dation under those conditions indicating that an interaction between acetaminophen and ascorbic acid [8] was not necessary for the acetaminophen effect.

Oxygen consumption is an early event in lipid peroxidation preceded only by free radical formation. TBA-reactive substance formation is a late event. If an antioxidant inhibits TBA-reactive substance formation to a greater extent than O_2 consumption, it is probably acting in the mid-portion of the lipid peroxidation process, perhaps interfering with peroxide breakdown. If both are inhibited to the same extent, the antioxidant is acting early, probably scavenging free radicals. Oxygen electrode experiments shown in Fig. 2B demonstrate that the stoichiometric relationship between TBA-reactive substance production and O_2 consumption was not changed by acetaminophen. This suggests that acetaminophen scavenges free radicals in the system before they can react with O_2.

Several pathways are active in the metabolism of acetaminophen, but a minor one which is dependent on cytochrome P-450 is responsible for the hepatotoxicity of the drug [1]. Recent studies have suggested that *N*-acetylimidoquinone may be the toxic intermediate of the drug [8] and that enzyme systems other than cytochrome P-450 can produce it [8, 9]. The present findings raise the possibility that acetaminophen can be metabolized non-enzymatically, i.e. by lipid peroxidation. Evaluation of this will require further study.

While acetaminophen is an antioxidant under the conditions used in these experiments, it can cause lipid peroxidation as well. Fasted mice given high doses of acetaminophen exhale large amounts of ethane, indicating that lipid peroxidation is occurring *in vivo* [10]. This observation would suggest that the chemical antioxidant effect of acetaminophen may have been overcome by metabolic alterations caused by the compound. Thus, because of its complex metabolic effects, acetaminophen is not a reliable *in vivo* antioxidant. However, consideration of its antioxidant properties would appear to be essential in interpreting the effects of acetaminophen in biological systems.

In summary, acetaminophen, added in subtoxic doses, protected isolated vitamin E-deficient hepatocytes against spontaneous lipid peroxidation and loss of viability. The compound can also inhibit non-enzymatic microsomal lipid peroxidation, indicating that its effect in the cells is due to intrinsic antioxidant properties.

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REFERENCES

1. J. R. Mitchell, D. J. Jollow, W. Z. Potter, D. C. Davis, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. Ther.* **187**, 185 (1973).
2. K. E. Hill and R. F. Burk, *J. biol. Chem.* **257**, 10668 (1982).
3. R. F. Burk, M. J. Trumble and R. A. Lawrence, *Biochim. biophys. Acta* **618**, 35 (1980).
4. S. Orrenius, G. Dallner and L. Ernster, *Biochem. biophys. Res. Commun.* **14**, 329 (1964).
5. T. C. Pederson and S. D. Aust, *Biochem. Pharmac.* **23**, 2467 (1974).
6. P. R. Miles, J. R. Wright, L. Bowman and H. D. Colby, *Biochem. Pharmac.* **29**, 565 (1980).
7. R. F. Burk, *Biochem. Pharmac.* **31**, 601 (1982).
8. S. D. Nelson, D. C. Dahlin, E. J. Rauckman and G. M. Rosen, *Molec. Pharmac.* **20**, 195 (1981).
9. J. A. Boyd and T. E. Eling, *J. Pharmac. exp. Ther.* **219**, 659 (1981).
10. A. Wendel and S. Feuerstein, *Biochem. Pharmac.* **30**, 2513 (1981).

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Actions of certain calcium channel blockers and calmodulin antagonists on inorganic phosphate-induced swelling and inhibition of oxidative phosphorylation of heart mitochondria

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Diltiazem, nifedipine and verapamil, members of the class of drugs known as calcium antagonists or calcium channel blockers [1, 2], were shown previously to mitigate the impairment of mitochondria in the isochemic myocardium [3–5]. Diltiazem and verapamil were also found to prevent inorganic phosphate-induced swelling of heart mitochondria *in vitro* [6]. Recently it was reported that calcium channel blockers bind to purified calmodulin [7, 8]. This observation raised the possibility that the effect of these drugs on mitochondria may be due to the inhibition of a calmodulin-mediated reaction.

This study was carried out to determine whether the effectiveness of calcium channel blocking drugs in preventing inorganic phosphate-induced swelling and inhibition of oxidative phosphorylation follows the rank and order of effectiveness in which they were found to bind to purified calmodulin [7, 8]. The effects of trifluoperazine and R 24 571, two well-known inhibitors of calmodulin mediated reactions [9–11], were also investigated to determine the possible involvement of calmodulin in P_i-induced swelling of mitochondria.

Methods

Mitochondria from rabbit heart [6] and rat liver [12] were isolated as described previously. The initial homogenization medium for rat liver, unlike the reported pro-

cedure [12], contained 1 mM EGTA*, but in the subsequent steps it was omitted from the medium. The protein concentration of the final mitochondrial suspension (30–40 mg/ml) was determined by the method of Lowry *et al.* [13]. Oxygen consumption by mitochondria was measured as described previously [6]. The rates of state 3 and state 4 respiration, ADP/0 ratios, and respiratory control index (RCI) were calculated according to Estabrook [14]. The oxidative phosphorylation rate (OPR) was calculated according to Edoute *et al.* [15]. Swelling of mitochondria was monitored by recording the light absorbance changes of the mitochondrial suspension at 540 nm in an Aminco model DW-2a spectrophotometer as described previously [6]. The isolated rabbit heart mitochondria used in the present study contained 2.35 ± 0.49 (N = 6) nmoles of calcium and 21.84 ± 0.19 nmoles (N = 6) of magnesium per mg mitochondrial protein as determined by atomic absorption spectroscopy [16]. The calmodulin content of isolated mitochondria was determined by a radioimmunoassay procedure [17], using a calmodulin radioimmunoassay kit purchased from the Caabco Corp., Houston, TX. The drugs were obtained from the following sources: diltiazem, a benzothiazepine derivative, from Marion Research Laboratories, Kansas City, KS; verapamil, a papaverine derivative, from Knoll Pharmaceuticals, Whippany, NJ; nisoldipine (Bay k 5552), a dihydropyridine derivative, from Miles Research Laboratory, New Haven, CT; Prenylamine and fendiline, two of the diphenylpropylamine derivatives, from the Chinoin Pharmaceutical Co., Budapest, Hungry; trifluoperazine, a phenothiazine, from Smith Kline & French, Philadelphia, PA; and dibucaine from the Sigma Chemical Co., St. Louis, MO. R 24 571 was obtained from Janssen Pharmaceutica, Beerse, Belgium.

Results

Incubation of rabbit heart mitochondria suspended in the assay medium containing 2 mM inorganic phosphate (a concentration usually found in the normoxic myocardium [18]) resulted in only a small decrease in light absorbance (Fig. 1), indicating minimal swelling. Measurement of the rate of state 3 respiration (Q^2O_2) after 3 min of preincubation with 2 mM P_i under these conditions demonstrated that the mitochondria retained normal respiratory function (Table 1). Elevation of the P_i concentration to 5 mM

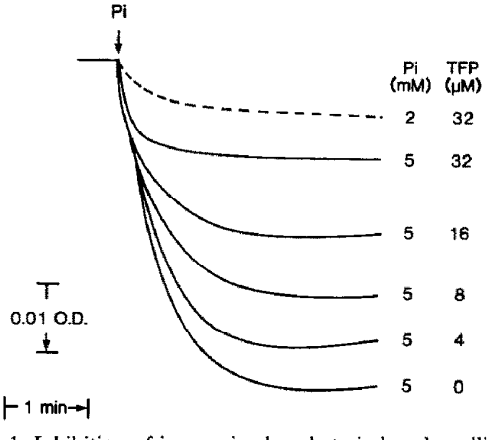

Fig. 1. Inhibition of inorganic phosphate-induced swelling of mitochondria by trifluoperazine (TFP). Swelling was determined spectrophotometrically at 540 nm as described under Methods. Inorganic phosphate (P_i) was added as indicated. TFP was added before P_i.

* Abbreviations: EGTA, ethyleneglycol-bis-(β-aminoethyl)*N*,*N*'-tetraacetic acid; BSA, bovine serum albumin; and TFP, trifluoperazine.

Table 1. Prevention of inorganic phosphate-induced inhibition of mitochondrial oxidative phosphorylation by trifluoperazine*

Inorganic phosphate (mM)	Trifluoperazine (μM)	Rate of respiration [nanoatoms oxygen · min^{-1} · mg protein)$^{-1}$] State 3	State 4	Respiratory control index (RCI)	ADP/O ratio	Oxidative phosphorylation rate (OPR) [nmoles ATP · min^{-1} · mg protein)$^{-1}$]
2	0	413 ± 23	55 ± 3	7.7 ± 0.8	2.89 ± 0.02	1190 ± 65
5	0	266 ± 25†	46 ± 2	5.8 ± 0.4†	2.78 ± 0.03†	739 ± 70†
5	4	316 ± 27	46 ± 3	7.0 ± 0.7	2.78 ± 0.01	882 ± 76
5	8	364 ± 28‡	45 ± 2	8.1 ± 0.6‡	2.84 ± 0.05	1042 ± 82‡
5	16	419 ± 31‡	44 ± 3	9.5 ± 0.8‡	2.95 ± 0.05‡	1244 ± 109‡
5	24	471 ± 20‡	50 ± 2	9.4 ± 0.2‡	2.87 ± 0.03	1355 ± 64‡
5	32	490 ± 26‡	47 ± 3	10.6 ± 0.7‡	2.91 ± 0.04‡	1412 ± 66‡
5	64	460 ± 12‡	61 ± 2‡	7.5 ± 0.2	2.83 ± 0.02	1301 ± 32‡

* Each value is the mean ± S.E. of six separate experiments.
† Significantly different from control (2 mM P_i), $P < 0.05$.
‡ Significantly different from 5 mM P_i, $P < 0.05$.

resulted in a large decrease in the light absorbance, indicating swelling of mitochondria. The magnitude of swelling was found to be dependent on P_i concentration as reported earlier [16]. This coincided with a significantly decreased rate of state 3 respiration, RCI, ADP/O ratios, and oxidative phosphorylation rate (OPR). Preincubation of mitochondria for 3 min with trifluoperazine (TFP) resulted in a dose-dependent inhibition of the swelling induced by 5 mM P_i (Fig. 1). TFP also prevented the inhibition of oxidative phosphorylation induced by 5 mM P_i (Table 1). The concentration of TFP which maintained the Q^3O_2 at the 2 mM P_i level was found to be about 16 μM. For unknown reasons, concentrations of TFP above 16 μM slightly increased the Q^3O_2 above the level at 2 mM P_i. However, concentrations of TFP above 32 μM produced uncoupling of mitochondria. These data indicate that TFP, at low concentrations, can prevent P_i-induced swelling and inhibition of oxidative phosphorylation. Dibucaine, a local anesthetic and also a calmodulin antagonist [19], had similar effects. The relative effectiveness of calmodulin antagonists and certain calcium channel blocking drugs in preventing P_i-induced inhibition of oxidative phosphorylation are presented in Table 2. R 24 571, which is reported to be relatively specific and much more potent than TFP in inhibiting calmodulin-mediated reactions [11], had no effect on P_i-induced swelling and inhibition of oxidative phosphorylation. Indeed, instead of a protective effect, this drug produced a further inhibition of state 3 oxygen consumption at higher concentrations. Concentrations of R 24 571 higher than 20 μM could not be used since the drug was found to be insoluble in the assay medium.

The addition of calmodulin (50 μg/mg protein) and Ca^{2+} (15 nmoles/mg protein) to mitochondria in an EGTA-free assay medium had no effect on P_i-induced swelling and inhibition of oxidative phosphorylation of mitochondria or on the protective action of TFP. EGTA exacerbated the P_i-induced swelling and inhibition of oxidative phosphorylation as we reported earlier [16]. The calmodulin content of the rabbit heart mitochondria was determined by radioimmunossay and compared with those of isolated rat liver mitochondria which have been reported to contain calmodulin [20] and calmodulin receptors [21]. The calmodulin content was 125 and 68 ng/mg protein of rabbit heart and rat liver mitochondria respectively.

Discussion

The results of this study show that calcium channel blocking drugs and certain calmodulin antagonists inhibited phosphate-induced swelling of heart mitochondria. The order of effectiveness of these compounds followed the order in which they are found to bind to purified calmodulin [7], with the exception of R 24 571. Thus, it would appear that a calmodulin-dependent process might be involved in the phosphate-induced swelling of heart mitochondria. However, the established criteria for calmodulin involvement [10] cannot be applied in the present study due to the permeability barrier to calmodulin imposed by the inner mitochondrial membrane. The inability of R 24 571, a specific inhibitor of calmodulin [11], to inhibit phosphate-induced swelling indicates that calmodulin is probably not involved in this process. In addition, the rather low levels of calmodulin we and others [22, 23, and this report] have found in isolated mitochondria are, in our opinion, insufficient to play any role in phosphate-induced swelling of mitochondria. Thus, we conclude that the inhibitory effect of calcium channel inhibitory drugs and certain calmodulin antagonists does not result from inhibition of a calmodulin-dependent process.

Table 2. Relative effectiveness of calcium channel blocking drugs and calmodulin inhibitors on inorganic phosphate-induced inhibition of the rate of state 3 respiration (Q^3O_2) of isolated heart mitochondria

Drugs	Q^3O_2 IC_{50} (μM)*
R 24 571	(−)†
Fendiline	4
Prenylamine	5
Trifluoperazine	10
Nisoldipine (Bay k 5552)	25
Dibucaine	30
Verapamil	50
Diltiazem	150

* Concentrations of the drugs that produced 50% of the maximum protective effect in the presence of 5 mM P_i.

† No protective effect up to 20 μM. A further inhibition of Q^3O_2 was observed in the presence of the drug.

Swelling of mitochondria *in vitro* induced by phosphate in KCl containing medium, as observed in the present study, probably involved in movement of K^+ and phosphate across the inner membrane [24, 25]. It may be postulated that the inhibition of swelling by the drugs used in the present study was due to the inhibition of K^+ and/or phosphate transport across the inner mitochondrial membrane. It is unlikely that these drugs interfere with the phosphate transport since the inhibition of this process would have resulted in the inhibition of oxidative phosphorylation which was not observed. Thus, a more plausible hypothesis for the inhibition of swelling of mitochondria is the inhibition of K^+ transport across the inner mitochondrial membrane. Support for this hypothesis comes from the observation that dibucaine, which inhibited phosphate-induced swelling of mitochondria, is also known to inhibit K^+ transport across the inner membrane [26, 27]. It is possible, therefore, that the calcium channel inhibitory drugs, dibucaine and trifluoperazine, act directly on K^+ transport or act indirectly by affecting divalent cation content of mitochondria since divalent cations are known to regulate monovalent cation transport [28, 29].

The pharmacological significance of the inhibition of phosphate-induced swelling of heart mitochondria is not known. It is possible that these drugs may be "beneficial" for mitochondria in the ischemic myocardium where accumulation of phosphate [18, 30] and swelling impairment of mitochondria [31–34] may occur. Any effect on mitochondria would of course depend upon the intracellular concentration of the drugs; information on that is unknown.

In summary, calcium channel inhibitory drugs, such as fendiline, prenylamine, nisoldipine (Bay k 5552), verapamil and diltiazem, and calmodulin antagonists, such as dibucaine and trifluoperazine, with the exception of R 24 571, were found to prevent phosphate-induced swelling of rabbit heart mitochondria *in vitro*. It is suggested that this effect of these drugs on swelling of mitochondria is probably due to the inhibition of K^+ transport across the inner mitochondrial membrane without involving calmodulin.

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REFERENCES

1. A. Fleckenstein, *A. Rev. Pharmac. Toxic.* **17**, 149 (1977).
2. A. Schwartz, *Am. J. Cardiol.* **49**, 497 (1982).
3. T. Nagao, M. A. Matlib, D. Franklin, R. W. Millard and A. Schwartz, *J. molec. cell. Cardiol.* **12**, 29 (1980).
4. L. R. Bush, Y-P, Li, M. Shlafer, J. R. Jolly and B. R. Lucchesi, *J. Pharmac. exp. Ther.* **218**, 653 (1981).
5. W. G. Nayler, R. Ferrari and A. Williams, *Am. J. Cardiol.* **46**, 242 (1980).
6. P. L. Vaghy, M. A. Matlib, L. Szekeres and A. Schwartz, *Biochem. Pharmac.* **30**, 2603 (1981).
7. J. D. Johnson, P. L. Vaghy, T. H. Crouch, J. D. Potter and A. Schwartz, in *Advances in Pharmacology and Therapeutics II* (Eds. H. Yoshida, Y. Hagihara and S. Ebashi), Vol. 3, p. 121. Pergamon Press, New York (1982).
8. P. M. Epstein, K. Fiss, R. Hachisu and D. M. Andrenyak, *Biochem. biophys. Res. Common.* **105**, 1142 (1982).
9. R. M. Leven and B. Weiss, *Biochim. biophys. Acta* **540**, 197 (1978).
10. W. Y. Cheung, *Science* **207**, 19 (1980).
11. H. V. Belle, *Cell Calcium* **2**, 483 (1981).
12. M. A. Matlib and P. A. Srere, *Archs Biochem. Biophys.* **174**, 705 (1976).
13. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
14. R. W. Estabrook, in *Methods in Enzymology* (Eds. R. W. Estabrook and M. E. Pullman), Vol. 10, p. 41. Academic Press, New York (1967).
15. Y. Edoute, J. C. N. Kotze and A. Lochner, *J. molec. cell. Cardiol.* **11**, 831 (1979).
16. P. L. Vaghy, M. A. Matlib and A. Schwartz, *Biochem. biophys. Res. Commun.* **100**, 37 (1981).
17. J. G. Chafouleas, J. R. Dedman, R. P. Munjaal and A. R. Means, *J. biol. Chem.* **254**, 10262 (1979).
18. E-G. Krause and A. Wollenberger, *Biochem. Z.* **342**, 171 (1965).
19. M. Volpi, R. I. Sha'afi, P. M. Epstein, D. M. Andrenyak and M. B. Feinstein, *Proc. natn. Acad. Sci. U.S.A.* **78**, 795 (1981).
20. O. Hatase, M. Tokuda, T. Itano, H. Matsui and A. Doi, *Biochem. biophys. Res. Commun.* **104**, 673 (1982).
21. R. L. Pardue, M. A. Kaetzel, S. H. Hahn, B. R. Brinkley and J. R. Dedman, *Cell* **23**, 533 (1981).
22. J. A. Smoake, S-Y. Song and W. Y. Cheung, *Biochim. biophys. Acta* **341**, 402 (1974).
23. E. Carafoli, V. Niggli, K. Malmstrom and P. Caroni, *Ann. N.Y. Acad. Sci.* **356**, 258 (1980).
24. G. P. Brierley, M. Jurkowitz, K. M. Scott and A. J. Merola, *Archs Biochem. Biophys.* **147**, 546 (1971).
25. S. Izzard and H. Tedeschi, *Archs Biochem. Biophys.* **154**, 527 (1973).
26. A. Azzi and A. Scarpa, *Biochim. biophys. Acta* **135**, 1087 (1967).
27. D. W. Jung and G. P. Brierley, *Archs Biochem. Biophys.* **193**, 76 (1979).
28. K. D. Garlid, *J. biol. Chem.* **255**, 11273 (1979).
29. G-Y. Shi, D. W. Jung, K. D. Garlid and G. P. Brierley, *J. biol. Chem.* **255**, 10306 (1979).
30. P. B. Garlick, G. K. Radda and P. J. Seeley, *Biochem. J.* **184**, 547 (1979).
31. A. Schwartz, J. M. Wood, J. C. Allen, E. P. Bornet, M. L. Entman, M. A. Goldstein, L. A. Sordahl and M. Suzuki, *Am. J. Cardiol.* **32**, 46 (1973).
32. R. B. Jennings and C. E. Ganote, *Circulation Res.* **34** and **35** (Suppl. III), 156 (1974).
33. R. B. Jennings and C. F. Ganote, *Circulation Res.* **38**, (Suppl. I), I–80 (1976).
34. 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).

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Modulation of prostanoid synthesis by antimicrobials

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To maintain cells or tissues in culture for extended periods of time often requires that antimicrobials be added to the culture medium. The influence of such agents, however, on the characteristics of the tissues must be considered. One characteristic of mammalian tissues which is of interest in many studies is the production of prostanoids (prostaglandins and thromboxanes). The modulation of prostaglandin synthesis by many drugs has been demonstrated [1–4], although antimicrobials have not been studied extensively in this respect. We have investigated, therefore, the effects on prostanoid synthesis of an antimicrobial mixture, containing penicillin, streptomycin, and amphotericin B, which is commonly used to inhibit microbial growth in culture media. The individual drugs in the mixture were tested and two other unrelated antimicrobials, tetracycline and metronidazole, were tested also.

The antibiotic–antimycotic mixture investigated in this study (No. 600-5240 Gibco Laboratories, Grand Island, NY) contained penicillin G (10,000 units/ml), streptomycin (10 mg/ml) and amphotericin B (25 μg/ml). The manufacturer recommends that this mixture be added to culture media in a concentration of 1% (v/v). Individual antimicrobial drugs were obtained from the Sigma Chemical Co., St. Louis, MO. The actions of the drugs on prostanoid biosynthesis were tested by use of a microsome-enriched preparation of bovine seminal vesicle (BSV) prostaglandin synthase that was obtained from Miles Laboratories, Elkhart, IN. The standard assay mixture contained 4 mg of BSV microsomal protein, sodium arachidonate (61 μM), and an appropriate amount of test drug dissolved in phosphate buffer (50 mM, pH 7.4) to give a final assay volume of 1.0 ml. The amphotericin B was soluble only to a small extent in aqueous media and was first dissolved in dimethyl sulfoxide (DMSO) (Baker Chemical, Phillipsburg, NJ) before being added to the assay mixture. Two concentrations of DMSO were used such that the final concentrations of DMSO in the test assay were 1% and 8.6% (w/v). DMSO at these concentrations, without amphotericin B, was also tested. After 20 min of incubation with gentle shaking at 37°, the reaction was stopped by adding 0.5 ml