Biochemical Pharmacology, Vol. 30, No. 18, pp. 2603–2610, 1981.
Printed in Great Britain.

0006-2952/81/182603–08 $02.00/0

PROTECTIVE EFFECTS OF VERAPAMIL AND DILTIAZEM AGAINST INORGANIC PHOSPHATE INDUCED IMPAIRMENT OF OXIDATIVE PHOSPHORYLATION OF ISOLATED HEART MITOCHONDRIA

PÁL L. VÁGHY,* MOHAMMED A. MATLIB, LÁSZLÓ SZEKERES† and ARNOLD SCHWARTZ‡
Department of Pharmacology and Cell Biophysics, University of Cincinnati, College of Medicine, Cincinnati, OH 45267, U.S.A.
and
†Department of Pharmacology, University Medical School of Szeged, Szeged, Hungary

(*Received* 1 *December* 1980; *accepted* 25 *February* 1981)

Abstract—The effects of verapamil and diltiazem on oxidative phosphorylation of isolated rabbit heart mitochondria were related to the experimental conditions employed. In an assay medium containing 250 mM sucrose, 1 mM pyruvate and 5 mM potassium phosphate buffer (pH 7) at 37° (sucrose medium), only a high concentration of verapamil (200–800 μM) or diltiazem (400–600 μM) affected mitochondria. State 4 respiration was stimulated, state 3 respiration was inhibited, and the ADP:O ratio was decreased by these drugs in sucrose medium. These effects resulted in a depression of the respiratory control index (RCI) and oxidative phosphorylation rate (OPR). On the other hand, in an assay medium containing 150 mM KCl, 1 mM pyruvate and 2 mM potassium phosphate buffer (pH 7) at 37° (KCl medium), the high rate of state 3 respiration and the normal value of the ADP:O ratio were not influenced significantly by diltiazem (400–800 μM) or verapamil (200–400 μM). These data indicate that neither verapamil nor diltiazem has an effect on the normal, functioning, isolated mitochondria in KCl medium. Elevation of inorganic phosphate (P_i) from 2 to 5 mM in the KCl medium induced a swelling of the mitochondria, inhibition of state 3 respiration, and a decrease in the ADP:O ratio, RCI and OPR. Under these conditions, a low concentration of verapamil (25–200 μM) or diltiazem (50–800 μM) inhibited the swelling effect of P_i and at the same time prevented the P_i-induced decrease in state 3 respiration, and the ADP:O ratio, RCI and OPR. In a medium containing 150 mM KCl, 1 mM pyruvate, 2 mM ADP and 10 μM palmitoyl-CoA, the addition of 5 mM P_i induced swelling of mitochondria and a decreased rate of state 3 respiration. Under these conditions, even a low concentration of verapamil (6–200 μM) or diltiazem (25–400 μM) inhibited swelling and prevented the inhibition of state 3 respiration. It is concluded that low concentrations of verapamil and diltiazem had no effect on unswollen heart mitochondria. An increase in the free P_i concentration induced swelling of mitochondria and resulted in an inhibition of oxidative phosphorylation, provided that the extramitochondrial potassium concentration was as high as that normally found in the cytosol. Under these conditions, a low concentration of verapamil and diltiazem was able to affect the mitochondrial membranes so as to prevent P_i-induced swelling and the related inhibition of oxidative phosphorylation.

It is well known that experimentally induced ischemia results in the development of several metabolic, functional, and morphological changes in the myocardium [1–5]. Among these, swelling of mitochondria and impairment of oxidative phosphorylation have been consistently demonstrated [6–8]. Recently, several groups have shown that some of the *in vivo* consequences of myocardial ischemia, particularly mitochondrial damage, can be mitigated by pretreatment of the heart with verapamil [9–13] or diltiazem [14, 15], two clinically useful drugs that are generally classified as "calcium channel blockers" or "calcium antagonists" [16]. In spite of extensive research, however, neither the mechanism of the ischemic damage to mitochondria nor the exact modes of action of these drugs are well established, although the mitochondrial protective effect has been ascribed to an inhibition of increased calcium influx across the sarcolemma [11, 14, 17]. There is evidence, however, that metabolites which accumulate in the ischemic myocardial cells may also be responsible for the mitochondrial damage. These metabolites are fatty acyl-CoA esters [18], Ca^{2+} [19] and inorganic phosphate (P_i) [20]. Although the inhibition of the "slow or calcium channel" in the sarcolemma by "calcium antagonists" is well established [16], the involvement of other sites, both extra- and intracellular, in the pharmacological actions of these drugs has not been excluded and, in fact, has recently been implicated [21, 22]. If this is to be considered, then the abilities of these drugs to enter the cell and their availabilities to the intracellular organelles in sufficiently high concentrations must be demonstrated. It was shown recently that verapamil is not only able to enter the myocardial

* Pál L. Vághy is a Merrell Research Fellow. Permanent address: Department of Pharmacology, University Medical School of Szeged, Szeged, Hungary.
‡ Author to whom all correspondence should be addressed: Dr. Arnold Schwartz, Department of Pharmacology and Cell Biophysics, College of Medicine, University of Cincinnati Medical Center, 231 Bethesda Avenue, Cincinnati, OH 45267, U.S.A.

cells but that its tissue concentration in beating heart cells may be about thirty times higher than in the extracellular compartment [23, 24]. The intracellular accumulation of verapamil indicates that a direct effect of this drug and, perhaps, other calcium antagonists on subcellular organelles may occur and have important therapeutic significance.

The "calcium antagonists" are a heterogenous class of considerably diverse chemical structures [25]. Most of them are lipophilic, some are positively charged, and others are neutral. It is not surprising, therefore, that these drugs may interact with hydrophobic sites not only in or on the cell membrane, but with intracellular membrane sites as well. Furthermore, the *process* of interaction most certainly depends upon pH, ions, metabolites and temperature. Myocardial ischemia produces an immediate and profound change in the affected cells, which includes a drop in pH, a rise in P_i, a drop in creatine phosphate and ATP, a rise in fatty acyl CoA derivatives, and changes in intracellular Na^+, Ca^{2+} and K^+. It is likely that these changes alter the reactivity of intracellular membranes to certain drugs.

The aim of our investigations was to study the possibility of a direct effect of verapamil or diltiazem, two representative "calcium antagonists", on isolated heart mitochondria under conditions which may simulate some of the changes that characterize the cytosol in myocardial ischemia. The use of isolated mitochondria is convenient because the complexities of effects on the cell membrane are avoided. On the other hand, we recognize the difficulty of extrapolating *in vitro* drug effects to *in vivo* conditions and present the data with that caution in mind. Our results indicate that relatively low concentrations of verapamil or diltiazem have negligible effects on the *normal* functioning mitochondria, but that they protect, significantly, heart mitochondria against the deleterious effects of free inorganic phosphate which is known to accumulate in ischemic myocardial cells [26]. Preliminary aspects of this study were recently reported by Vághy *et al.* [27].

METHODS

Isolation of mitochondria. Male and female albino rabbits weighing 2–3 kg were employed. The chest was opened after cervical dislocation, and the heart was quickly removed and immediately placed in ice-cold physiological saline solution to wash it free from blood. The heart was then transferred to an ice-cold medium that contained 180 mM KCl, 10 mM ethyleneglycol-bis-(β-aminoethyl ether) *N,N'*-tetraacetic acid (EGTA) and 0.5% bovine serum albumin (BSA Fraction V, Sigma Chemical Co., St. Louis, MO), pH 7.4, at 4° (KEA medium). All subsequent steps were carried out in a cold room at 4°. The ventricular tissue was cut free from fat, large vessels and atria, the weight was determined, and then the tissue was minced with scissors. Approximately 1.5 to 2.0 g of tissue was homogenized in 10 ml of KEA medium with the aid of an electrically driven teflon pestle at 2500 rpm in a glass homogenizing vessel immersed in iced-saline solution. Three times three passes were made with a 30-sec rest period after three passes. Then 25 ml of KEA medium was added to the homogenate and three times three passes were made again as described above. The homogenate was centrifuged at 500 *g* for 10 min using a Beckman J-21C type centrifuge fitted with a JA-20 rotor. The supernatant fraction was filtered through two layers of cheesecloth into centrifuge tubes and was centrifuged at 8000 *g* for 10 min. The resultant pellet was washed by rinsing, resuspension and centrifugation at 8000 *g* for 10 min. The washing procedure was repeated and the final mitochondrial pellet was resuspended in a small volume of KEA medium to make a mitochondrial protein concentration of 20–30 mg per ml. This isolation method resulted in a yield of 20 mg mitochondrial protein/g wet tissue. These mitochondria were qualitatively similar to those isolated by the Polytron method [28], which yields about 10 mg mitochondrial protein/g wet tissue (authors' unpublished observation). The protein concentration of the final mitochondrial suspension was determined by the method of Lowry *et al.* [29], with bovine serum albumin as a standard.

Measurement of oxidative phosphorylation. The oxygen consumption of mitochondria was determined polarographically with the aid of a Clark type of oxygen electrode fitted to an Oxygraph model K-IC (Gilson Medical Electronics). The assays were carried out either at 30° or at 37° using 1 mg mitochondrial protein in 1.5 ml assay medium. The exact compositions of the various assay media are indicated in the legends of the figures and tables. State 3 and state 4 respiration and the RCI and the ADP:O ratio, were calculated according to Estabrook [30]. State 4 respiration was determined after the ADP was phosphorylated. The rate of respiration was expressed in nanoatoms oxygen consumed by 1 mg mitochondrial protein per min. The oxidative phosphorylation rate (OPR) was calculated according to Edoute *et al.* [31] and was expressed as nmoles ATP synthesized by 1 mg mitochondrial protein per min.

Determination of the osmotic volume changes. Two milligrams of mitochondrial protein was suspended in 3 ml of 150 mM KCl medium containing 1 mM pyruvate. The sample was stirred continuously with a magnetic stirrer and incubated at 37°; the changes in the light absorbance before and after the addition of P_i were recorded continuously at 640 nm with the aid of an Aminco model DW 2a spectrophotometer. Other additions are indicated in the legends of the figures. A decrease in light absorbance was evaluated as swelling of mitochondria [32, 33].

RESULTS

The temperature dependence of mitochondrial respiration and oxidative phosphorylation in a sucrose medium was examined (Table 1). When the temperature was increased from 30° to 37° both state 3 and state 4 respirations increased. The RCI and the ADP:O ratio, however, were not influenced significantly. As a result of these changes, the calculated oxidative phosphorylation rate (OPR), which is a measure of the rate of ATP production, was significantly higher at 37° than at 30°. All the subsequent experiments were carried out at 37°. In the sucrose medium, both verapamil and diltiazem

Table 1. Effect of temperature on respiration and oxidative phosphorylation of isolated heart mitochondria*

Temperature	N	State 3	State 4	RCI	ADP:O	OPR
30°	6	222†	18.4†	12.1	2.83	628†
		±9.4	±0.47	±0.28	±0.06	±27.4
37°	4	312.5†	26.8†	11.8	2.92	911†
		±19.1	±1.4	±0.5	±0.05	±53

* The assay medium contained 250 mM sucrose, 1 mM pyruvate, and 5 mM potassium phosphate buffer (pH 7). The experiments were started by the addition of 1 mg of mitochondrial protein to 1.5 ml of test medium. State 3 respiration was induced by the addition of 500 nmoles ADP. Statistical significances were calculated by Student's *t*-test. States 3 and 4 are expressed in nanoatoms oxygen consumed by 1 mg of mitochondrial protein per min. OPR is the oxidative phosphorylation rate expressed as nmoles ATP synthesized by 1 mg of mitochondrial protein per min. RCI is the respiratory control index (the ratio of state 3 : state 4 oxygen consumption).

† Values with the same symbols are significantly different ($P < 0.05$).

Table 2. Effects of verapamil on respiration and oxidative phosphorylation of isolated heart mitochondria in sucrose medium*

Verapamil (μM)	N	State 3	State 4	RCI	ADP:O	OPR
0	4	312.5	26.8	11.8	2.92	911
		±19.1	±1.4	±0.5	±0.05	±53
200	4	216.8†	32.3†	6.8†	2.81	608†
		±8.6	±1.3	±0.1	±0.04	±15
400	4	162.5†	37.8†	4.2†	2.75†	445†
		±9.6	±1.5	±0.2	±0.04	±23
800	4	134.5†	59.0†	2.3†	2.42†	325†
		±3.2	±1.8	±0.03	±0.06	±14

* The assay medium consisted of 250 mM sucrose, 1 mM pyruvate and 5 mM potassium phosphate buffer at 37° and pH 7. Values are means ±S.E. Statistical significances were calculated by Student's *t*-test. See Table 1 for definitions.

† Values are significantly different from control, no verapamil ($P < 0.05$).

Table 3. Effects of diltiazem on respiration and oxidative phosphorylation of isolated heart mitochondria in sucrose medium*

Diltiazem (μM)	N	State 3	State 4	RCI	ADP:O	OPR
0	4	312.5	26.8	11.8	2.92	911
		±19.1	±1.4	±0.5	0.05	±53
400	4	228.5†	29.8	7.9†	2.94	671†
		±9.5	±2.7	±0.9	±0.04	±23
800	4	216.0†	51.0	4.6†	2.82	608†
		±9.1	±10.6	±0.6	±0.05	±22
1600	4	180.8†	75.0†	2.45†	2.62†	474†
		±11.9	±8.0	±0.12	±0.04	±33

* Conditions are the same as indicated in the legend of Table 2.

† Values are significantly different from control, no diltiazem ($P < 0.05$).

Table 4. Effects of verapamil on respiration and oxidative phosphorylation of isolated heart mitochondria in KCl medium containing 2 mM P_i*

Verapamil (μM)	N	State 3	State 4	RCI	ADP:O	OPR
0	4	583.4 ±52.8	53.8 ±3.5	11.1 ±1.2	2.87 ±0.03	1675 ±164
200	4	567.8 ±36	48.1 ±3.0	11.9 ±0.6	2.99 ±0.06	1696 ±95
400	4	519.3 ±43.8	57.8 ±3.2	9.0 ±0.5	2.91 ±0.04	1505 ±115

* The assay medium contained 150 mM KCl, 1 mM pyruvate, and 2 mM potassium phosphate buffer (pH 7) at 37°. The experiments were started by the addition of 1 mg of mitochondrial protein to 1.5 ml of medium. State 3 respiration was induced by the addition of 527 nmoles ADP after 3 min. Values are means ±S.E. Statistical significances were calculated by Student's *t*-test. The values are not different significantly ($P > 0.05$)

inhibited state 3 respiration, increased state 4 respiration and decreased the RCI and OPR, but only when high concentrations of the drugs were used (Tables 2 and 3). The ADP:O ratio was also depressed by 400–800 μM verapamil and by 1600 μM diltiazem.

In KCl medium, state 3 respiration and the ADP:O ratio were largely dependent on the extramitochondrial concentration of inorganic phosphate. Normal ADP:O ratios and high rate state 3 respiration were obtained when only 2 mM P_i was present. In this condition, neither verapamil (200–400 μM) nor diltiazem (400–800 μM) had a significant effect (Tables 4 and 5).

When the P_i concentration was raised from 2 to 5 mM in the KCl medium, an inhibition of state 3 respiration occurred which was accompanied by a significant decrease in the RCI and a slightly depressed ADP:O ratio (Tables 6 and 7). Under these conditions, verapamil, in concentrations of 25–200 μM, exerted a dose-dependent *protective* effect, i.e., it prevented the decrease in state 3 respiration, the RCI and the ADP:O ratio (Table 6). In a similar manner, diltiazem, in a concentration range of 50–800 μM, protected mitochondria against the P_i-induced impairment of oxidative phosphorylation (Table 7). The OPR was considerably depressed by increasing the P_i in the KCl medium

Table 5. Effects of diltiazem on respiration and oxidative phosphorylation of isolated heart mitochondria in KCl medium containing 2 mM P_i*

Diltiazem (μM)	N	State 3	State 4	RCI	ADP:O	OPR
0	9	553.4 ±28.1	54.4 ±2.1	10.3 ±0.7	2.92 ±0.04	1618 ±86
400	6	581.7 ±40.8	49.0 ±1.3	11.9 ±0.7	3.00 ±0.04	1742 ±116
800	4	612.8 ±41.3	53.3 ±4.9	11.7 ±0.9	2.96 ±0.05	1811 ±105

* Conditions are the same as indicated in the legend of Table 4.

Table 6. Effects of P_i and verapamil on respiration and oxidative phosphorylation of isolated heart mitochondria in KCl medium*

P_i (mM)	Verapamil (μM)	N	State 3	State 4	RCI	ADP:O
2	0	6	583 ±36	53 ±2.4	11.1 ±0.8	2.89 ±0.04
5	0	6	450† ±56	54 ±1.5	8.3† ±1.0	2.71† ±0.04
5	25	6	514‡ ±46	54 ±0.9	9.6‡ ±0.9	2.83‡ ±0.05
5	50	6	558‡ ±44	49‡ ±0.9	11.5‡ ±0.9	2.86‡ ±0.03
5	100	6	605‡ ±41	47‡ ±2.3	13.1‡ ±0.9	2.96‡ ±0.03
5	200	6	629‡ ±49	47‡ ±2.4	13.3‡ ±0.6	2.99‡ ±0.01

* The assay medium contained 150 mM KCl, 1 mM pyruvate, and different concentrations of potassium phosphate buffer (P_i), pH 7, at 37°. The experiments were started by the addition of 1 mg of mitochondrial protein to 1.5 ml of medium. State 3 respiration was induced by the addition of 527 nmoles ADP after 3 min. Values are means ±S.E. Statistical significances were calculated by Student's paired *t*-test.

† Values are significantly different from 2 mM P_i ($P < 0.05$).

‡ Values are significantly different from 5 mM P_i, no verapamil ($P < 0.05$).

from 2 to 5 mM (Fig. 1). Low concentrations of both verapamil and diltiazem exerted a dose-dependent protective effect against the P_i-induced diminution of OPR.

Earlier studies suggested that the P_i-induced impairment of oxidative phosphorylation in the KCl

Table 7. Effects of P_i and diltiazem on respiration and oxidative phosphorylation of isolated heart mitochondria in KCl medium*

P_i (mM)	Diltiazem (μM)	N	State 3	State 4	RCI	ADP:O
2	0	10	551.5 ±25.2	56.5 ±2.8	10.2 ±0.7	2.93 ±0.04
5	0	10	415.8† ±36.3	58.7 ±4.9	7.4† ±0.7	2.62† ±0.07
5	25	6	425.3 ±63.5	62.0 ±11.6	7.3 ±1.2	2.61 ±0.06
5	50	7	436.3‡ ±38.6	59.9 ±6.6	7.4 ±0.8	2.63 ±0.05
5	100	6	500.3‡ ±36.8	68.2 ±12.3	7.9‡ ±0.9	2.70‡ ±0.06
5	200	6	555.8‡ ±32.3	63.8 ±10.1	9.6‡ ±1.2	2.88‡ ±0.05
5	400	7	621.7‡ ±44.5	54.3 ±1.4	11.5‡ ±1.0	2.97‡ ±0.03
5	800	6	625.0‡ ±45	53.6 ±1.3	11.6‡ ±0.7	3.00‡ ±0.04

* The conditions are the same as indicated in the legend of Table 6.

† Values are significantly different from 2 mM P_i ($P < 0.05$).

‡ Values are significantly different from 5 mM P_i, no diltiazem ($P < 0.05$).

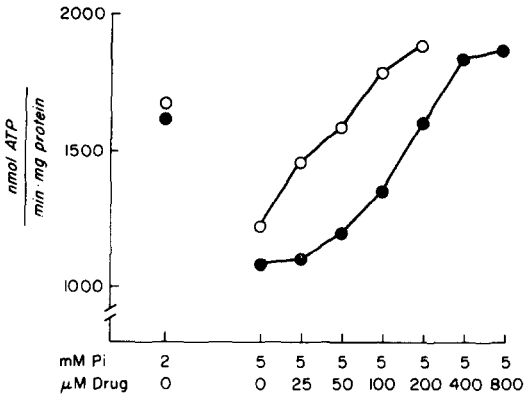

Fig. 1. Effects of verapamil and diltiazem on the oxidative phosphorylation rate of mitochondria in KCl medium containing 5 mM P_i. Conditions are the same as indicated in the legend of Table 6. Key: (○——○) effect of verapamil; and (●——●) effect of diltiazem.

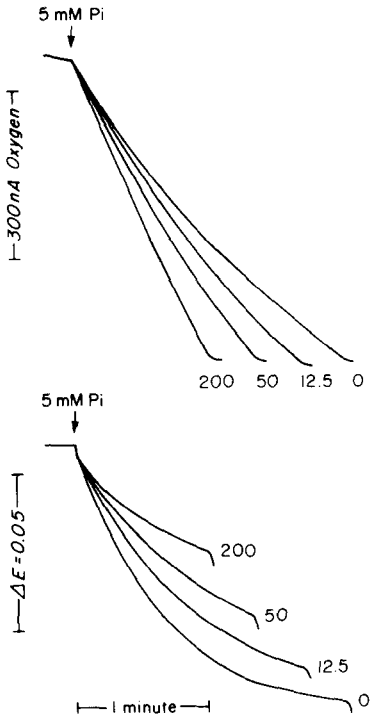

Fig. 3. Effects of verapamil on P_i-induced state 3 respiration and swelling of mitochondria. The assay medium contained 150 mM KCl, 1 mM pyruvate, 2 mM ADP and 10 μM palmitoyl-CoA (pH 7) at 37°. Mitochondria (1 mg/1.5 ml) were preincubated for 2 min in the absence of P_i; then 5 mM P_i was added. Upper panel: oxygen consumption of mitochondria in the absence and presence of verapamil; lower panel: changes in the light absorbance (extinction) measured at 640 nm in the absence and presence of verapamil. Numbers indicate the concentration of the drug in μM.

medium is related to the ability of P_i to induce swelling of mitochondria [20]. To confirm this possibility and to challenge the swelling with Ca^{2+}-blocking drugs, the volume changes of mitochondria were determined photometrically in substrate-supplied KCl medium. Swelling was initiated by the addition of various concentrations of P_i. Although 2 mM P_i induced only negligible changes, the addition of 5 mM P_i resulted in considerable swelling of the mitochondria (Fig. 2A), and both verapamil and diltiazem effectively inhibited this swelling process [Fig. 2(B and C)].

Addition of 5 mM P_i to mitochondria that had been preincubated for 2 min in KCl medium containing 2 mM ADP and 10 μM palmitoyl-CoA induced a slow rate of state 3 respiration and, at the same time, considerable swelling of mitochondria occurred. Verapamil or diltiazem in even lower concentrations inhibited the swelling effect of P_i in a dose-dependent manner and prevented the progressive inhibition of the P_i-induced state 3 respiration (Figs. 3 and 4). The lowest effective concentrations of verapamil and diltiazem in this condition were 6 and 12 μM respectively.

To provide easier comparison of the absolute values obtained with the different assay conditions, we have presented only those results which were obtained with 1 mM pyruvate as an oxidizable substrate. The 1 mM pyruvate in our assay medium is about 200 times higher than the K_m of heart mitochondria for pyruvate [34] and about twenty to fifty

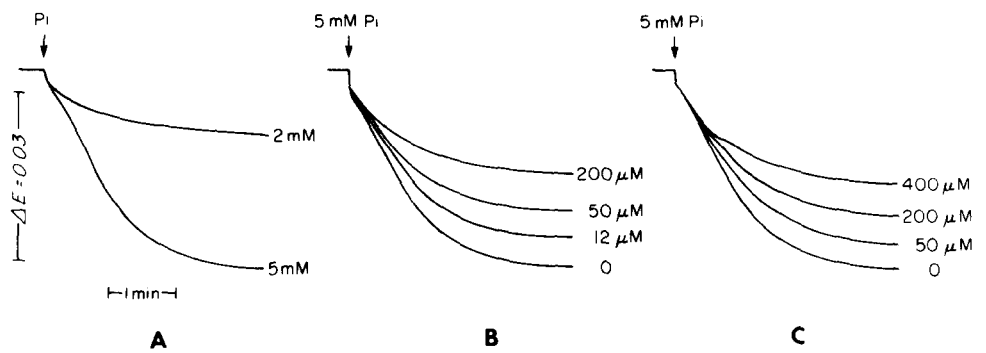

Fig. 2. Effects of verapamil and diltiazem on the inorganic phosphate induced swelling of mitochondria. The assay medium contained 150 mM KCl and 1 mM pyruvate (pH 7) at 37°. Two milligrams of mitochondrial protein was added to 3 ml of medium and the changes in light absorbance (extinction) were recorded at 640 nm. Inorganic phosphate (P_i) was added 2 min later. (A) Effects of 2 and 5 mM P_i. (B) Effects of verapamil and (C) effects of diltiazem on the swelling induced by 5 mM P_i.

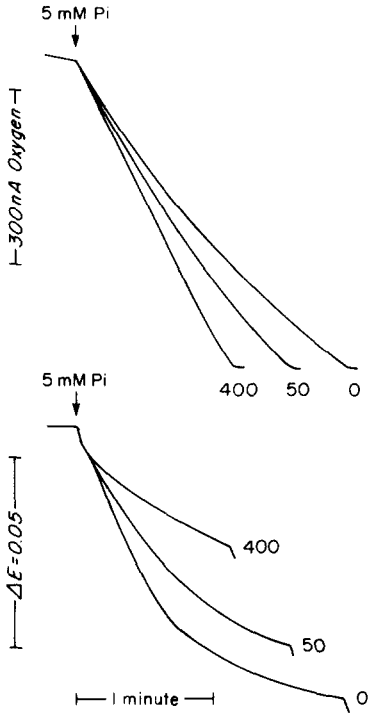

Fig. 4. Effects of diltiazem on the P_i-induced state 3 respiration and swelling of mitochondria. Conditions are the same as indicated in the legend for Fig. 3.

times higher than the concentration of this substrate in cardiac tissues [2, 35]. Furthermore, qualitatively similar changes were obtained using other NAD-linked substrates such as glutamate and malate.

DISCUSSION

Oxidative phosphorylation of isolated mitochondria is conventionally studied in isosmotic sucrose medium containing inorganic phosphate buffer, pH 7.2 to 7.4, and some type(s) of oxidizable substrate(s) at room temperature or in some cases at 30°. In spite of the fact that in these conditions the mitochondria are stable and are characterized by a rather high RCI, studies carried out in sucrose medium have shortcomings. First, respiration and ion transport in the mitochondrial preparation are inhibited at temperatures lower than the physiological 37°. Second, the function of the mitochondria is studied under nonphysiological conditions, since sucrose is not a component of the intracellular space. Third, pH 7.4 corresponds to the blood pH rather than to the pH of the cytosol where the mitochondria are located. Recent studies have revealed an intracellular pH very close to the 7.0 in the normoxic myocardium [36, 37]. Our data show that state 3 respiration and, therefore, the OPR are considerably higher at 37° and pH 7 than at 30°, but the RCI and ADP:O are unchanged. Consistent with previous reports [13, 14], we have found that low concentrations of verapamil or diltiazem do not affect oxidative phosphorylation when mitochondria are suspended in sucrose medium. It is not surprising that high concentrations of these drugs cause inhibition and uncoupling of oxidative phosphorylation. This is due, probably, to a disruptive effect on membranes, characteristic of high concentrations of lipophilic substances [38]. This type of effect cannot be related to any therapeutic actions of verapamil or diltiazem because the concentrations necessary to produce impairment of oxidative phosphorylation *in vitro* are probably much higher than those that are present in the cytosol at "therapeutic" blood concentrations, although these have not as yet been definitely determined [39]. Furthermore, an inhibition of ATP production could hardly be classified as "beneficial" under any circumstances. Our data indicate that, in the conventionally used sucrose medium, only the potentially damaging effects of these drugs on mitochondrial oxidative phosphorylation can be studied.

In order to study the possibility of a direct "therapeutic" effect of drugs on mitochondria of ischemic tissue, an *in vitro* model having characteristics somewhat similar to those that are found to be present *in vivo* under pathological conditions is needed. Accordingly, we changed the composition of the assay media to attempt to simulate what may occur in the cytosol of the ischemic myocardial cells. Because the potassium concentration in the cytosol is around 150 mM, the unphysiological sucrose component of the assay medium was replaced by KCl and the experiments were carried out at physiological pH and temperature. We observed a high rate of state 3 respiration in this medium when only 2 mM P_i was present, which corresponds approximately to the tissue P_i concentration of the normoxic myocardium [40]. In this condition the ADP:O ratio was at a normal level and the RCI was around 10. For this rather high rate of state 3 respiration, not surprisingly a high OPR [more than 1600 nmoles $ATP \cdot min^{-1} \cdot (mg\ protein)^{-1}$] was calculated. Furthermore, when the extramitochondrial P_i concentration was increased in the KCl medium in order to simulate the elevated tissue concentration of free P_i that has been reported in the ischemic myocardium [26], a significant decrease in state 3 respiration, RCI, ADP:O and OPR occurred. This observation is contrary to those which were demonstrated in the sucrose medium where stimulation of state 3 respiration resulted from an elevation of P_i from 1 to 8 mM at a constant ADP concentration (Refs. 41 and 42, and the authors' unpublished observations). The interpretation of these data is difficult and the important issue as to whether mitochondrial respiration is controlled by adenine nucleotide translocation [42] or by the phosphate potential [41] remains to be settled (for review see Ref. 43). To understand the different responses of mitochondria to the elevation of extramitochondrial P_i in various assay media, several factors need to be considered. First, there is a higher absolute value of state 3 respiration in KCl medium than in sucrose medium. This indirectly indicates that in a sucrose medium, where the extramitochondrial K^+ concentration is much lower than the K^+ concentration in the matrix of mitochondria (~ 140 mM [44]), oxidative phosphorylation is inhibited. We have observed that the increase in extramitochondrial KCl concentration in the

sucrose medium which contained 2 mM P_i results in a stimulation of state 3 respiration (unpublished observations). Increasing the P_i concentration in sucrose medium by adding more and more of the potassium phosphate salt results not only in an increase in the extramitochondrial P_i but also in the extramitochondrial K^+ concentration. This decreases the outward directed potassium concentration gradient across the inner membrane, which was initially established by placing mitochondria in the very low K^+ containing sucrose medium. Our data suggest that the state 3 respiration of mitochondria depends not only on the extramitochondrial concentration of P_i but also on the extramitochondrial K^+ concentration. It is also important to note that sucrose confers stability on mitochondria and prevents the swelling effect of inorganic phosphate. Therefore, it is not surprising that a relatively high rate of state 3 respiration can be measured even when 10 mM P_i is present in the sucrose medium [28].

The elevation of P_i induced a swelling of mitochondria in KCl medium not only in the absence but also in the presence of excess amounts of ADP and partially inhibitory concentrations of palmitoyl-CoA. As the swelling progressed, the rate of state 3 respiration diminished, indicating that the P_i-induced inhibition of state 3 respiration in KCl medium is closely related to the swelling effect of P_i in mitochondria. Neither verapamil nor diltiazem had a significant effect on oxidative phosphorylation, even at very high concentrations when only 2 mM P_i was present in the KCl medium. Moreover, instead of inhibitory and uncoupling effects, which were obtained in the presence of high drug concentrations in the sucrose medium, these drugs at considerably lower concentrations protected oxidative phosphorylation against the deleterious effects of elevated P_i in KCl medium. Accordingly, the sensitivity of heart mitochondria to these drugs increased considerably when the components of the assay medium were modified in order to simulate what occurs in the cytosol of the ischemic myocardial cells. In all conditions, verapamil was found to be more potent than diltiazem.

Although the effective concentrations of verapamil ranged from 6 to 25 μM, we still are unable to relate this "beneficial" effect to the *in situ* situation because therapeutic blood or tissue levels of verapamil in animals or humans in myocardial ischemia have not been determined. In one recent study, a new method for assay of verapamil was described [39]. Various infusions were correlated with blood levels in human serum and these were about 1 μM, but no relationship to any therapeutic effect was described. If the blood concentration *in vivo* indeed is in the micromolar range, the results reported in our study would be relevant only if some type of concentration process existed. Recently, a 30-fold accumulation of verapamil in working cardiac tissue has been demonstrated [23, 34].

The question also arises whether the effects of these "calcium antagonists" on mitochondria are related to the transport of calcium in mitochondria or are the result of a nonspecific membrane stabilizer action. We have demonstrated recently that, like verapamil and diltiazem, ruthenium red, a specific inhibitor of calcium transport in mitochondria, protects oxidative phosphorylation against the deleterious effects of elevated P_i concentration [45]. These data suggest that calcium may be involved in the P_i-induced damage to heart mitochondria and that the effects of low concentrations of "calcium antagonists" may be related to an inhibition of P_i-induced calcium movement in mitochondria.

Acknowledgements—We thank Dr. M. Ashraf for the electron microscopical examination of the mitochondrial preparations and Ms. Gwen Kraft for her excellent drawing of the figures. We also appreciate the valuable criticism of Drs. William Rouslin and Dennis Epps and the assistance of Mr. Billy Joe Rice in the animal experiments. Diltiazem was supplied by the Marion Research Laboratories, Inc., Kansas City, KS, verapamil was a gift from the Knoll Pharmaceutical Co., Whippany, NJ. This work was supported by NIH Grant PO1 HL 22619-03 (4B) and a grant from the Marion Research Laboratories Inc. Dr. Pál L. Vághy was supported by a fellowship from the Merrell Research Center, Cincinnati, OH.

REFERENCES

1. B. E. Sobel, *Circulation Res.* **34–35**, Suppl. III-173 (1974).
2. L. H. Opie, *Circulation Res.* **38**, Suppl. I-52 (1976).
3. B. F. Trump, W. J. Mergner, M. W. Kahng and A. J. Saladino, *Circulation* **53**, Suppl. I-17 (1976).
4. J. M. Wood, H. G. Hanley, M. L. Entman, C. J. Hartley, J. A. Swain, U. Busch, C. H. Chang, R. M. Lewis, W. J. Morgan and A. Schwartz, *Circulation Res.* **44**, 52 (1979).
5. J. W. De Jong, in *The Pathophysiology of Myocardial Perfusion* (Ed. W. Schaper) p. 179. Elsevier, Amsterdam (1979).
6. R. B. Jennings, H. M. Sommers, P. B. Herdson and J. P. Kaltenbach, *Ann. N.Y. Acad. Sci.* **156**, 61 (1969).
7. R. B. Jennings and C. E. Ganote, *Circulation Res.* **34–35**, Suppl. III-156 (1974).
8. W. Rouslin and R. W. Millard, *J. molec. cell. Cardiol.* **12**, 639 (1980).
9. W. G. Nayler, A. Grau and A. Slade, *Cardiovas. Res.* **10**, 650 (1976).
10. W. G. Nayler, E. Fassold and C. Yepez, *Cardiovas. Res.* **12**, 152 (1978).
11. W. G. Nayler, R. Ferrari and A. Williams, *Am. J. Cardiol.* **46**, 242 (1980).
12. K. A. Reimer, J. E. Lowe and R. B. Jennings, *Circulation* **55**, 581 (1977).
13. I. P. Clements, R. E. Vliestra, J. D. Dewey and C. E. Harrison, Jr., in *Proceedings of the Third International Symposium on Coronary Heart Disease* (Ed. M. Kaltenbach), p. 284. George Their Vilog, Stuttgart (1978).
14. A. Schwartz, T. Nagao and M. A. Matlib, in *New Drug Therapy with a Calcium Antagonist* (Ed. R. J. Bind), p. 480 Excerpta Medica, Amsterdam (1979).
15. T. Nagao, M. A. Matlib, D. Franklin, R. W. Millard and A. Schwartz, *J. molec. cell. Cardiol.* **12**, 29 (1980).
16. A. Fleckenstein, *A. Rev. Pharmac. Toxic.* **17**, 149 (1977).
17. J. A. Watts, C. D. Koch and K. F. LaNoue, *Am. J. Physiol.* **238**, H908 (1980).
18. A. L. Shug, E. Shrago, N. Bittar, J. D, Folts and J. R. Koke, *Am. J. Physiol.* **228**, 689 (1975).
19. A. C. Shein and R. B. Jennings, *Am. J. Path.* **67**, 441 (1972).
20. P. L. Vághy, P. Bor and L. Szekeres, *Biochem. Pharmac.* **29**, 1385 (1980).

21. J. Church and T. T. Zsotér, *Can. J. Physiol. Pharmac.* **58**, 254 (1980).
22. T. T. Zsotér, *Am. Heart J.* **99**, 805 (1980).
23. H. Lüllmann, P. B. M. W. M. Timmermans and A. Ziegler, *Eur. J. Pharmac.* **60**, 277 (1979).
24. H. Lüllmann, P. B. M. W. M. Timmermans, G. M. Weikert and A. Ziegler, *J. med. Chem.* **23**, 560 (1980).
25. D. J. Triggle and V. C. Swamy, *Chest* **78**, Suppl. 174 (1980).
26. E.-G. Krause and A. Wollenberger, *Biochem. Z.* **342**, 171 (1965).
27. P. L. Vághy, M. A. Matlib, M. Ashraf and A. Schwartz, *Circulation* **62**, Suppl. III-177 (1980).
28. L. A. Sordahl, C. Johnson, Z. R. Blailock and A. Schwartz, in *Methods in Pharmacology* (Ed. A. Schwartz), Vol. 1, p. 247. Meredith, New York (1971).
29. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
30. R. W. Estabrook, in *Methods in Enzymology* (Eds. R. W. Estabrook and M. E. Pullman), Vol. 10, p. 41. Academic Press, New York (1967).
31. Y. Edoute, J. C. N. Kotzé and A. Lochner, *J. molec. cell. Cardiol.* **11**, 831 (1979).
32. G. P. Brierley, M. Jurkowitz, K. M. Scott and A. J. Merola, *Archs Biochem. Biophys.* **147**, 545 (1971).
33. S. Izzard and H. Tedeschi, *Archs Biochem. Biophys.* **154**, 527 (1973).
34. S. V. Pande and R. Parvin, *J. biol. Chem.* **253**, 1565 (1978).
35. J. R. Williamson, *J. biol. Chem.* **241**, 5026 (1966).
36. P. A. Poole-Wilson, *J. molec. cell. Cardiol.* **10**, 511 (1978).
37. P. B. Garlick, G. K. Radda and P. J. Seeley, *Biochem. J.* **184**, 547 (1979).
38. A. Helenius and K. Simons, *Biochim. biophys. Acta* **415**, 29 (1975).
39. T. M. Jaouni, M. B. Leon, D. R. Rosing and H. M. Fales, *J. Chromat.* **182**, 473 (1980).
40. A. Wollenberger, E.-G. Krause and B. E. Wahler, *Pflügers Arch. ges. Physiol.* **270**, 413 (1960).
41. A. Holian, C. S. Owen and D. F. Wilson, *Archs Biochem. Biophys.* **181**, 164 (1977).
42. J. J. Lemasters and A. E. Sowers, *J. biol. Chem.* **254**, 1248 (1979).
43. K. F. LaNoue and A. C. Schoolwerth, *A. Rev. Biochem.* **48**, 871 (1979).
44. G. P. Brierley, *Molec. cell. Biochem.* **10**, 41 (1976).
45. P. L. Vághy, M. A. Matlib and A. Schwartz, *Biochem. biophys. Res. Commun.* **100**, 37 (1981).