Biochemical Pharmacology, Vol. 38, No. 8, pp. 1231–1239
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

DIFFERENTIAL EFFECTS OF AMIODARONE AND PROPRANOLOL ON LIPID DYNAMICS AND ENZYMATIC ACTIVITIES IN CARDIAC SARCOLEMMAL MEMBRANES

P. CHATELAIN,*† R. LARUEL,* P. VIC‡ and R. BROTELLE*

*SANOFI, Centre de Recherche Labaz-Sanofi, avenue de Béjar I B-1120 Brussels, Belgium, and ‡SANOFI, Centre de Recherche Clin-Midy, rue du Professeur J. Blayac F-34082 Montpellier, France

(*Received* 27 *May* 1988; *accepted* 19 *October* 1988)

Abstract—The amphiphilic cationic cardioactive drugs (pindolol, propranolol and amiodarone) were tested for their effects on lipid dynamics (measured by fluorescence depolarization) and on enzymatic activities up to 1 mM in purified cardiac sarcolemmal vesicles from adult rat. The vesicles were enriched 12- to 37-fold (with respect to tissue homogenate) in Na^+/K^+ ATPase, K^+-stimulated *p*-nitrophenylphosphatase, 5'nucleotidase and adenylate cyclase, all of which are believed to be components of sarcolemma. Phospholipids and cholesterol content were enriched 5- and 13-fold respectively. There was very little contamination of the sarcolemmal vesicles by sarcoplasmic reticulum (as judged by Ca^{2+} ATPase and glucose-6-phosphatase activities) or mitochondria (as judged by cytochrome-*c*-oxidase activity). Pindolol had no effect on lipid dynamics and enzyme activities except for the isoproterenol-stimulated adenylate cyclase. The latter was also totally inhibited at 1 μM by propranolol which inhibited Mg^{2+} ATPase and increased fluidity above 20 μM. Amiodarone affected all the enzyme activities (except Na^+/K^+ ATPase): isoproterenol-stimulated adenylate ($IC_{50} = 30\ \mu M$), Mg^{2+} ATPase ($IC_{50} = 20\ \mu M$) and K^+-stimulated-*p*-nitrophenylphosphatase were inhibited; 5'nucleotidase was activated above 2 μM. By contrast with propranolol, amiodarone decreased lipid mobility. The effect was linear with the concentration of the drug above 1 μM.

The cell-plasma membrane of the heart, the sarcolemma, plays a key role in the mediation of metabolic, electrophysiological and contractile functions of myocytes. The preparation of pure sarcolemmal membranes, which is a prerequisite to ascertain the function of the sarcolemma in physiology and pathology, has remained a methodological challenge.

In the present study, a cardiac sarcolemmal membrane preparation of high purity from adult rat has been developed for use in studies of the mechanisms of action of hormones and cardioprotective drugs. Among these latter compounds, we focused our at ention on propranolol and amiodarone, two antianginal and anti-arrhythmic drugs [1, 2]. Most of the pharmacological properties of propranolol appear to be determined by its β-adrenergic receptor-blocking properties [3]. The electrophysiological characteristics of the drug correspond to Class II antiarrhythmics according to Vaughan-Williams [4]. Amiodarone has, among its pharmacological properties, non-competitive α- and β-adrenergic blocking properties [5, 6] and has the electrophysiological characteristics of a Class III antiarrhythmic agent [7]. On the other hand, propranolol displays a non-specific membrane stabilizing effect [3, 8] which may interplay with β-adrenergic blockade in the complex pharmacological properties of the drug. Amiodarone displays significant hydrophobic behaviour as evidenced by micelle formation [9], a high degree (>95%) of fixation to serum proteins [10], a high value of the octanol/buffer partition coefficient and a pronounced effect on lipid dynamics in purified synaptic membranes [11]. Due to their hydrophobic behaviour, the lipid components have been implicated in the ability of propranolol [8, 12] and amiodarone [11] to interact with the membrane which in turn produces alterations in the functional properties of membrane enzymes.

In this communication, we report characteristics of the sarcolemmal membrane preparation and describe the effects of amiodarone, propranolol and pindolol on several enzymatic activities (Na^+/K^+ ATPase, K^+ PNPase/§, Mg^{2+} ATPase, 5'-nucleotidase and basal and isoproterenol-stimulated adenylate cyclase) as well as on lipid dynamics tested in identical experimental conditions.

MATERIALS AND METHODS

Isolation of sarcolemma-enriched fraction from cardiac muscle. Male Sprague–Dawley rats weighing 200–300 g were killed by decapitation. The fractionation procedure outlined in Fig. 1 was carried out at 4° unless otherwise stated. Hearts were quickly removed and placed in oxygenated buffer containing 124 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 20 mM Na_3PO_4, 10 mM glucose, pH 7.4 (buffer A). Ventricular tissue was minced with a Delepine press

† To whom correspondence should be addressed.

§ Abbreviations used: PMSF, phenylmethylsulfonyl fluoride; DPH, 1,6-diphenyl-1,3,5-hexatriene; *P*, degree of fluorescence depolarization; K^+PNPase, K^+-stimulated para-nitrophenyl-phosphatase; IC_{50}, concentration of drug necessary to inhibit 50% of enzymatic activity.

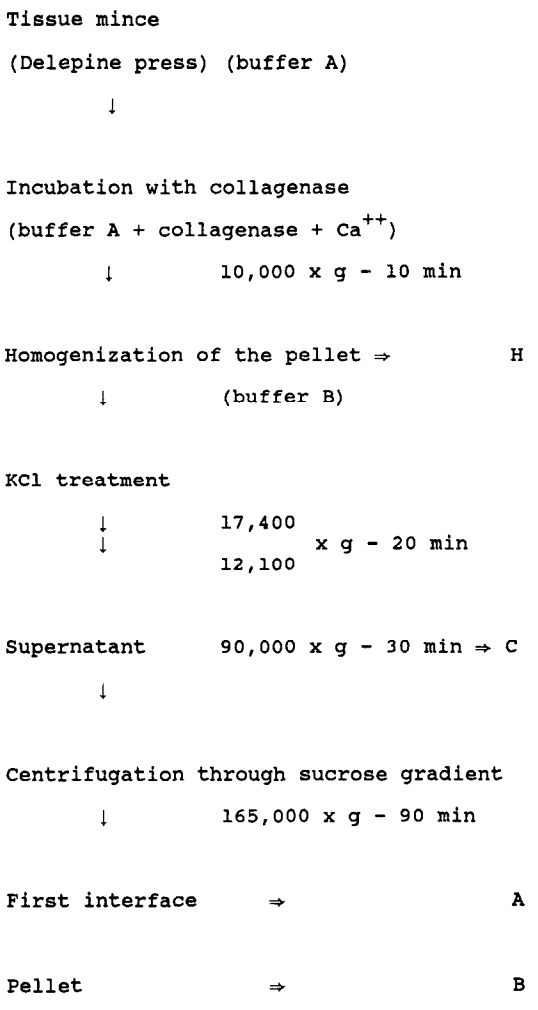

Fig. 1. Procedure for preparing sarcolemma-enriched fraction from rat cardiac muscle.

(Arthur Thomas, Philadelphia, PA) equipped with a grid with 1 mm^2 mesh. A 20% (w/v) suspension of the minced tissue in buffer A containing 25 μM $CaCl_2$ and 0.5 mg/ml collagenase was incubated at 37°. After an incubation time of 15 min, the suspension was centrifuged at 10,000 *g* for 10 min. The 20% w/v suspension of the pellet in 1 mM $NaHCO_3$, 1 mM PMSF, 1 mM 1,4-dithioerythritol, pH 8.0 (buffer B), was homogenized using a motor-driven glass–Teflon homogenizer (8 passes at 650 rpm). The resulting homogenate was filtered through nylon (0.4 mm^2 mesh). To the homogenate (H), an equal volume of the buffer containing 1.2 M KCl, 100 mM Na pyrophosphate, 1 mM $NaHCO_3$, 1 mM PMSF, 0.1 mM 1,4-dithioerythritol, pH 8.0 was added. The homogenate was gently stirred for 30 min. It was then centrifuged at 17,400 *g* for 20 min. The pellet was resuspended in buffer B and re-centrifuged at 12,100 *g* for 20 min. The pooled supernatants were centrifuged at 90,000 *g* for 30 min. The resulting pellet (C) was washed twice in 2.5 mM $MgCl_2$ 25 mM Na pyrophosphate, 1 mM $NaHCO_3$, 0.1 mM 1,4-dithioerythritol, pH 8.0. The washed pellet was resuspended by hand in 30% (w/v) sucrose, 300 mM KCl, 50 mM Na pyrophosphate, 100 mM Tris, pH 7.6, at the approximate concentration of 6 mg protein/ml. In a 5 ml tube there were successively layered 3 ml of the homogenate obtained as described above, 1 ml of 25% (w/v) sucrose, 300 mM KCl, 50 mM Na pyrophosphate, 100 mM Tris, pH 7.6, and 1 ml of 250 mM sucrose (8.5% w/v), 120 mM KCl, 20 mM Na pyrophosphate, 30 mM NaCl, 0.1 mM PMSF, 30 mM imidazole, pH 6.8. The sucrose gradient was centrifuged at 220,000 *g* for 90 min. The upper interface (A) and the pellet (B) were collected. After dilution in 5 mM Tris, 0.1 mM 1,4-dithioerythritol, 1 mM EDTA, pH 7.4, the material was collected by centrifugation at 145,000 *g* for 30 min. The final pellets were homogenized in 250 mM sucrose, 0.1 mM 1,4-dithioerythritol, 5 mM Tris, pH 7.4 (buffer C) and stored in liquid nitrogen. For determination of membrane purification, samples of the homogenate (H) and the pellet (C) were resuspended in buffer C and stored in liquid nitrogen.

Analytical methods. K^+-stimulated para-nitrophenyl phosphatase activity (K^+ PNPase) was measured as described by Tashima *et al.* [13] in a medium containing 5 mM Tris-nitrophenylphosphate, 1 mM $MgCl_2$; 20 μM EDTA, 0.05% mercaptoethanol, 25 mM Tris, pH 7.4, with or without 0.5 mM KCl. ATPase activity (Na^+/K^+ ATPase and Mg^{2+} ATPase) was assayed according to Bers [14] as described by Chatelain *et al.* [15] in a medium containing 100 mM NaCl, 15 mM KCl, 1 mM EGTA, 4 mM $MgCl_2$, 50 mM Tris–HCl, 5 mM ATP–Tris, pH: 7.4 (total ATPase) or 1 mM EGTA, 4 mM $MgCl_2$, 50 mM Tris, 5 mM ATP–Tris, pH 7.4 (Mg^{2+} ATPase). The free inorganic phosphate was determined according to Fiske and Subbarow [16]. 5′Nucleotidase activity was measured as described by Campbell [17] in a medium containing 10 mM AMP, 5 mM $MgCl_2$, 40 mM barbital, pH 7.5, with and without 10 mM $NiCl_2$. Adenylate cyclase activity was measured as described by Minneman *et al.* [18] in a medium containing 500 μM [α-^{32}P]ATP, 5 mM $MgCl_2$, 500 μM EGTA, 1 mM cAMP, 500 μM theophylline, 10 mM phospho(enol)pyruvate, pyruvate kinase (30 μg/ml) and 30 mM Tris–HCl, pH 7.5. Ca^{2+} ATPase activity was measured as described by Jones *et al.* [19] in a medium containing 50 mM histidine, 3 mM $MgCl_2$, 100 μM $CaCl_2$ (instead of 50 μM), 100 mM KCl and 3 mM ATP, pH 7.4. Ca^{2+} ATPase activity was taken as that activity inhibitable by 1 mM Tris–EGTA. In all assays, the divalent cation ionophore A 23187 (3 μg/ml) was included in the assay medium to eliminate calcium accumulation spaces. Glucose-6-phosphatase activity was measured as described by Baginski [20] in a medium containing 10 mM glucose-6-phosphate, 250 μM EDTA, 62.5 mM saccharose and 25 mM cacodylate, pH 6.5. Cytochrome oxidase activity was measured as described by de Duve *et al.* [21] in a medium containing 17 μM cytochrome *c*, 6 mM EDTA, 30 mM phosphate, pH 7.4. Protein was assayed by the method of Lowry *et al.* [22] using bovine serum albumin as standard. When assayed for drug effects, the sarcolemmal membranes (0.1 mg/ml in the specific assay medium) were incubated 20 min at 25° with the solvent (DMSO) or the desired amount of

drug dissolved in the solvent so that the DMSO final concentration did not exceed 2% [23]. The membranes were then diluted 2–5-fold for enzymatic assays.

For electron microscopy freshly isolated membrane fractions were fixed in 2% glutaraldehyde for 1 hr at 4° followed by 2 hr at room temperature. Then they were postfixed in osmium tetroxide for 1 hr and dehydrated in graded ethanol. After Epon embedding, thin sections were prepared using a Reichert ultra microtome. After staining (uranyl acetate and lead citrate), the grids were examined through Jeol 100 Electron microscope.

Lipids were extracted according to Bligh *et al.* [24] with minor modifications as described by Okumura *et al.* [25]. Individual phospholipids were separated by two-dimensional thin-layer chromatography. The first and second developing solvents consisted respectively of $CHCl_3/CH_3OH/NH_4OH$ (13:5:1) and of $CHCl_3/CH_3OH/CH_3COCH_3/CH_3COOH/H_2O$ (6:2:8:2:1). Phosphorus content was determined according to Itaya and Ui [26]. Cholesterol content was determined by means of an enzymatic color test (Boehringer, Ingelheim, F.R.G.) as modified by Ott *et al.* [27].

Fluorescence measurements. Sarcolemmal membranes were labeled with 1,6-diphenyl-1,3,5-hexatriene (DPH) according to Shinitzky and Inbar [28]: membranes (0.1 mg/ml in 134 mM NaCl, 20 mM Tris, pH 7.4 buffer) were incubated with 1 μM DPH (taken from a 1 mM stock solution in tetrahydrofuran) at 25° for 20 min. This was found to be the minimum time required to reach a steady-state level of fluorescence intensity. Membrane and probe concentrations were shown to be low enough to avoid depolarization artifacts and probe interaction respectively. After labeling, the sarcolemmal membranes incubated 20 min at 35° with the solvent (DMSO) or the desired amount of drug dissolved in the solvent so that the DMSO final concentration did not exceed 2% [23]. The membranes were then diluted 2–5-fold for fluorescence measurements.

Steady-state measurements of the degree of fluorescence depolarization (P) and excited-state lifetimes (τ) of DPH were performed on a SLM 4800 spectrofluorimeter (Urbana-Champaign, IL) working in the T format. The polarized emitted light ($\lambda_{ex} = 356$ nm) was detected in two independent cross-polarized channels passing through cut-off filters ($\lambda_{em} > 418$ nm) or a monochromator ($\lambda_{em} = 450$ nm) [11]. The steady-state fluorescence depolarization (P) is defined as follows:

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} - I_{\perp}}$$

where $I_{\parallel}$ and $I_{\perp}$ are the intensities of the light emitted with its polarization plane parallel ($\parallel$) and perpendicular ($\perp$) to that of the exiting beam.

Chemicals. Chemicals were reagent of analytical grade and obtained from Merck (Darmstadt, F.R.G.). Biochemicals were obtained from Sigma (St Louis, MO) unless otherwise stated. Alamethicin was a generous gift from Dr. R. L. Keene (Infectious Diseases Research, Upjohn, Kalamazoo, MI). Propranolol and pindolol were obtained from ICI Ltd. (Alderly Park, U.K.) and Sandoz Ltd. (Basel, Switzerland) respectively.

RESULTS

Sarcolemmal membranes preparation and characterization

The activity of several typical marker enzymes determined at various stages of the sarcolemmal membranes preparation is listed in Table 1 together with protein recovery. Fraction A which contains 0.38% of the proteins of the homogenate (H) is clearly enriched in K^+ PNPase, Na^+/K^+ ATPase, 5'nucleotidase and adenylate cyclase, all considered as sarcolemmal marker enzymes [29]. By contrast, there is little contamination by sarcoplasmic reticulum as judged by the low specific activity of glucose-6-phosphatase and the absence of Ca^{2+} ATPase activity. As checked by others [30, 31], the collagenase digestion has no effect on the enzymatic activities.

A more direct comparison of the degree of purification can be made from the comparison of the enrichment (Table 1). Enrichment of the pellet C which contains 6% of the proteins of the homogenate is relatively low as compared to the homogenate. The main enrichment is observed in the discontinuous sucrose gradient where (1) Na^+/K^+ ATPase, K^+ PNPase, 5'nucleotidase and adenylate cyclase are enriched 12–37-fold, and (2) Ca^{2+} ATPase and cytochrome *c* oxidase are eliminated from the upper interface and found in the pellet B. The specific activity of Na^+/K^+ ATPase should correspond to that of K^+ PNPase since K^+ PNPase represents one step of the sequence of ATP hydrolysis by Na^+/K^+ ATPase [13]. This discrepancy can be partially resolved by the introduction into the assay medium of reagents such as alamethicin or sodium dodecyl sulfate [32], which increase membrane permeability and unmask latent enzyme activity. Alamethicin when present at 0.1 mg/mg protein leads to an increase of 75% in the Na^+/K^+ ATPase activity. The enrichment in Na^+/K^+ ATPase activity is then close to that obtained for K^+ PNPase activity (Table 1). The effect of alamethicin suggests that part of the sarcolemmal membranes are present under the form of closed vesicles. This conclusion is confirmed by the electron transmission micrographs which indicate a high degree of vesiculation of the membrane present in fraction A (Fig. 2). The membrane vesicles are of varying sizes, they are generally empty although occasionally small vesicles are entrapped in the larger vesicles. Note that the micrographs are devoid of intact mitochondria confirming the biochemical data (Table 1) on the relative purity of fraction A.

The lipid composition of the sarcolemmal membranes was characterized. The purified membranes contain 0.73 ± 0.07 μmol P_i/mg of protein and 0.19 ± 0.03 μmol cholesterol/mg of protein (mean ± SEM of five determinations). This constitutes a 5- and 13-fold enrichment respectively as compared to the homogenate which contains 0.15 ± 0.01 μmol P_i and 0.015 ± 0.002 μmol cholesterol per mg of protein (mean ± SEM of 8 determinations). The cholesterol–phospholipid

Table 1. Enzymatic activity and enrichment of several positive and negative marker enzymes of the sarcolemmal membrane

Enzymes or proteins	Membranous fractions			
	H	C	A	B
Protein %	100	6.0 ± 5.6	0.385 ± 0.039	3.01 ± 0.38
Mg^{2+} ATPase	34.7 ± 2.5 (1×)	50.7 ± 11.0 (1.5×)	121.8 ± 14.2 (3.5×)	19.8 ± 4.4 (0.6×)
Na^+/K^+ ATPase	5.5 ± 1.0 (1×)	12.4 ± 2.7 (2.2×)	68.1 ± 10.4 (12.4×)	5.4 ± 0.7 (1×)
K^+ PNPase	0.54 ± 0.04 (1×)	2.93 ± 0.71 (5.3×)	20.2 ± 1.9 (37.2×)	0.72 ± 0.23 (1.3×)
Adenyl. cyclase	1.36 ± 0.28 (1×)	5.2 ± 0.8 (3.8×)	50.4 ± 10.0 (37×)	1.8 ± 0.3 (1.3×)
5'nucleotidase	0.65 ± 0.18 (1×)	3.4 ± 1.4 (5.0×)	7.9 ± 3.0 (12.7×)	0.22 ± 0.04 (0.4×)
Ca^{2+} ATPase	2.04 ± 0.65 (1×)	10.83 ± 6.55 (5.3×)	ND (0)	3.46 ± 0.14 (1.7×)
Glucose-6-P	0.222 ± 0.020 (1×)	0.624 ± 0.072 (2.8×)	0.791 ± 0.084 (3.6×)	0.539 ± 0.003 (2.4×)
Cyt. *c* oxidase	37.4 ± 2.9 (1×)	82.7 ± 12.2 (2.2×)	18.8 ± 8.0 (0.5×)	119.6 ± 1.5 (3.2×)

H, C, A and B represent respectively the homogenate, the pellet and the first interface and the pellet of the discontinuous sucrose gradient (see Materials and Methods).

The results are the mean ± SD of three separate preparations. Enzymatic activities are expressed as μmol.mg protein^{-1}.hr^{-1} except the adenylate cyclase activity which is expressed as nmol cAMP.mg protein^{-1}.hr^{-1}. The adenylate cyclase is stimulated by 10 nM NaF.

Degree of enrichment given in parentheses.

ND: not detectable.

molar ratio of the sarcolemmal membranes averaged 0.26 which constitutes a 2.5-fold enrichment as compared to the homogenate. The mean phospholipid composition of the homogenate and the sarcolemmal fractions is listed in Table 2. The major phospholipids in the sarcolemmal membranes are phosphatidylcholine and phosphatidylethanolamine which represent 76% of the total. The former occurs in greater quantities than the latter. Among the minor components, sphingomyelin and phosphatidylserine + phosphatidylinositol are enriched 2–3-fold over the homogenate while the content of cardiolipin decreases 3-fold. The other phospholipid components which represent 1% or less (in particular lyso-compounds) are unchanged in the sarcolemmal membranes as compared to the homogenate. These results are in general in agreement with previous data [19, 29].

Effects of drugs on enzymatic activities in sarcolemmal membranes. The effects of increasing amounts of amiodarone, propranolol and pindolol on the activity of Na^+/K^+ ATPase, K^+ PNPase, Mg^{2+} ATPase, basal and stimulated adenylate cyclase and 5'nucleotidase were investigated. Neither propranolol nor pindolol has any effect on Na^+/K^+ ATPase activity up to 1 mM, while amiodarone at 1 mM inhibits the enzyme activity by 25% (not shown). This observation is confirmed by the effects of the drugs on K^+ PNPase activity: propranolol and pindolol have no effect up to 1 mM whereas at 1 mM amiodarone inhibits the enzyme by 50% with a threshold dose of 30 μM (Fig. 3). The

Table 2. Lipid composition of rat heart homogenate and sarcolemmal membrane

Phospholipids	% Homogenate	Sarcolemma
Lysophosphatidylcholine	0.9 ± 0.2	1.0 ± 0.4
Sphingomyelin	0.9 ± 0.2	1.7 ± 0.4
Lysophosphatidylethanolamine	0.7 ± 0.2	0.5 ± 0.2
Phosphatidylcholine	39.2 ± 1.1	44.5 ± 1.6
Phosphatidylserine + phosphatidylinositol	4.5 ± 0.8	12.7 ± 0.4
Phosphatidylethanolamine	35.1 ± 0.7	31.4 ± 0.8
Phosphatidylglycerol	0.9 ± 0.2	1.0 ± 0.4
Cardiolipin	16.5 ± 0.6	5.1 ± 0.8
Phosphatidic acid	0.7 ± 0.2	0.7 ± 0.4
Bisphosphatidic acid	0.4 ± 0.2	0
Unidentified	0.3 ± 0.2	0.7 ± 0.3

The results are the mean ± SEM of eight determinations for the homogenate and five determinations for the sarcolemmal membrane.

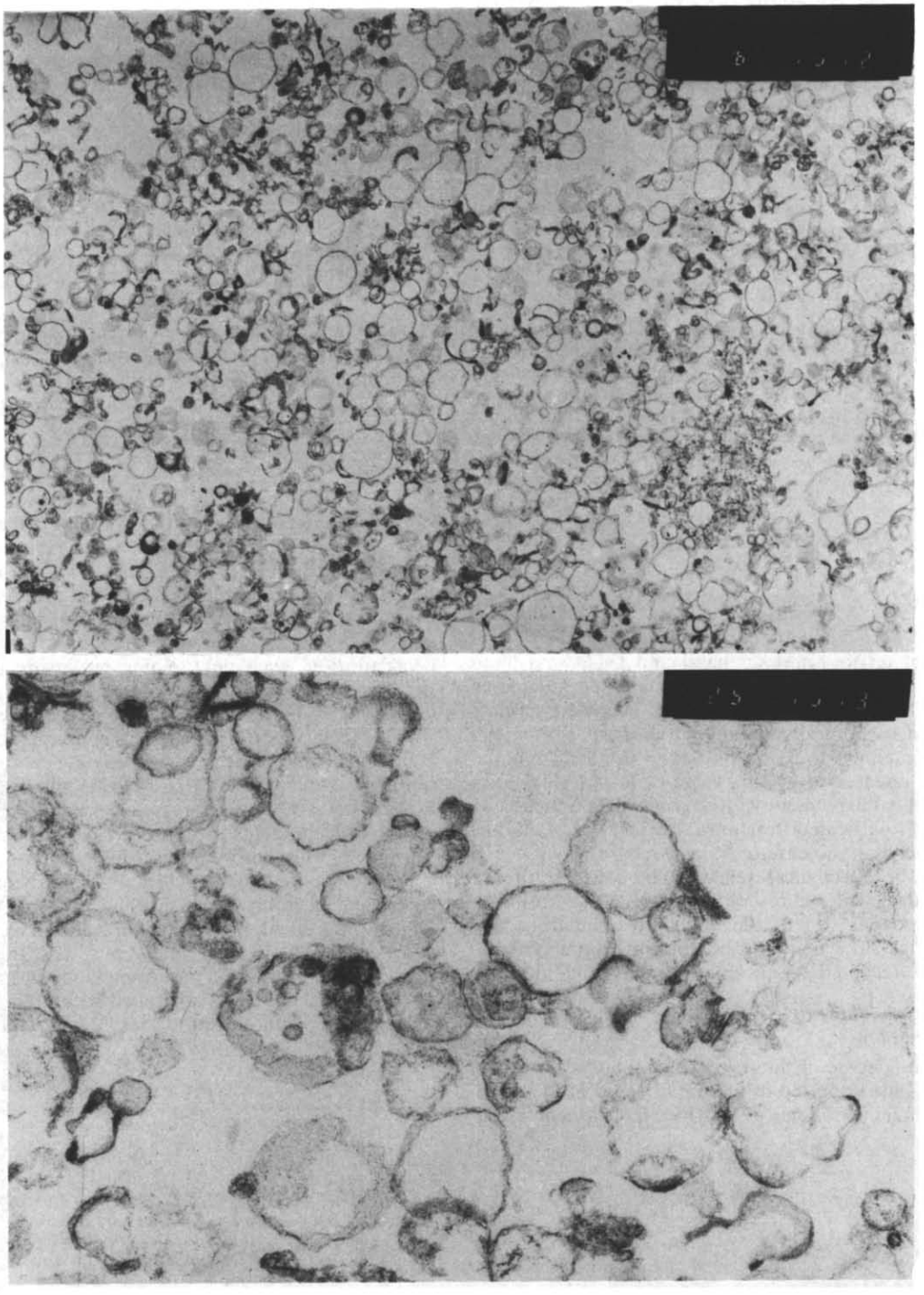

Fig. 2. Representative transmission electron micrograph showing the morphology of rat heart sarcolemmal membranes (fraction A). Magnification 12,000 (upper panel) and 50,000 (lower panel).

inhibitory effects of the drugs on Mg^{2+} ATPase activity are different from the above and from each other. Amiodarone exerts a marked effect, inhibiting enzyme activity by 50% (IC_{50}) at a concentration of about 20 μM. Next comes propranolol which has an estimated IC_{50} of about 0.8 mM and then pindolol which has no effect at all (Fig. 4). With respect to adenylate cyclase, the effects of the three drugs were tested on the basal and GTP-, fluoride- and isoproterenol- (in the presence of GTP) stimulated enzyme. The concentrations of the various stimuli and the corresponding activity (expressed as nmol cAMP produced.mg protein^{-1}.hr^{-1} at 37°, mean ± SEM of 3–5 determinations) were: GTP

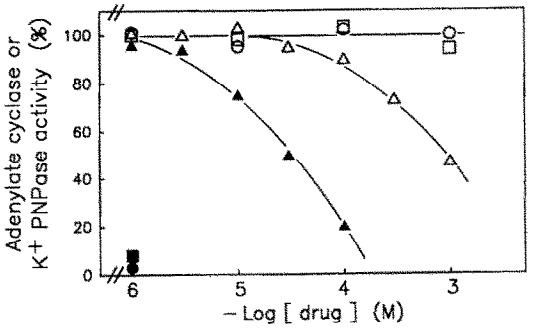

Fig. 3. Effects of amiodarone (△,▲), propranolol (○,●) and pindolol (□,■) on K^+PNPase activity (open symbols) and adenylate cyclase activity (closed symbols). For adenylate cyclase, isoproterenol and GTP concentrations were 0.1 mM and 10 μM respectively. The results represent the mean of three experiments, performed in duplicate, on sarcolemmal membranes from different preparations. In Figs 3–6, the SD is less than 10% and was omitted for clarity.

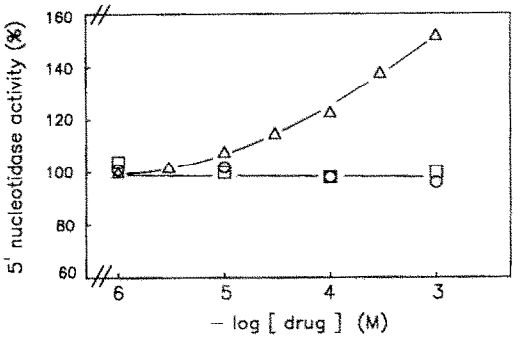

Fig. 5. Effects of amiodarone (△), propranolol (○) and pindolol (□) on 5'nucleotidase activity. The results represent the mean of four experiments, performed in duplicate, on sarcolemmal membranes from different preparations.

10 μM, 4.9 ± 0.4, NaF 10 mM, 50.4 ± 4.7, isoproterenol 0.1 mM, 18.6 ± 1.8. Basal activity was 2.3 ± 0.2. None of the drugs had any significant effect up to 1 mM on basal and GTP- and NaF-stimulated enzyme (not shown). When stimulated via the β-adrenergic receptor, adenylate cyclase activity was totally inhibited by 1 μM of propranolol or pindolol (Fig. 3). Amiodarone also had an inhibitory effect with an IC_{50} value of 30 μM (Fig. 3) in excellent agreement with previous data obtained on a KCl particulate fraction [6]. Finally, amiodarone induced an increase of 5'nucleotidase activity (Fig. 5). The effect increased with the concentration of the drug with a threshold dose of 3 μM. Among the compounds tested, the effect of amiodarone was specific since neither pindolol nor propranolol had any effect on the 5'nucleotidase activity up to 1 mM.

Effects of the drugs on lipid dynamics in sarcolemmal membranes

The degree of fluorescence depolarization (P) of DPH incorporated in the lipid bilayer of the sarcolemmal membranes was 0.216 ± 0.002 (mean ± SD, N = 5) at 37°. This value was unaffected when the sarcolemmal membranes were suspended in the various assay mediums used when measuring the enzyme activity. Furthermore, the degree of fluorescence depolarization is constant in a range of protein concentration from 0.02 to 0.1 mg/ml. That range corresponds to the adequate range of protein concentration when determining enzymatic activities, so that the determination of the degree of fluorescence depolarization and of the enzymatic activities can be performed in the same experimental conditions with respect to protein concentration, buffer and temperature. The effects of the three drugs tested are shown in Fig. 6 where the variation of fluorescence depolarization (ΔP) is plotted as a function of drug concentration. Amiodarone induced a decrease in lipid fluidity which was linearly related to its concentration with a threshold dose of 1 μM. On the other hand, propranolol induced an increase in lipid fluidity which was curvilinear as a function of the concentration of the drug. Pindolol had no effect on lipid dynamics as probed with DPH up to the highest concentration tested, i.e. 1 mM.

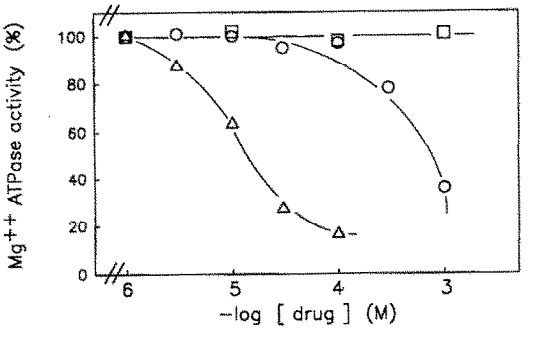

Fig. 4. Effects of amiodarone (△), propranolol (○) and pindolol (□) on Mg^{2+} ATPase activity. The results represent the mean of 2–3 experiments, performed in duplicate, on sarcolemmal membranes from different preparations.

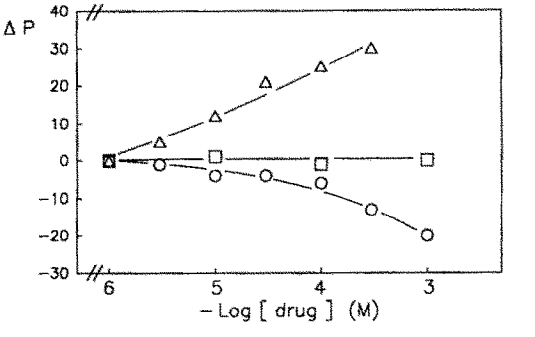

Fig. 6. Dose–response curves of the effects of amiodarone (△), propranolol (○) and pindolol (□) on the degree of fluorescence depolarization (P) of DPH embedded in the hydrocarbon core of the lipid bilayer of sarcolemmal membranes. ΔP is defined as $\Delta P = P_d - P$ where P_d and P represent the degree of fluorescence depolarization in presence (P_d) and in absence (P) of a given drug. The results represent the mean of 3–5 experiments.

Expressed in absolute values, the divergent effects of amiodarone on lipid dynamics were twice as great as those of propranolol.

DISCUSSION

To prepare and characterize heart sarcolemma is a difficult task exposed to uncertainties and considerable controversy [29, 14]. This is best illustrated by the diversity of the procedures developed to isolate heart sarcolemma [21, 14]. Although progress has been made in the last few years, most procedures have technical limitations which restrict their use: isolation of myocytes as starting material [30], long centrifugation time [19], complex operations such as heart slices incubation [31] or heart perfusion [33] with collagenase, which decrease the amount of material engaged in the procedure.

In the present study, we have developed a procedure of purification of sarcolemmal membranes of adult rat heart which aims at, *inter alia*, overcoming some of these limitations. To avoid unfavourable homogenization conditions, the procedure starts with the disruption of heart-tissue by proteolytic digestion followed by differential centrifugations, KCl–pyrophosphate treatment and sucrose gradient fractionation. The main improvement is obtained by use of the Delepine press which allows to obtain rapidly large amounts of minced tissue. The size of the mince is such that it allows an efficacious digestion by collagenase and a mild homogenization. The entire procedure can be completed within a day and can be scaled up to 30 g of starting ventricular tissue. As compared to some of the most powerful procedures of preparation of heart sarcolemmal membranes [30, 31, 33, 34], the sarcolemmal membranes prepared in this work were obtained in the same yield and with an intermediate degree of purification of several marker enzymes.

The three drugs tested for their effects on several enzymatic activities in the purified sarcolemmal membranes have very distinct patterns of action. The activity of pindolol is highly selective since, in the range of concentration studied, the drug had no effect on the various enzymes tested, including adenylate cyclase stimulated at various levels with the exception of the β-adrenergic-stimulated enzyme (Fig. 3). The latter inhibition, which is total at 1 μM, is in agreement with the β-adrenergic blocking properties of the drug which occurs in tme nanomolar range. The same property is observed with propranolol (Fig. 3) in agreement with the β-adrenergic blocking activity of the latter [3, 6]. In addition, propranolol exerts an effect on Mg^{2+} ATPase (Fig. 4). With the exception of Na^+/K^+ ATPase and the basal, GTP- and fluoride-stimulated adenylate cyclase, amiodarone affects the other enzymes studied in the micromolar concentration range. The lack of effect of amiodarone tested up to 100 μM on Na^+/K^+ ATPase is in agreement with the lack of effect of K^+ PNPase, an inhibition of the latter occurring above 100 μM (Fig. 3). These results contrast with previous studies which reported inhibition of Na^+/K^+ ATPase activity in guinea-pig heart particulate fraction with a K_i of $\simeq$ 64 μM [35] and in rat brain synaptic membranes with IC_{50} values of $\simeq$ 20 μM [15] and of $\simeq$50 μM [36]. In the present work, we verified that amiodarone has no effect on Na^+/K^+ ATPase activity in the homogenate (H) and the pellet (C) so that the absence of effect of the drug is independent of the degree of purification of the membranes. These apparent discrepancies could be reconciled in view of a recent work [37] which compares the species variations in the ouabain sensitivity of cardiac Na^+/K^+ ATPase. Beef and rat Na^+/K^+ ATPase was included for comparison. The ouabain "sensitive" group includes guinea-pig heart and rat brain; the ouabain "insensitive" group includes rat heart. Thus, amiodarone inhibits Na^+/K^+ ATPase as a function of the sensitivity of the enzyme to ouabain. The reason for the species variation of Na^+/K^+ ATPase sensitivity is not understood [37]. However, Abeywardena *et al.* [37] tentatively propose that acyl chain characteristics play a major role in the modulation of Na^+/K^+ ATPase to inhibition by ouabain since a good correlation between the unsaturation index of membrane lipids and the IC_{50} value for ouabain was observed. Thus amiodarone could inhibit the enzyme activity via an interaction with the acyl chain of the lipids rather than a direct action of the polypeptidic chains of the protein. The inhibition of isoproterenol-stimulated adenylate cyclase activity and the inhibition of selective β-adrenergic antagonist (iodocyanopindolol) binding are both non-competitive [6] suggesting that amiodarone acts at some sites distinct from the β-adrenergic recognizing site of the receptor. Amiodarone induces an increase of 5'nucleotidase activity. 5'Nucleotidase is an intrinsic protein located in the external half of the bilayer; its activity is highly dependent on lipid–protein interaction [38, 39]. When tested on the solubilized enzyme, amiodarone has no effect.

In order to interpret the signal of DHP (Fig. 6) in terms of lipid dynamics, we verified that none of the drugs had any direct effect on the probe itself by measuring the fluorescence lifetime of the excited state (τ). The latter had a value of 9.5 ± 0.5 nsec (mean $\pm$ SD, N = 3) in the control membranes. This value was not modified by the highest concentration of each drug tested, which is in agreement with previous results obtained in rat brain synaptic membranes [11] and multilamellar vesicles of natural or synthetic phospholipids [15]. First of all, the variations of P will reflect the presence of a given compound in the lipid matrix of the sarcolemmal membranes. In this respect, pindolol which does not affect P is not present in the membrane, or is present to such a low degree that it does not influence the lipid dynamics and indirectly the various enzymes embedded in the bilayer. Propranolol has an apparent partition coefficient 200 times higher than pindolol [8]. In the same range of concentration as pindolol, propranolol modifies P which is indicative of its presence in a bilayer in an amount sufficient to modify the lipid dynamics and in turn some enzymatic activity such as Mg^{2+} ATPase (Fig. 4). Like the majority of amphiphilic cationic compounds [40], the effect of propranolol is to increase lipid mobility ("fluidity"). Amiodarone has an apparent partition coefficient of 17,000 [23] which is 1500 times higher than propranolol. The effect of the drug on lipid dynamics occurs at a lower concentration than

propranolol. Contrasting with propranolol (this work) and most of the amphiphilic cationic drugs [40], amiodarone decreases the lipid mobility, i.e. reduces lipid movements (decrease fluidity), in agreement with the effect observed on rat-brain synaptic membranes [11] and multilamellar vesicles [15]. This suggests a profound interaction between amiodarone and the acyl chains of the phospholipids which implies the location of the drug along the fatty acid chains. Compared to the effects of propranolol, that indicates that large portions of the two molecules are not located in the same region of the phospholipid bilayer.

When measured in identical experimental conditions, the effects of amiodarone on lipid dynamics occurs in the same range of concentration as the various effects on several enzymatic activities. Since, most of the enzymatic activities tested depend, at least in part, on interaction with the surrounding lipids, it is tempting to suggest that at least some of the effects of amiodarone on the enzymatic activities measured in this work are due to effects of the drug on lipid dynamics either by a gross reduction of lipid mobility or by more subtle redistribution of lipids within the plane of the bilayer. Finally it must be stressed that effective treatment of arrhythmias in the human may be achieved at a plasma concentration of 1.5 to 3.8 μM [41, 42], i.e. in the range of concentration for which interactions with the lipid bilayer and modifications of some enzymatic activities are observed in purified sarcolemmal membranes.

Acknowledgements—We thank Mrs M. Gremel and C. Roque for their technical assistance and Mrs E. Corbisier for expert secretarial work. This work was partly supported by a grant from IRSIA (Belgium).

REFERENCES

1. Charlier R, *Antianginal Drugs*, Springer Verlag, Berlin, 1971.
2. Prichard BNC and Vrhovac B, Pharmacology of Antianginal drugs. In: *Arteries and Veins*. (Eds. Harcus AW and Adamson L), pp. 266–280. Churchill Livingstone, London, 1975.
3. Barrett AM and Cullum VA, The biological properties of the optical isomers of propranolol and their effects on cardiac arrhythmias. *Br J Pharmacol* **34**: 43–55, 1968.
4. Vaughan-Williams EM, Classification of antidysrhythmic drugs. *Pharmacol Ther* **1**: 115–138, 1975.
5. Charlier R, Deltour G, Baudine A and Chaillet F, Pharmacology of amiodarone, an antianginal drug with new biological profile. *Arzneim-Forsch* **18**: 1408–1417, 1968.
6. Gagnol JP, Devos C, Clinet M and Nokin P, Amiodarone. Biochemical aspects and haemodynamic effects. *Drugs* **29**: 1–10, 1985.
7. Singh NB, Amiodarone: Histological development and pharmacological profile. *Am Heart J* **106**: 788–797, 1983.
8. Wiethold G, Hellebrecht D, Lemmer B and Palm D, Membrane effects of beta-adrenergic blocking agents: investigations with the fluorescence probe 1-anilino-8-naphtalene sulfonate (ANS) and antihemolytic activities. *Biochem Pharmacol* **22**: 1437–1449, 1973.
9. Warren RJ, Stedman RJ, Shami EG, Rattie ES and Ravin LJ, Observations on the micelle formation of 2-butyl-3-benzofuranyl-4-(2-diethylamino)ethocy)-3,5-diiodophenyl ketone hydrochloride (SK & F 33134-A) by NMR spectroscopy. *J Pharm Sci* **59**: 1357–1358, 1970.
10. Lalloz MPA, Byfield PGH and Himsworth RL, Binding of amiodarone by serum proteins and the effects of drugs, hormones and other interacting ligands. *J Pharm Pharmacol* **36**: 366–372, 1984.
11. Chatelain P, Laruel R and Gillard M, Effect of amiodarone on membrane fluidity and Na^+/K^+ ATPase activity in rat-brain synaptic membranes. *Biochem Biophys Res Commun* **129**: 148–154, 1985.
12. Godin DV, Wan NG and Tucheck JM, Studies on the interaction of propranolol with erythrocyte membranes. *Biochim Biophys Acta* **436**: 757–773, 1976.
13. Tashima Y, Hasegawa M, Mizunuma H and Sakagishi V, Specific effects of spermine on ouabain-sensitive and potassium-dependent phosphatase activity of kidney plasma membranes. *Biochim Biophys Acta* **482**: 1–10, 1977.
14. Bers DM, Isolation and characterization of cardiac sarcolemma. *Biochim Biophys Acta* **555**: 131–146, 1979.
15. Chatelain P, Ferreira J, Laruel R and Ruysschaert JM, Amiodarone-induced modifications of the phospholipid physical state. A fluorescence polarization study. *Biochem Pharmacol* **35**: 3007–3013, 1986.
16. Fiske CH and Subbarow Y, The colorimetric determination of phosphorus. *J Biol Chem* **66**: 375–400, 1925.
17. Campbell DM, Determination of 5′-nucleotidase in blood serum. *Biochem J* **82**: 34P, 1962.
18. Minneman KP, Hegstrand LR and Molinoff PB, The pharmacological specificity of beta-1 and beta-2 adrenergic receptors in rat heart and lung *in vitro*. *Mol Pharmacol* **16**: 21–33, 1979.
19. Jones LR, Besch HR, Fleming JW, McConnanghey MM and Watanabe AM, Separation of vesicles of cardiac sarcolemma from vesicles of cardiac sarcoplasmic reticulum. *J Biol Chem* **254**: 530–539, 1979.
20. Baginski ES, Foa PP and Zak B, Glucose-6-phosphatase. In: *Methoden der enzymatischen Analyse* (Ed. Bergmeyer HU), pp. 839–844. Verlag Chemie GmbH, Weinheim, 1970.
21. de Duve C, Pressman BG, Granetto R, Wattiaux R and Appelmans F, Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem J* **60**: 604–617, 1955.
22. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
23. Chatelain P and Laruel R, Amiodarone partitioning with phospholipid bilayers and erythrocyte membranes. *J Pharm Sci* **74**: 783–784, 1985.
24. Bligh EG and Dyer WJ, A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37**: 911–917, 1959.
25. Okumura K, Ogawa K and Sabake T, Pretreatment with chlorpromazine prevents phospholipid degradation and creatine kinase depletion in isoproterenol-induced myocardial damage in rats. *J Cardiovasc Pharmacol* **5**: 983–988, 1983.
26. Itaya K and UI M, A new micromethod for the colorimetric determination of organic phosphate. *Clin Chim Acta* **14**: 361–366, 1966.
27. Ott P, Binggeli Y and Brodbeck URS, A rapid and sensitive assay for determination of cholesterol in membrane lipid extracts. *Biochim Biophys Acta* **685**: 211–213, 1982.
28. Shinitsky M and Inbar M, Microviscosity parameters and protein mobility membranes. *Biochim Biophys Acta* **433**: 133–149, 1976.

29. Dhalla NS and Pierce GN, Isolation and characterization of the sarcolemmal membrane from the heart. In: *Methods in Studying Cardiac Membranes* (Ed. Dhalla NS), pp. 3–18. CRC Press, Boca Raton 1984.
30. Weglicki WB, Owens K, Kennett F, Kessner A, Harris L and Wise RM, Preparation and properties of highly enriched cardiac sarcolemma from isolated adult myocytes. *J Biol Chem* **255**: 3605–3609, 1980.
31. Cramb G and Dow JW, Two sites binding of bepridil and modulation of adenylate cyclase in cardiac sarcolemmal membranes. *Biochim Biophys Acta* **736**: 99–108, 1983.
32. Jones LR, Maddock SW and Besch HR, Unmasking effect of alamethicin on the (Na^+, K^+) ATPase, β-adrenergic receptor-coupled adenylate cyclase and cAMP dependent protein kinase activities of cardiac sarcolemmal vesicles. *J Biol Chem* **255**: 9971–9980, 1980.
33. Mansier P, Charlemagne D, Rossi B, Preteseille M. Swynghedauw B and Lelievre L, Isolation of impermeable inside-out vesicles from an enriched sarcolemma fraction of rat heart. *J Biol Chem* **258**: 6628–6635, 1983.
34. Lamers JMJ and Stimis JT, Phosphorylation of low molecular weight proteins in purified preparations of rat heart sarcolemma and sarcoplasmic reticulum. *Biochim Biophys Acta* **624**: 443–459, 1980.
35. Broekhuysen J, Clinet M and Delisee C, Action of amiodarone on guinea-pig heart sodium and potassium activated adenosine triphosphatase. Comparison with ouabain. *Biochem Pharmacol* **21**: 2951–2960, 1972.
36. Prasadara KS, Rao SB, Camus PH and Menhendale HM, Effect of amiodarone on Na^+ K^+-ATPase and Mg^{2+}-ATPase activities in rat brain synaptosomes. *Cell Biochem Funct* **4**: 143–151, 1986.
37. Abeywardena MY, McMurchie EJ, Russell GR and Charnock JS, Species variation in the ouabain sensitivity of cardiac Na^+/K^+ ATPase. A possible role for membrane lipids. *Biochem Pharmacol* **33**: 3646–3654, 1984.
38. Merisko EM, Ojakian GK and Widnell CC, The effects of phospholipids on the properties of hepatic 5′-nucleotidase. *J Biol Chem* **256**: 1983–1993, 1981.
39. Dipple I, Gordon LM and Houslay MD, The activity of 5′-nucleotidase in liver membranes is affected by an increase in bilayer fluidity achieved by anionic drugs but not by cationic drugs. *J Biol Chem* **257**: 1811–185, 1982.
40. Goldstein DB, The effects of drugs on membrane fluidity. *Ann Rev Pharmacol Toxicol* **24**: 43–64, 1984.
41. Canada AT, Lesko LJ and Haffajee CI, Disposition of amiodarone in patients with tachyarrhythmias. *Curr Ther Res* **30**: 968–974, 1981.
42. Latini R, Tognoni G and Kates RE, Clinical pharmacokinetics of amiodarone. *Clin Pharmacokinetics* **9**: 136–156, 1984.