+)$-ATPase activity 2.5-fold. Under these conditions

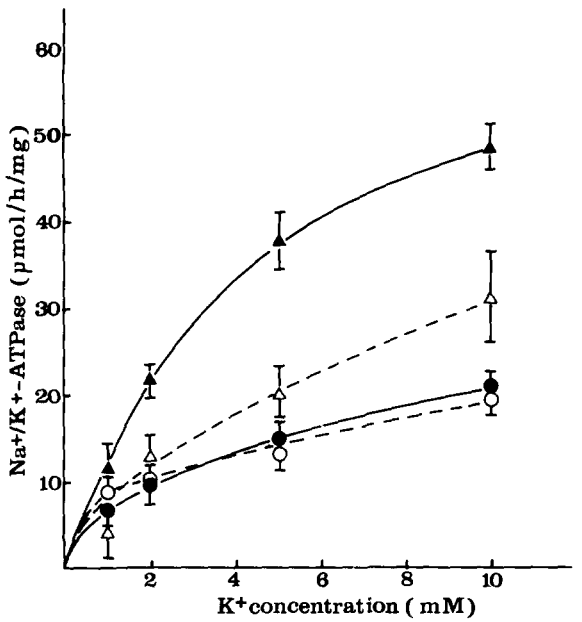


Fig. 4. Effect of palmitoylcarnitine on sarcolemma ($Na^+ + K^+$)-ATPase measured in either control or SDS-treated vesicles. ($Na^+ + K^+$)-ATPase was determined in sarcolemma vesicles preincubated either without (●, ○) or with SDS (▲, △) as described under Methods. The activities represent the values obtained by subtraction of the amount of ATP hydrolysis observed in the presence of 1 mM ouabain. This is done for all different conditions. Palmitoylcarnitine was tested at 25 μM concentration in either the native (○) or the SDS (1 μmol/mg membrane protein)-treated (△) vesicles. Each point represents the mean of five experiments. Vertical bars indicate the standard errors.

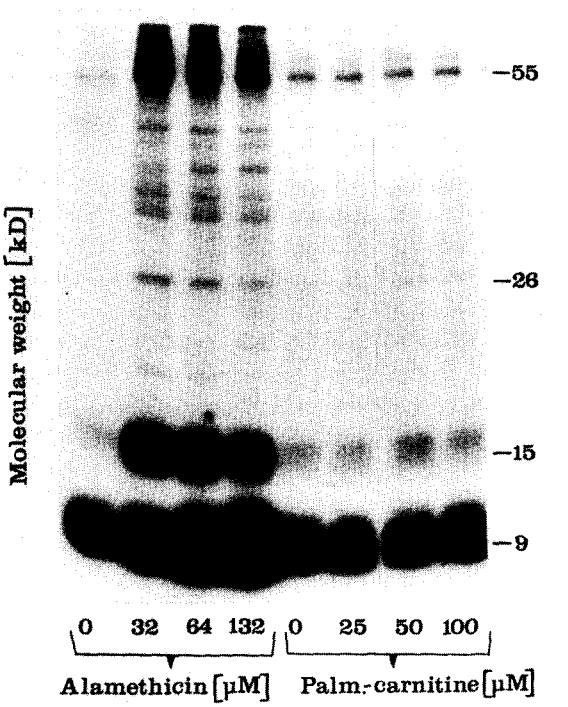


Fig. 5. Effect of alamethicin and palmitoylcarnitine on the intrinsic cyclic AMP-dependent protein kinase of cardiac sarcolemma vesicles. Phosphorylation reactions, SDS-polyacrylamide gel electropheresis and autoradiography were carried out as described under Methods. Protein standards, used for estimating molecular weights of sarcolemma phosphoproteins, are described elsewhere [23,24]. Molecular mass expressed in kDa.

37% inhibition was found by the addition of 25 μM palmitoylcarnitine at 10 mM $K^+$ (Fig. 4). Apparently, the detergent effect of SDS may be sufficient for the lipid intermediate to enter the lipid bilayer and to affect the ($Na^+ + K^+$)-ATPase. These results are also in agreement with those obtained by the group of Adams et al. [3], who studied effect of palmitoylcarnitine on ($Na^+ + K^+$)-ATPase in a partially solubilized sarcolemma preparation. The fact that no effect was observed of the carnitine derivative on the ($Na^+ + K^+$)-ATPase activity in native sarcolemma vesicles could be due to an unmasking of enzyme activity concomitantly occurring with the inactivation. Therefore the ability of palmitoylcarnitine to unmask the intrinsic cyclic AMP-dependent protein kinase was tested using again as a substrate [$\gamma$-$^{32}$P]ATP. As is demonstrated in Fig. 5 the peptidic ionophore alamethicin activates cyclic AMP-dependent $^{32}$P-incorporation into several sarcolemma proteins: the phospholamban-like protein (9 kDa) and some minor proteins of 55, 26, and 15 kDa. These proteins have been shown to be substrates of intrinsic cyclic AMP-dependent protein kinase (cf. also Refs. 19 and 21). Palmitoylcarnitine was not able to unmask the protein kinase up to concentrations of 50 and 100 μM (0.4 and 0.8 μmol/mg membrane protein). In other experiments it was shown that no concomitant inactivation of cyclic AMP-dependent protein kinase occurred. This was tested by adding palmitoylcarnitine to alamethicin treated sarcolemma vesicles (results not shown). In conclusion, palmitoylcarnitine at relatively high concentrations (50–100

$\mu$M), did not increase the permeability of the sarcolemma vesicles for ATP and cyclic AMP which excluded the possibility that it can unmask latent $(Na^+ + K^+)$-ATPase activity.

*Effect of oleate-albumin complexes on sarcolemma $Ca^{2+}$ permeability and $(Na^+ + K^+)$-ATPase*

Fatty acid was added to the sarcolemma vesicles in the form of fatty acid-albumin complexes. Albumin has two very strong binding sites and a number of weak binding sites for free fatty acid. Thus with high fatty acid-albumin ratios the concentration of unbound fatty acid increases exponentially. These concentrations have been previously determined for oleate-albumin complexes under slightly different conditions as used in the present transport and enzyme assays. For oleate-albumin complexes of molar ratio 1, 5 and 7 the concentration of free oleate amounts to 0.0055, 1.72 and 6.36 $\mu$M, respectively [27]. It can be seen from Fig. 6 that the oleate-albumin complex of molar ratio 7 markedly increased the $^{45}Ca$ release rate of sarcolemma vesicles loaded with $^{45}Ca$ by the operation of the $Na^+/Ca^{2+}$ antiporter. It should be noted that the free oleate over membrane protein ratio amounted to 0.5 $\mu$mol/mg when using the oleate-albumin complex with a molar ratio of 7. Thus in comparing the results presented in Figs. 2 and 6, it can be concluded that free fatty acid and acylcarnitine are similarly effective in increasing the $Ca^{2+}$ permeability of cardiac sarcolemma vesicles.

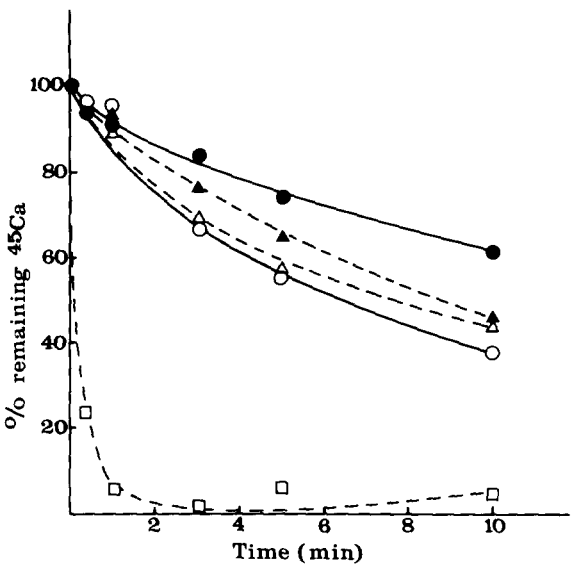


Fig. 6. Effect of oleate-albumin complexes of different molar ratios on the passive $Ca^{2+}$ release from cardiac sarcolemma vesicles. Passive efflux from preloaded vesicles was estimated by dilution of a 50 $\mu$l sample (13.0 $\mu$g sarcolemma protein) of a 2 min $Na^+$-$Ca^{2+}$ exchange reaction into 1.2 ml medium containing 160 mM KCl, 20 mM Mops and 0.1 mM EGTA. The control efflux (●) is compared with the amount of efflux in medium containing additionally oleate-albumin complexes of molar ratios 0 (○), 1 (△), 5 (▲) and 7 (□). Albumin was chosen at a fixed concentration of 0.14 mM. Each point represents the mean of three experiments.

TABLE II

EFFECT OF OLEATE-ALBUMIN COMPLEXES OF DIFFERENT MOLAR RATIOS ON CARDIAC SARCOLEMMA $(Na^+ + K^+)$-ATPase MEASURED EITHER IN CONTROL OR IN SDS-TREATED VESICLES

Values represent means ± S.E. of four experiments. For details of determination of ouabain-sensitive $(Na^+ + K^+)$-ATPase, preparation of oleate-albumin complexes and conditions for SDS treatment of sarcolemmal membranes, is referred to the legends of Fig. 5 and Materials and Methods.

| Albumin (mM) | Oleate (mM) | Ouabain-sensitive $(Na^+ + K^+)$-ATPase (% of control) | |
|---|---|---|---|
| | | Control | +SDS |
| 0 | 0 | 100 | 211 ± 25 |
| 0.14 | 0 | 101 ± 4 | 201 ± 27 |
| 0.14 | 0.14 | 107 ± 11 | 225 ± 30 |
| 0.14 | 0.70 | 94 ± 9 | 239 ± 29 |
| 0.14 | 0.98 | 43 ± 14 [a] | 29 ± 14 [a] |

[a] $P < 0.025$ versus Control.

Albumin (0.14 mM) itself increased slightly but reproducibly the $Ca^{2+}$ permeability, as is illustrated in Fig. 6. This effect may be attributed to the contamination of the albumin preparation with $Ca^{2+}$ or $Na^+$ which might have been present albeit extensive dialysis. These cations could have initiated $Ca^{2+}$ release by operation of $Ca^{2+}$-$Ca^{2+}$ or $Ca^{2+}$-$Na^+$ exchange [28]. It is interesting to note that oleate-albumin complex of ratio 5 somewhat decreased the $Ca^{2+}$ permeability of the sarcolemma vesicles compared to the situation of albumin alone (Fig. 6). This effect of low concentration of free oleate (0.1 $\mu$mol/mg membrane protein) may be due to inhibition of $Ca^{2+}$ release from membranes, as was observed for skeletal muscle sarcoplasmic reticulum by the group of Katz [29].

TABLE III

$^{22}Na$ RELEASE FROM CARDIAC SARCOLEMMA VESICLES

$^{22}Na$ efflux was initiated by adding a 5 μl $^{22}NaCl$-preloaded vesicle suspension to 200 μl medium containing 160 mM KCl, 20 mM Mops (pH 7.4) as described under Methods. *n*, the number of experiments. The sampling times after the $^{22}Na$ efflux was started were 10 s, 1, 2, and 45 min.

| | *n* | $^{22}Na$ content in nmol/mg | | | |
|---|---|---|---|---|---|
| | | 10 s | 1 min | 2 min | 45 min |
| Control | 7 | 320 ± 25 | 278 ± 18 | 225 ± 17 | 36 ± 13 |
| 0.025 mM palmitoylcarnitine | 7 | 340 ± 25 | 283 ± 29 | 244 ± 25 | 27 ± 10 |
| 0.050 mM palmitoylcarnitine | 5 | 263 ± 21 [a] | 211 ± 23 [a] | 181 ± 11 [a] | – |
| 1 mM oleate-albumin (7:1) | 3 | 259 ± 42 | 209 ± 33 [a] | 168 ± 29 [a] | 17 ± 5 [a] |
| 1 mM $CaCl_2$ | 7 | 285 ± 28 | 217 ± 32 [a] | 172 ± 29 [a] | 6 ± 1 [a] |
| 0.055 mM alamethicin | 4 | 10 ± 2 [a] | 6 ± 1 [a] | 6 ± 1 [a] | – |

[a] Values differ significantly from the corresponding control values ($P < 0.05$).

It was shown previously by us that fatty acid-albumin molar ratios higher than 5 were inhibitory to sarcolemma ($Na^+ + K^+$)-ATPase which was estimated in a sarcolemma preparation partially solubilized by using desoxycholate. Similar results are obtained in the presently used native sarcolemma preparation (Table II). The complex of molar ratio 7 (0.1 μmol free oleate per mg membrane protein) was producing a strong inhibition of ouabain-sensitive ($Na^+ + K^+$)-ATPase in either control or SDS-treated sarcolemma vesicles (Fig. 6). From the results shown in Fig. 4 and Table II can be concluded that the potency of free fatty acid to inhibit ($Na^+ + K^+$)-ATPase in native sarcolemma vesicles is much greater than that of acylcarnitine.

*Effect of the lipid intermediates on the $Na^+$ permeability of sarcolemma*

It is generally believed that the $Na^+/K^+$-pumping ATPase has an important contribution in regulating the $Ca^{2+}$ gradient across the sarcolemma by its influence on the rate and net direction of the $Na^+/Ca^{2+}$ antiporter. Not only a depression of the ($Na^+ + K^+$)-ATPase activity but also an increase of the sarcolemma permeability to monovalent cations may result into a derangement of regulation of in vivo $Ca^{2+}$ movements. Therefore the possibility that acylcarnitine or free fatty acid alters the sarcolemma permeability to sodium ions was investigated by $^{22}Na$ flux studies. For this purpose sarcolemma vesicles were preloaded with $^{22}Na$ by preincubating them in 160 mM $^{22}NaCl$ for 20 h at 0–4°C. The $^{22}Na$ efflux was initiated by rapid 40-fold dilution in 160 mM KCl medium. First the ability of the sarcolemma fraction to exchange $^{22}Na$ for external $Ca^{2+}$ was studied. As can be seen from the results in Table III, 1 mM $CaCl_2$ caused 20% more $^{22}Na$ release than in the control situation at the 1 min time point which indicates that still the $Na^+/Ca^{2+}$ antiporter can operate in these preincubated sarcolemma vesicles. It corresponds with 70 nmol $Na^+$ per mg sarcolemma protein released, and, if 3 $Na^+$ for 1 $Ca^{2+}$ exchange is assumed (cf. Refs. 17, 22, 30, 31), 24 nmol $Ca^{2+}$/mg sarcolemma protein are taken up by the sarcolemma vesicles. Thus, no more $Na^+$ release can be expected on base of the maximum $^{45}Ca$ uptake observed before (compare Figs. 1 and 2). The ionophore alamethicin was able to release almost all $^{22}Na$ within 10 s, the first time point taken in the Millipore filtration stop method (Table III). Both palmitoylcarnitine at 50 μM and oleate-albumin complex of molar ratio 7 were able to stimulate $^{22}Na$ release. If the lipid intermediate over membrane protein ratio (for palmitoylcarnitine and oleate this ratio amounted to 0.30 and 0.04 μmol/mg, respectively), is taken into account, it can be concluded that at least oleate seems to be more effective in increasing $Na^+$ permeability than increasing $Ca^{2+}$ permeability.

## Discussion

In the present study it has been demonstrated that an important in vitro effect of endogenously occurring free fatty acids and acylcarnitines is the increase of the $Na^+$ and $Ca^{2+}$ permeability of cardiac sarcolemma. These findings are in agreement with previous reports on the in vitro effect of fatty acids and palmitoylcarnitine on $Ca^{2+}$ permeability of the sarcoplasmic reticulum [33,34]. It is still unclear whether the concentrations of these lipid intermediates used in in vitro experiments are indeed present under ischemic conditions in the heart. From the values given by Idell-Wenger et al. [7] it was calculated that the cytosol content of acylcarnitine in normal and ischemic heart is 150 and 780 nmol/g wet weight. It is important to recognize that the affinity of palmitoylcarnitine is such that there is very little free palmitoylcarnitine either within the cell or in in vitro sarcolemma vesicle suspensions [12]. Thus it would be expected that this 'cytosol' palmitoylcarnitine would be associated with sarcolemma and network sarcoplasmic reticulum Pitts et al. [32] estimated the ratio palmitoylcarnitine to sarcoplasmic reticulum protein by assuming the presence of 3 mg sarcoplasmic reticulum per g wet weight of heart and a contribution of 60% of the total cellular membrane area by the network sarcoplasmic reticulum. On the basis of these assumptions values of 30 and 160 nmol acylcarnitine per mg sarcoplasmic reticulum protein in normal and ischemic hearts were calculated, respectively [32]. If these estimations would also be true for the sarcolemma it is evident from the present study that acylcarnitine concentrations in the ischemic heart are high enough to affect significantly the permeability of the surface membrane. On the other hand, if one would consider the tissue phospholipids (homogenate and isolated sarcolemma have been found to contain 150 and 1500 nmol phospholipid/mg protein, respectively, unpublished results) as the main dissolving and binding compartment into which acylcarnitine distributes, it can be calculated that acylcarnitine accumulating up to 780 nmol/g wet weight [7] will be present in a molar ratio of 1/25 to tissue phospholipids. This is also close to the 1/10 molar ratio reached if 150 nmoles of palmitoylcarnitine/mg sarcolemma protein are added in the in vitro vesicle studies. At any rate, these calculations are valuable for determining whether the amounts of palmitoylcarnitine added to the sarcolemma membranes are in the right order of magnitude rather than a hundred times too high or too low in comparing to the amounts reached in vivo under pathological conditions.

Theoretical determination of the amount of free fatty acid per mg membrane protein from known values of the tissue free fatty acid contents in ischemia should be done with more caution than in the case of acylcarnitine. In the in vitro experiments oleate was added to the sarcolemma membranes in the form of oleate-albumin complexes as these are similarly offered from the plasma to the cardiac cell in vivo. Although fatty acid-albumin ratios of 4 or more are very usual in the plasma, even in pathological states, interstitial accumulation (due to low albumin concentration in the interstitium compared to that of plasma, compare Ref. 35) and intracellular accumulation of free fatty acid due to a hampered $\beta$-oxidation may very likely lead to high concentrations of unbound fatty acid. Other factors which are contributing to free fatty acid accumulaton are the increased intracellular lipolysis and interstitial apolipoprotein C-II-dependent lipoprotein lipase action. Pronounced accumulation of nonesterified fatty acids, up to 200 nmol/g wet weight, has been observed in biopsies taken from ischemic areas of the ventricular wall of dog hearts [9]. In some pilot experiments in our laboratory the left anterior coronary artery was ligated for 3 h in anesthetized pigs and we measured a total nonesterified fatty acid concentration of $590 \pm 170$ nmol/g wet weight (S.E., $n = 4$) in biopts taken from the left ventricle, which concentration is higher than that observed in dog hearts after 2 h ischemia by Van der Vusse et al. [9]. A significant part of this fatty acid may be present in the interstitium making calculations of the concentration of unbound free fatty acid in compartments close to the sarcolemma doubtful [36].

Previously, it was shown by us and others [2,16,37,38] that free fatty acids are potent inhibitors of $(Na^+ + K^+)$-ATPase. This effect of free fatty acids is confirmed in the present investigation concerning the findings with SDS-treated sarcolemma vesicles. A very important observation

was that the inhibitory action of fatty acid could also be shown in a native sarcolemma preparation. This appeared not to be true for palmitoylcarnitine which at 25 $\mu$M was not equally effective in inhibiting $(Na^+ + K^+)$-ATPase in the native compared with the SDS-treated sarcolemma vesicles. This result is in agreement with those of Owens et al. [12] who obtained no effect of palmitoylcarnitine on sarcolemma $(Na^+ + K^+)$-ATPase using concentrations of the lipid intermediate up to 10 $\mu$mol/mg membrane protein whereas considerable inhibitory potency for palmitoylcarnitine has been reported against the activity of deoxycholate-treated $(Na^+ + K^+)$-ATPase [3,38]. Apparently the detergent effect of deoxycholate or SDS may be sufficient for the lipid intermediate to enter the lipid bilayer to readily affect the $(Na^+ + K^+)$-ATPase. An alternative explanation would be that the presently used sarcolemma preparation, containing mainly rightside-out oriented vesicles, is affected by the lipid intermediates in a different manner as a sarcolemma (compare Ref. 19) preparation containing inside-out oriented vesicles. This would imply that palmitoylcarnitine can only inhibit the vectorial enzyme $(Na^+ + K^+)$-ATPase at its cytosolic side of the sarcolemma membrane. However, from another point of view, it should be questioned whether the vesicle sidedness is important for studying the effect of lipid intermediates on vectorial membrane properties: the amphiphiles studied are lipophilic in character and therefore may rapidly pass the membrane having access to both sides. But a preferential binding of palmitoylcarnitine to the inner part of the sarcolemma in vivo may be expected due to its positive charge assuming that more acidic phospholipids are localized at the cytosolic side of the surface membrane.

In conclusion, it is not clear whether the observed inhibition of $(Na^+ + K^+)$-ATPase by palmitoylcarnitine in SDS-treated sarcolemma vesicles, has any relevance to pathological situations.

The effects of free fatty acid and palmitoylcarnitine on either $(Na^+ + K^+)$-ATPase or $Ca^{2+}$ and $Na^+$ permeability of cardiac sarcolemma membrane may have significance for the cause of derangements of cardiac cell function as seen in ischemia [5,6]. Inhibition of the $(Na^+ + K^+)$-ATPase pump and an increase in $Na^+$ permeability of the sarcolemma membrane may lead to elevation of intracellular $Na^+$, which increases the net $Ca^{2+}$ influx via $Na^+$-$Ca^{2+}$ exchange. Also the effect on $Ca^{2+}$ permeability may have consequences for the steepness of the $Ca^{2+}$ gradient across the sarcolemma membrane. The concomitant increase of $Ca^{2+}$ currents possibly explains the increased excitability of the ventricular cells during an ischemic period [15]. It is likely that cellular $Ca^{2+}$ overload plays a major role in the development of cardiac failure and necrosis in ischemia [13,14,39,40].

## Acknowledgement

The authors wish to thank Miss Cecile Hanson for typing the manuscript.

## References

1 Kurien, V.A., Yates, P.A. and Oliver, M.F. (1971) Eur. J. Clin. Invest. 1, 225–241
2 Lamers, J.M.J. and Hülsmann, W.C. (1977) J. Mol. Cell. Cardiol. 9, 343–346
3 Adams, R.J., Cohen, D.W., Gupte, S., Johnson, J.D., Wallick, E.T., Wang, T. and Schwartz, A. (1979) J. Biol. Chem. 254, 12404–12410
4 Katz, A.M. and Messineo, F.C. (1981) Circ. Res. 48, 1–16
5 Opie, L.H. (1979) Am. Heart J. 97, 375–388
6 Liedtke, A.J. (1981) Progr. Cardiovasc. Dis. 23, 321–336
7 Idell-Wenger, J.A., Grotyohann, L.W. and Neely, J.L. (1978) J. Biol. Chem. 253, 4310–4319
8 Shug, A.L., Thomsen, J.H., Folts, J.D., Bittar, N., Klein, M.I., Koke, J.R. and Huth, P.J. (1978) Arch. Biochem. Biophys. 187, 25–33
9 Van der Vusse, G.J., Roemen, T.H.M., Prinzen, F.W., Coumans, W.A. and Reneman, R.S. (1982) Circ. Res. 50, 538–546
10 Karnowsky, M.J. (1979) Am. J. Pathol. 97, 212–221
11 Inoue, D. and Pappano, A.J. (1983) Circ. Res. 52, 625–634
12 Owens, K., Kennett, F.F. and Weglicki, W.B. (1982) Am. J. Physiol. 242, H456–H461
13 Dhalla, N.S., Das, P.K. and Sharma, G.P. (1978) J. Mol. Cell. Cardiol. 10, 363–385
14 Farber, J.L. (1981) Life Sci. 29, 1289–1295
15 Clusin, W.T., Buchbinder, M. and Harrison, D.C. (1983) Lancet i, 272–273
16 Wood, J.W., Bush, B., Pitts, B.J.R. and Schwartz, A. (1977) Biochem. Biophys. Res. Commun. 74, 677–684
17 Lamers, J.M.J. and Stinis, J.T. (1981) Biochim. Biophys. Acta 640, 521–534
18 Caroni, P. and Carafoli, E. (1981) J. Biol. Chem. 256, 3263–3270

19 Lamers, J.M.J. and Stinis, J.T. (1982) in Advances in Studies on Heart Metabolism (Caldarera, C.M. and Harris, P., eds.), pp. 41–47, CLUEB, Bologna
20 Lamers, J.M.J. and Stinis, J.T. (1983) Cell Calcium 4, 281–294
21 Jones, L.R., Maddock, S.W. and Hathaway, D.R. (1980) J. Biol. Chem. 255, 9971–9980
22 Reeves, J.P. and Sutko, J.L. (1980) Science 208, 1461–1464
23 Lamers, J.M.J., Stinis, J.T. and De Jonge, H.R. (1981) FEBS Lett. 127, 139–143
24 Lamers, J.M.J. and Stinis, J.T. (1980) Biochim. Biophys. Acta 624, 443–459
25 Lamers, J.M.J. and Stinis, J.T. (1979) Life Sci. 24, 2313–2320
26 Flockerzi, V., Mewes, R., Ruth, P. and Hofmann, F. (1983) Eur. J. Biochem. 135, 131–142
27 Spector, A.A. and Fletcher J.E. (1978) in Disturbances in Lipid and Lipoprotein Metabolism (Dietschy, J.M., Gotto, A.M. and Ontko, J.A., eds.), pp. 229–249, Am. Physiol. Soc., Bethesda
28 Slaughter, R.S., Sutko, J.L. and Reeves, J.P. (1983) J. Biol. Chem. 258, 3183–3190
29 Katz, A.M. Messineo, F.C., Miceli, J. and Nash-Adler, P.A. (1981) Life Sci. 28, 1103–1107
30 Pitts, B.J.R. (1979) J. Biol. Chem. 254, 6232–6235
31 Bers, D.M., Philipson, K.D. and Nishimoto, A.Y. (1980) Biochim. Biophys. Acta 601, 358–371
32 Pitts, B.J.R., Tate, C.A., Van Winkle, B., Wood, J.M. and Entman, M.L. (1978) Life Sci. 23, 391–402
33 Messineo, F.C., Pinto, P.B. and Katz, A.M. (1982) in Advances in Myocardiology (Chazov, E., Smirnov, V. and Dhalla, N.S., eds.), Vol. 3, pp. 407–415, Plenum Publishing, New York
34 Cheah, A.M. (1981) Biochim. Biophys. Acta 648, 113–119
35 Landis, E.M. and Rappenheimer, J.R. (1963) in Handbook of Physiology, Section 2: Circulation (Hamilton, W.F. and Dow, P., eds.), Vol. 2, pp. 96–1034, Am. Physiol. Soc., Washington
36 Stam, H. and Hülsmann, W.C. (1981) Biochem. Int. 2, 477–484
37 Ahmed, K. and Thomas, B.S. (1971) J. Biol. Chem. 246, 103–109
38 Adams, R.J., Pitts, B.J.R., Woods, J.M., Gende, O.A., Wallick, E.T. and Schwartz, A. (1979) J. Mol. Cell. Cardiol. 11, 941–959
39 Dhalla, N.S. (1976) J. Mol. Cell. Cardiol. 8, 661–667
40 Hearse, D.J. (1977) J. Mol. Cell. Cardiol. 9, 605–616