Biochemical Pharmacology, Vol. 26, pp. 1775–1781. Pergamon Press, 1977. Printed in Great Britain.

BINDING OF THE POLYFLAVANE P13 ON VARIOUS PIG MYOCARDIAL MEMBRANES—EFFECTS ON PROTEIN RELEASE AND ON Ca^{2+} AND Mg^{2+} MOVEMENTS

ATTILIO DI PIETRO, CATHERINE GODINOT, CHRISTIAN VIAL and DANIELE C. GAUTHERON

Laboratoire de Biologie et Technologie des Membranes-CNRS, Université Claude Bernard de Lyon I, 43, Bd du 11 Novembre 1918, 69621 Villeurbanne, France

and

PIERRE FOULHOUX

Centre de Recherches Pierre Fabre, 17, avenue Jean Moulin, 81106-Castres, France

(*Received* 4 *January* 1977: *accepted* 9 *March* 1977)

Abstract—P13, a polyflavane compound, bound to myocardial membranes with various degrees of efficiency: sarcolemma > mitochondria > sarcoplasmic reticulum. The binding was enhanced by added cations. Partial release of bound P13 was obtained by washing and the same cations which enhanced the binding, inhibited the release. Washing-induced protein release from membranes was prevented by bound P13. The binding of P13 did not modify respiratory activity and energy-linked Ca^{2+} accumulation in intact mitochondria, but retained a better coupling activity in aged mitochondria by preventing cation efflux which occurs during ageing. P13 behaved like a membrane stabilizer since its binding diminished membrane leakage and therefore prevented protein and cation passive efflux through aged membranes.

Ultrastructural and metabolic changes induced by myocardial infarction are being extensively studied. It has been well established that the energetic stores of creatine phosphate [1] and glycogen [2] are considerably lowered; oxygen deprivation is accompanied by an important enzyme release [3] and cation efflux [4, 5], probably due to an increased membrane permeability, since alterations and lesions have been observed throughout the myocardial cell.

Previous studies with P13, a polyflavanic polymer, indicated promising effects on energetic metabolism of hypoxic myocardium, since it favored maintenance of higher energy stores [7]; it has also been shown in the same study that the $(Na^+ + K^+)$ ATPase of sarcolemma, which controls sodium and potassium movements across the membrane, was strongly inhibited by P13. On the other hand, experiments on the electrical activity of a myocardial fiber have suggested that P13 decreased potassium efflux*. By these various properties, P13 may protect the myocardial cell against anoxia.

The present paper describes the binding of P13 on myocardial membranes: sarcoplasmic reticulum, mitochondrial and sarcolemmal membranes. In the case of mitochondria and sarcolemma, the binding appeared cation-dependent and protected the membranes against protein release. Moreover P13 binding prevented mitochondrial membrane ageing as tested on their coupling properties and the Ca^{2+} and Mg^{2+} movements.

In addition, according to Carafoli [6], mitochondria are implicated in myocardium contraction process by accumulating calcium to initiate relaxation while the release of Ca^{2+} initiates contraction. Therefore the effects of P13 on calcium movements through mitochondrial membranes have been investigated: it has been shown that P13 did not affect the influx of Ca^{2+} and the concomittant respiratory stimulation while it decreased the uncoupler-induced Ca^{2+} efflux.

* Y. M. Gargouïl, personal communications.

MATERIAL AND METHODS

P13 was obtained as described earlier [7] from the "Centre de Recherches Pierre Fabre" and used in pH-controlled aqueous solutions.

Preparation of myocardial membranes

Known procedures were used as described earlier to prepare whole mitochondria [8], purified sarcolemma [9, 10] or purified sarcoplasmic reticulum [11, 12] from pig heart.

P13 binding

Membranes were incubated for 30 min with increasing P13 concentrations at 0–4° and centrifuged at 135,000 *g* for 90 min (sarcoplasmic reticulum), 36,000 *g* for 10 min (mitochondria) or 12,000 *g* for 5 min (sarcolemma) in an Eppendorf centrifuge. This centrifugation of the sarcolemma in 25 mM imidazole 1 mM EDTA, pH 7.0, buffer, was sufficient to eliminate the protein from the supernatant fluid. Bound P13 was calculated by subtracting the amount of P13 present in the supernatant fluid from the initially added P13; P13 content in the supernatant fluid was determined by simultaneous measurement of optical density at 275 and 505 nm. Indeed P13 spectrum

showed a high 275 nm absorption band ($E^{1\%}_{1cm} = 90$) and a minor 505 nm band ($E^{1\%}_{1cm} = 4.8$); moreover, it was verified that the protein and nucleotide release was too low to modify significantly P13 determination.

Incubation media (1 ml final volume) were 0.25 M sucrose, 10 mM Tris–HCl, pH 7.4 (mitochondria and sarcoplasmic reticulum) or 25 mM imidazole-HCl, 1 mM EDTA, pH 7.0 (sarcolemma) with or without added cations such as Ca^{2+}, Mg^{2+} or $(Na^+ + K^+)$ at the indicated concentrations. Standard P13 curves were obtained in the absence of membranes and with the same medium and cations when added.

Results are followed by the standard error of the mean.

Reversal of P13 binding

To determine the reversibility of P13 binding, the membranes were first incubated (1 ml final volume) for 30 min with a high concentration of P13 (75 μg/ml), spun down as mentioned above and then the obtained pellets were washed once or twice: they were homogenized in 1 ml incubation medium and immediately spun down; P13 released in the supernatant washings was measured as previously described.

Protein determination

The procedure of Lowry *et al.* [13] was used to measure protein release during the washings; other determinations were made with the biuret method of Gornall *et al.* [14].

Mitochondrial respiration

The respiratory activity of intact, freshly prepared, or aged mitochondria was studied by oxypolarography [15] incubating 2.4 mg mitochondria in 2 ml 16 mM Tris–HCl, 112 mM KCl, 1.25 mM potassium phosphate, pH 7.4 at 28°. The respiratory substrate was 10 mM glutamate; ADP, when added, was 0.2 mM. P13 effects were studied on state 4 respiration and on respiratory control ratio (RCR) as defined by Chance & Williams [15]. RCR values were calculated by the (state 3/initial state 4) ratio. P13 was added in the incubation medium for freshly prepared mitochondria or during storage for aged mitochondria. Ca^{2+} stimulation of state 4 respiration was studied by adding 1.5 mM $CaCl_2$.

Cation determination

Mitochondrial Mg^{2+} and Ca^{2+} were measured by atomic absorption spectrophotometry. When cation efflux occuring during respiration was examined, mitochondria were incubated for 5 min, in 1 ml of the same medium used for oxypolarographic studies with or without 10 mM cation. The reaction was stopped by rapid centrifugation with an Eppendorf centrifuge. Mitochondrial cations were then extracted by 1 ml 5% perchloric acid; after centrifugation, supernatant fluids were diluted in 0.5% La—0.1 N HCl and their cation content was analysed on a Perkin Elmer model 403 atomic absorption spectrophotometer. When mentioned, 10^{-4} M 2,4-dinitrophenol (2,4-DNP) was added at 5 min and the reaction proceeded for 5 more min; finally, it was stopped by rapid centrifugation.

Mitochondrial Ca^{2+} accumulation was also determined using the metallochromic indicator murexide [16] and measuring murexide Ca^{2+} complex absorption with a dual-wavelength Aminco-Chance spectrophotometer. Mitochondria (2.4 mg) were incubated in 3 ml 225 mM mannitol, 55 mM sucrose, 10 mM Tris–HCl, 1.67 mM potassium phosphate, 10 mM glutamate, 50 μM murexide, pH 7.4 buffer at 30°. The reaction was initiated by 0.333 mM $CaCl_2$. When indicated, Mg^{2+} was 6.6 mM and 2,4-DNP was 0.66×10^{-4} M.

Assay of ATPase activity

ATPase activity was measured by the previously described colorimetric method [17]. Mitochondria (0.8 mg) were incubated in 0.8 ml 31 mM Tris–HCl, 0.25 M sucrose, 3 mM ATP, 3 mM Mg Cl_2, 1.25 mM ammonium acetate, pH 7.4 at 30° for 1 min. The reaction was stopped by 75 μl 50% ice cold trichloracetic acid and the inorganic phosphate liberated was measured by the Sumner method [18].

RESULTS

P13 binding on myocardial membranes

Figure 1 shows that all membranes exhibit a great affinity for P13 and that in the studied concentration range, the binding in the absence of added cation is a linear function of P13 concentration; concentrations over 80 μg P13/ml were discarded as beyond the physiological and pharmacological range. In the presence of cations, a slight curvature appeared.

The amount of P13 bound depended on the membrane type and on cation content of the medium. The lowest binding was obtained for sarcoplasmic reticulum since only 35 ± 2.5 percent of the P13 added to the medium remained bound to the membrane (Fig. 1a). The Scatchard plots (insert Fig. 1a) indicate that these membranes have two classes of sites with very different affinities.

The binding was greater with mitochondria since a 46.5 ± 1.5% value was obtained in the absence of added cations; this percentage was considerably enhanced by 3 mM Ca^{2+} or Mg^{2+}: 82 ± 4% up to 40 μg P13/ml and 78 ± 4% at 75 μg/ml with Ca^{2+}; 89 ± 2% up to 40 μg/ml and 80 ± 2% at 75 μg/ml with Mg^{2+} (Fig. 1b). Scatchard plots (insert—Fig. 1b) show that the presence of either Mg^{2+} or Ca^{2+} increase the affinity of mitochondrial membranes for P13, apparently without modification of the number of sites which in every case are about 125 μg P13/mg protein. The binding was apparently unaffected by ageing since stored and frozen mitochondria bound the same amounts of P13 as intact mitochondria.

The largest P13 binding was obtained with sarcolemma: 87.5 ± 2% up to 20 μg/ml and 78 ± 2% at 75 μg/ml in the absence of added cations; 100% up to 20 μg/ml and 85 ± 2% at 75 μg/ml with 100 mM Na^+ + 10 mM K^+ (Fig. 1c). Scatchard plots (insert Fig. 1c) show that there are sites of very high affinity besides other sites; because of the very affine sites, the Scatchard are difficult to interpret. This binding of P13 was not modified if the membranes were stored or freshly prepared.

Reversal of P13 binding

Table 1 indicates that P13 binding to mitochondria or sarcolemma was reversible (at least partially) since

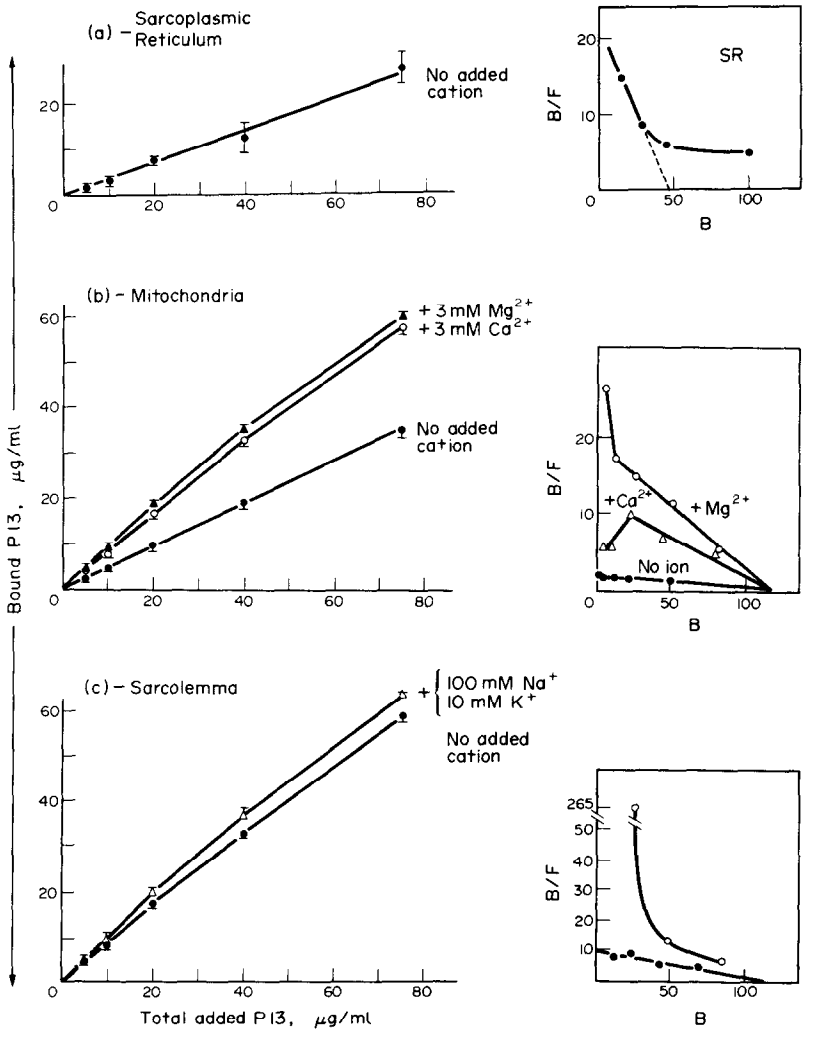

Fig. 1. P13 binding to myocardial membranes. Incubation and P13 determination are described in Methods. The indicated cations were added to the incubation medium. a—Sarcoplasmic reticulum: 0.27 mg protein/1 ml; b—mitochondria: 0.72 mg protein/1 ml; c—Sarcolemma: 0.75 mg protein/1 ml. Each curve is the average of four different experiments; vertical bars indicate S.E.M. Insert: B,F expressed as μg P13/mg protein.

about 20 per cent bound P13 were released by one washing and 30 per cent by two washings in relatively small volumes of 1 ml (cf. legend of Table 1); great volumes could not be used for washings, because the molecular absorbance of P13 is too low for accurate spectrophotometric measurements in too dilute solutions. However dilution by 50 volumes of washing medium completely released the inhibition of ATPase activities [7] with both types of membranes.

The same cations which enhanced P13 binding (Ca^{2+} or Mg^{2+} for mitochondria, $Na^+ + K^+$ for sarcolemma) partially prevented against P13 release, when added during binding. If they were added only in the washing medium, the protection was even better. Curiously enough, P13 release also decreased by about 50 per cent when EGTA or EDTA was added to the washing medium indicating that Ca^{2+} or Mg^{2+} might form a complex with P13, since they were no longer accessible to their chelating agents.

Such a cation P13 complex formation was observed in other experiments when the addition of Ca^{2+} to aqueous solution of P13 gave rise to a "gel" and therefore modified P13 spectrum. This gel could also be obtained with $AlCl_3$ instead of Ca^{2+} and might be related to chelate formation.

Effects of bound P13 on protein release during washing

Table 2 shows that a low (5 per cent) but a measurable amount of protein was released during washing of mitochondria. This amount did not depend on the presence of added cations in the incubation medium, but was greatly increased by high concentrations of chelating agents like EGTA or EDTA. P13 efficiently decreased protein release during washing: it totally prevented any loss of mitochondrial protein in the absence of added chelating agents and diminished the large release induced by EGTA or EDTA.

Effects of bound P13 on mitochondrial respiration and respiration-driven cation accumulation

Ca^{2+} accumulation. Respiration-driven energy can be used by the mitochondrion to accumulate Ca^{2+};

Table 1. Release of membrane-bound P13 by washing

	Mitochondria Washing	Bound P13 μg/ml	% Released P13		Sarcolemma Washing	Bound P13 μg/ml	% Released P13
No cation added during binding	Before washing	49		No cation added during binding	Before washing	54	
	1 Washing	39.7	19		1 washing	44.8	17
	2 Washings	34.3	30		2 Washings	39	28
+ 3 mM Ca^{2+} during binding	Before washing	67			1 Washing Na^+, K^+	49.7	7
	1 Washing	57.8	14		2 Washings Na^+, K^+	47.3	10
	2 Washings	54.7	18				
	1 Washing EGTA	59.8	11				
	2 Washings EGTA	57	15				
+ 3 mM Mg^{2+} during binding	Before washing	67		100 mM Na^+ 10 mM K^+ during binding	Before washing	65.3	
	1 Washing	59.3	13		1 Washing	58.3	11
	2 Washings	54	20		2 Washings	53.7	18
	1 Washing EDTA	60.6	10		1 Washing Na^+, K^+	60.6	7
	2 Washings EDTA	58.3	13		2 Washings Na^+, K^+	58.0	11

Protein amounts: Mitochondria 0.72 mg/ml. Sarcolemma 0.75 mg/ml. In all experiments, P13 binding was carried out by adding 75 μg P13 to 1 ml incubation medium (see Methods) with or without Ca^{2+} or Mg^{2+} for mitochondria, with or without $Na^+ + K^+$ for sarcolemma. P13 bound was measured after spinning down the membranes as a pellet. Washings were performed by homogenizing the pellet in 1 ml medium for 1 mn, and spinning down again; the P13 released was estimated in the corresponding supernatant fluid. When added to the washing medium, EGTA or EDTA were 10 mM, and Na^+, 100 mM, K^+ 10 mM. The percent of P13 released was calculated by comparison with P13 bound before washing.

this energy-linked cation movement stimulates state 4 respiration. If high Ca^{2+} amounts (1.5 mM) were added, the respiratory stimulation was followed by an irreversible inhibition in the presence of various substrates. The stimulation can be attributed to Ca^{2+} influx, but the inhibition has not yet been explained. Fig. 2a shows that increasing P13 concentrations did not modify Ca^{2+} stimulation of respiration but did modify the inhibition. In the presence of added Mg^{2+}, Ca^{2+} stimulation was lowered; in that case, P13 slightly increased Ca^{2+} stimulation of state 4 (Fig. 2b). Therefore P13 seemed to slightly enhance Ca^{2+} influx when it was lowered by Mg^{2+}, and did not affect it when it reached an optimal rate.

Table 2. Effect of mitochondria-bound P13 on washing-induced protein release

Washing		Released proteins (μg) −P13	+P13
No cation added during binding	1 Washing	36	0
+ 3 mM Ca^{2+}	1 Washing	32	0
	1 Washing with 10 mM EGTA	88	58
+ 3 mM Mg^{2+}	1 Washing	34	0
	1 Washing with 10 mM EDTA	56	7

Experimental conditions are the same as those described in Table 1 (0.72 mg mitochondrial protein). The same supernatant fluids used for P13 release were analysed for protein release by the method of Lowry *et al.* [13].

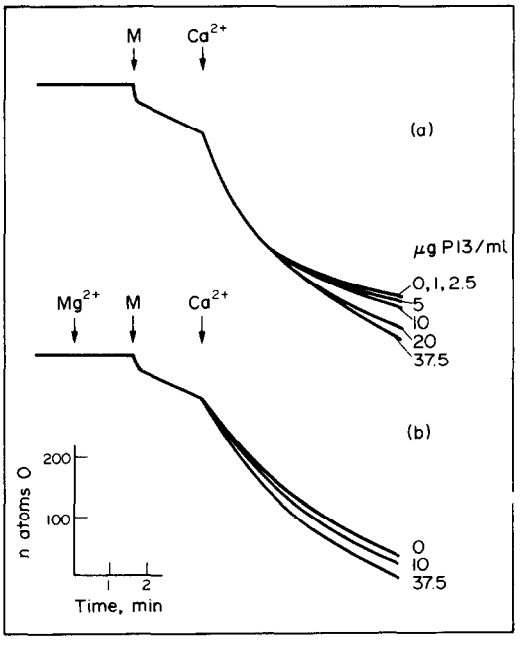

Fig. 2. Comparative effects of P13 and Mg^{2+} on Ca^{2+} state 4 respiration enhancement of mitochondria, measured by oxypolarography. (a)—Intact mitochondria (M) were incubated as described in Methods. The indicated P13 concentrations were added before the mitochondria. (b)—Same conditions plus 5 mM Mg^{2+} added before the mitochondria. Graphs have been directly reproduced from one experiment. Results were reproducible with three different mitochondria preparations.

The Ca^{2+} accumulation by mitochondria was studied with glutamate as respiratory substrate either by atomic absorption spectrophotometry or by the murexide technique. The first method indicated that P13 did not modify the amount of Ca^{2+} accumulated (290 nmoles Ca^{2+}/mg protein) but partially prevented 2,4-DNP-induced Ca^{2+} efflux since 230 nmoles Ca^{2+}/mg protein were recovered with P13 instead of 200 nmoles Ca^{2+}/mg protein without P13. However this method was not convenient to study the kinetics of Ca^{2+} accumulation and P13 effects; the murexide technique was thus used in all further studies.

Fig. 3a shows that in the conditions used, mitochondria accumulated about 400 nmoles Ca^{2+}/mg protein. When Mg^{2+} was added, the initial rate of incorporation was lowered, but the final accumulation level remained unchanged. This process was energy-linked and required a permeant anion like phosphate, since much lower amounts of Ca^{2+} of about 40–70 nmoles Ca^{2+}/mg protein were accumulated in the absence of either added substrate or phosphate. Once the Ca^{2+} had been accumulated, it was possible to induce its efflux by uncouplers such as 2,4-DNP. Fig. 3a indicates that P13 had no effect on Ca^{2+} influx; in the presence of added Mg^{2+} the slight increase in Ca^{2+} influx is probably not significant. On the contrary, P13 inhibited 2,4-DNP-induced Ca^{2+} efflux (Fig. 3b). A similar inhibition was produced by Mg^{2+} and these two effects were additive. The Ca^{2+} accumulation driven by ATP hydrolysis was studied with and without acetate as permeant anion instead of phosphate, since the latter was unusable in the colorimetric method used for determination of the released phosphate. Figure 4 shows that increasing P13 concentrations stimulated mitochondrial ATPase activity as previously described [7]. When Ca^{2+} was added, higher ATPase activity was

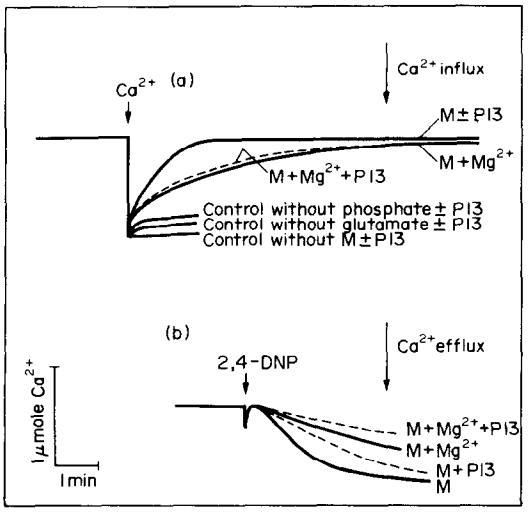

Fig. 3. P13 and Mg^{2+} effects on Ca^{2+} movements across the mitochondrial membrane. Ca^{2+} accumulation or efflux was measured with murexide (see Methods). (a)—P13 and Mg^{2+} effects on respiration-driven accumulation of Ca^{2+}; (b)—P13 and Mg^{2+} effects on 2,4-DNP induced efflux of accumulated Ca^{2+}. P13 concentration was 22 µg/ml. Mitochondria: 2,4 mg protein/3 ml. Graphs have been directly reproduced from one experiment and were reproduced three times.

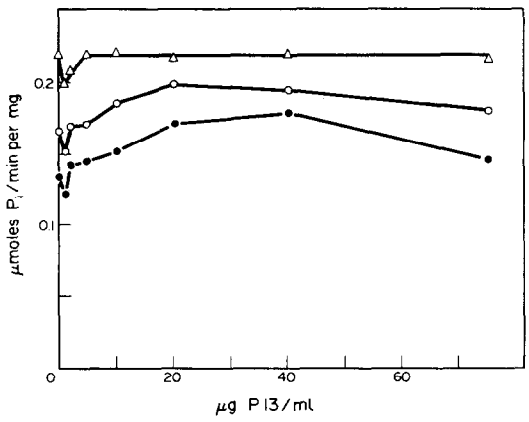

Fig. 4. P13 effects on Ca^{2+} stimulated ATPase activity. Mitochondrial ATPase activity was measured as described in Methods. ●: no addition; ○: + Ca^{2+}; △: + Ca^{2+} + ammonium acetate

obtained but the curve profile remained unchanged. When Ca^{2+} plus acetate were present, a maximum ATPase activity equal to that obtained with uncouplers, was obtained. This maximum activity was no longer modified, whatever the P13 concentration was.

The combined results (Figs 2, 3 and 4) indicate that P13 did not affect mitochondrial Ca^{2+} accumulation

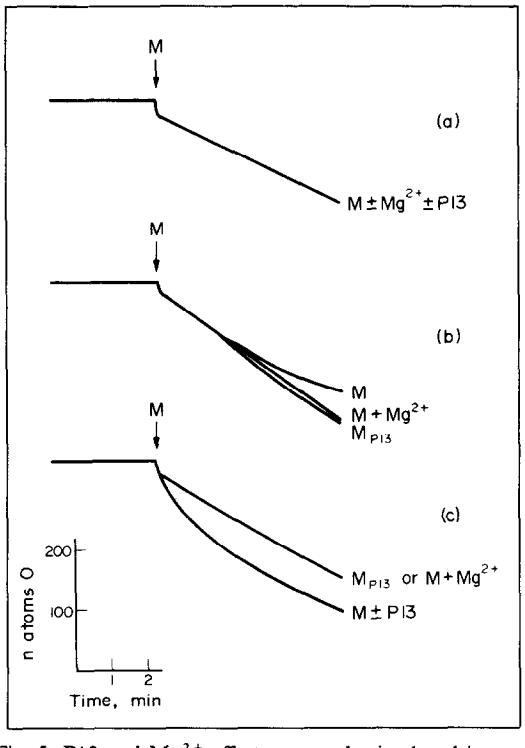

Fig. 5. P13 and Mg^{2+} effects on aged mitochondria state 4 respiration. State 4 respiration of mitochondria was measured by oxypolarography with or without 5 mM Mg^{2+} added before the mitochondria. (a)—Intact mitochondria: 37.5 µg P13/ml were added in the assay; (b)—Mitochondria stored at 0–4° for 24 hr with (M_{P13}) or without (M) 12.5 µg P13/mg protein; (c)—frozen mitochondria stored with (M_{P13}) or without (M) 12.5 µg P13/ml. Graphs have been directly reproduced from one experiment and were reproduced three times.

Table 3. P13 and Mg^{2+} effects on aged mitochondria RCR

	Intact mitochondria	Stored at 0–4° for 24 hr	Frozen mitochondria
M	4.1	1.5	1
M + Mg^{2+}	4.1	2	2
M + P13	4.1		
M_{P13}		1.9	1.5
M_{P13} + Mg^{2+}		2	2

M + P13: mitochondria + P13 added during the RCR assay (1 to 56 μg/ml). M_{P13}: mitochondria stored at 0–4° or frozen with or without 12.5 μg P13/mg proteins for 24 hr.

Mg^{2+} when added was 5 mM. RCR was measured as described under Methods. The experimentation was repeated twice and gave the same results.

when it occurred at a maximal rate, but slightly enhanced it when it was lowered; P13, like Mg^{2+}, inhibited 2,4-DNP induced Ca^{2+} efflux.

Effects on aged mitochondria respiration. In a previous report [7], it was shown that, with glutamate as a substrate, P13 did not affect state 4 respiration of intact mitochondria; Mg^{2+} did not modify oxygen consumption either (Fig. 5a). On the contrary, when mitochondria were stored at 0° (Fig. 5b), or frozen (Fig. 5c), with or without P13 and were then assayed for respiration, P13 showed a protective effect against ageing: the rate of respiration remained a linear function of time, while a time decreasing rate of respiration was observed with mitochondria stored or frozen without P13. Similar respiratory rates were obtained by adding Mg^{2+} in the assay. Therefore P13 might act as Mg^{2+} or by preventing Mg^{2+} release during ageing.

The RCR values of aged mitochondria were very low. P13, added during storage, partially prevented this lowering (Table 3) and the effect was almost equivalent to that produced by added Mg^{2+}.

Effects on cation content. Mitochondria, aged by storage or by freezing, lose part of their cations: 25% Mg^{2+} by storage and 31% by freezing; 55% Ca^{2+} by storage and 45% by freezing (Table 4). P13, added during storage, lowered these losses to 14% and 25% for Mg^{2+}, 35% and 38% for Ca^{2+}. Several experiments were performed giving the same variations. Respiration (Table 5) hardly affected cation contents but 2,4-DNP severely decreased Ca^{2+} level and P_{13} partly prevented this loss.

DISCUSSION

The results presented here, have shown that P13 binds very efficiently all the tested pig myocardial membranes. Sarcolemmal and mitochondrial membranes were the most sensitive to the drug and thus appear as the possible targets *in situ*; it is obvious that, when administered, P13 should easily reach sarcolemma, while the question arises to know if it will attain mitochondrial membranes.

The binding of P13 is enhanced by those cations related to the physiological function of the ATPases, specific of each myocardial membrane. It is therefore interesting to correlate this binding of P13 to its

Table 4. Effects of storage and freezing of mitochondria with P13 on their Mg^{2+} and Ca^{2+} content

	Intact mitochondria		Stored at 0–4° for 24 hr		Frozen mitochondria	
	−P13	+P13	−P13	+P13	−P13	+P13
Mg^{2+} nmoles/mg	15.4	15.4	11.6	13.2	10.6	11.5
Ca^{2+} nmoles/mg	22	22	9.9	14.2	12	13.6

Mitochondria were stored at 0–4° or frozen with or without 12.5 μg P13/mg proteins for 24 hours in 0.25 M sucrose, 10 mM Tris–HCl, pH 7.4 buffer. At this time, the mitochondria were spun down and their Mg^{2+} and Ca^{2+} contents were measured by atomic absorption spectrophotometry. Results represent a typical experiment; similar variations were observed three times.

Table 5. Effect of storage and freezing of mitochondria with P13 on Mg^{2+} and Ca^{2+} efflux during respiration

	Intact mitochondria		Stored at 0–4° for 24 hours		Frozen mitochondria	
	−P13	+P13	−P13	+P13	−P13	+P13
Mg^{2+} nmoles/mg	13.4	13.9	11.3	12	9.4	9.9
+ 2,4-DNP	12.5	13.3	10.1	10.1	8.4	9.5
Ca^{2+} nmoles/mg	19	19	17.1	17	18	16.2
+ 2,4-DNP	7	8.7	3.2	9.7	4.9	9.6

Intact or aged mitochondria were incubated for 5 or 10 min in the presence of glutamate as described in Methods. The remaining mitochondrial cations were determined by atomic absorption spectrophotometry. Results represent a typical experiment, three different experiments were performed.

effects previously observed on the membrane ATPases [7] and to the physiological cation movements.

The ATPase activities of sarcolemmal and sarcoplasmic reticulum membranes were always inhibited by P13 in the concentration ranges used here. The mitochondrial ATPase was inhibited by P13 when measured on the isolated enzyme or on the membrane broken by sonication, while it was slightly activated when measured on fresh mitochondria; in this latter case the main effect of P13 is likely to increase the access of ATP to the enzyme, but P13 however does not probably reach easily the active center, since the activation of the ATPase activity is limited. The same slight activation was observed in Fig. 4 with fresh mitochondria.

For every membrane, the inhibition of ATPase was easily reversed by large dilution. In the present study we have shown that washing reverses at least partly, P13 binding. It means that P13 binds reversibly to membrane sites very close to ATP sites, or even to the ATP sites on the ATPase itself, since the binding is accompanied by a competitive inhibition between ATP and P13. This does not exclude that some other membrane sites bind P13 almost irreversibly.

Cations that stimulate P13 binding also prevent P13 release from the membrane. It is interesting to recall that Gargouïl* has demonstrated that P13 diminishes K^+ efflux of sarcolemmal membranes. We focussed our attention on the effects of P13 on Ca^{2+} and Mg^{2+} permeability of mitochondrial membranes. These effects appeared quite complex. If P13 did not affect the rate of Ca^{2+} influx and the amount of accumulated Ca^{2+}, however an interesting result was that the concomitant presence of P13 and Mg^{2+} almost abolished the uncoupler-induced Ca^{2+} efflux (Fig. 3b) and P13 alone partially prevented this efflux.

This fact that EGTA or EDTA did not affect the protection respectively enhanced by Ca^{2+} or Mg^{2+} against the reversal of P13 binding, clearly indicates that these cations are no longer accessible to their chelating agents and therefore that they give a very strong association with the membrane in the presence of P13. This association could be a phospholipid-cation-protein ternary complex, which, according to Van Deenen [20] might play an important role in membrane functions.

According to Carafoli [6] mitochondria accumulate calcium to initiate muscle relaxation; as P13 does not change Ca^{2+} accumulation, relaxation should not be modified by the presence of P13. The release of Ca^{2+} initiates contraction [6]; if the uncoupler-induced Ca^{2+} efflux can be compared to the exit of Ca^{2+} initiating contraction, P13 by diminishing the rate of Ca^{2+} efflux should slow down contraction and diminish the cardiac work. This is in agreement with the previously described [7] saving of energy stores and would also explain how animals submitted to hypoxic conditions survive for a longer time when treated with P13 [19] which allows them to slow down their cardiac work.

In addition, P13 protects mitochondria against ageing: the presence of P13 during storage of mitochondria prevented the diminution of their respiratory control ratio as observed by Romani and Monadjem [21] when adding Mg^{2+} to the storage medium of mitochondria. Thus P13 mimics the effects of Mg^{2+} may be by partly preventing the loss of mitochondrial Mg^{2+} (Table 4) during storage. It has been shown that the release of Mg^{2+} leads to a loss of the functional impermeability of mitochondria [22]. Binet and Volfin [23] have also shown that the mitochondrial Mg^{2+} is essential for the maintenance of the functions and the stability of the membrane structure.

The effect of P13 on the permeability of the membrane is also illustrated on the protein release from the membrane (Table 2), since P13 prevents the washing-induced protein release from mitochondria.

In conclusion, these results have shown that P13 seems to act by preventing the leakage of some components of the cell (proteins, cations) thus maintaining the integrity of the cardiac membranes and their functions as suggested earlier [24]. A previous paper [7] showed that P13 preserves the energy stores of the myocardial cell. By now, nobody can tell what the primary effect of P13 is; but both effects contribute to lower the cardiac work.

Acknowledgement—Thanks are due to the French C.N.R.S. for a special help via ATP 2304.

REFERENCES

1. S. Gudbjarnason, *Cardiology* **56**, 232 (1971).
2. J. Caulfield and B. Klionsky, *Am. J. Path.* **35**, 489 (1959).
3. D. J. Hearse and E. B. Chain, *Biochem. J.* **128**, 1125 (1972).
4. R. B. Case, *Cardiology* **56**, 245 (1971).
5. R. B. Jennings, *Am. J. Cardiol.* **24**, 753 (1969).
6. E. Carafoli, in *Myocardial Biology* (Ed. N. S. Dahalla) p. 393. University Park Press (1974).
7. A. Di Pietro, C. Godinot, D. C. Gautheron and P. Foulhoux, *Biochem. Pharmac.* **26**, 0000 (1977).
8. D. C. Gautheron, R. Durand, N. Pialoux and Y. Gaudemer, *Bull. Soc. Chim. Biol.* **46**, 645 (1964).
9. H. Matsui and A. Schwartz, *Biochim. biophys. Acta* **128**, 380 (1966).
10. B. J. Pitts and A. Schwartz, *Biochim. biophys. Acta* **401**, 184 (1975).
11. R. J. Mead, M. B. Peterson and J. D. Welty, *Cir. Res.* **29**, 14 (1971).
12. J. M. Smoly, T. Wakabayashi, A. D. F. Addink and D. E. Green, *Archs Biochem. Biophys.* **143**, 6 (1971).
13. O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
14. A. G. Gornall, C. J. Bardawill and M. M. David, *J. biol. Chem.* **177**, 751 (1949).
15. B. Chance and G. R. Williams, *J. biol. Chem.* **217**, 383 (1955).
16. L. Mela and B. Chance, *Biochemistry* **7**, 4059 (1968).
17. A. Di Pietro, C. Godinot, M. L. Bouillant and D. C. Gautheron, *Biochimie* **57**, 959 (1975).
18. J. B. Sumner, *Science, N.Y.* **100**, 413 (1944).
19. M. C. Auclair, S. Dechezlepretre, H. Lauressergues, M. Lemeignan and P. Lechat, *Therapie* **31**, 647 (1976).
20. L. L. M. Van Deenan, *FEBS Symposium on the Biochemistry of Membrane Transport*, Zürich, L9 (1976).
21. R. J. Romani and A. Monadjem, *Proc. Natn. Acad. Sci. U.S.A.* **66**, 869 (1970).
22. E. Kun, E. B. Kearney, I. Wiedemann and N. M. Lee, *Biochemistry* **8**, 4443 (1969).
23. A. Binet and P. Volfin, *FEBS Lett.* **49**, 400 (1975).
24. M. C. Auclair, M. Lemeignan and A. Rodellec, *C. R. Acad. Sci.* (Serie D) **283**, 695 (1976).

* Y. M. Gargouïl, personal communication.