Biochemical Pharmacology, Vol. 37, No. 21, pp. 4197–4203, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

PROTECTION OF CARDIAC MEMBRANE PHOSPHOLIPID AGAINST OXIDATIVE INJURY BY CALCIUM ANTAGONISTS

DAVID R. JANERO,* BARBARA BURGHARDT and RENE LOPEZ
Department of Pharmacology and Chemotherapy, Roche Research Center, Hoffmann-La Roche Inc., Nutley, NJ 07110, U.S.A.

(*Received* 12 *February* 1988; *accepted* 28 *April* 1988)

Abstract—Calcium antagonists representative of the four major chemical classes were assessed for their abilities to prevent peroxidation of rat heart membrane lipids through xanthine oxidase-dependent, superoxide-driven, iron-promoted oxygen radical chemistry. The dihydropyridines nifedipine and nitrendipine did not affect peroxidation, even at a concentration (500 μM) approaching their solubility limit. The benzothiazepine diltiazem did protect the cardiac lipids against oxidative injury, but at high micromolar concentrations: 50% inhibition of peroxidation (antiperoxidant IC_{50}) required 510 μM diltiazem. The phenylalkylamines verapamil and gallopamil (D-600) were likewise weak antiperoxidants (~35% inhibition of peroxidation at 500 μM). In contrast, two other alkylamines, bepridil and prenylamine, were very effective membrane lipid protectants with respective antiperoxidant IC_{50} values of 55 and 75 μM. The diphenylpiperazines flunarizine (IC_{50} = 190 μM) and cinnarizine (IC_{50} = 180 μM) displayed moderate antiperoxidant activity. No Ca^{2+} antagonist inhibited xanthine oxidase under conditions whereby 10 μM allopurinol inhibited enzyme activity by 50%. The effects of the Ca^{2+} antagonist-antiperoxidants on the kinetics of cardiac membrane lipid peroxidation indicate that they inhibit peroxidation by intercepting oxy- and/or lipid free radical intermediates. These data raise the possibility that antiperoxidant action may contribute to the spectrum of pharmacologic and therapeutic activities of certain Ca^{2+} antagonists.

Oxidative damage to membrane lipids is increasingly recognized as a pathogenic factor in a variety of diseases [1]. Experimental evidence has implicated membrane peroxidation mediated by Fenton-type, metal-promoted oxygen chemistry [2] in the development of ischemic and reperfusion heart damage [3]. Key roles have been assigned to xanthine oxidase (XOD†; xanthine:oxygen oxidoreductase, EC 1.2.3.2) as enzymatic source of superoxide anion radical (O_2^-) and to iron (Fe^{2+}/Fe^{3+}) as redox couple in the formation of the oxygen and fatty-acyl lipid radicals being identified and spin-trapped in ischemic myocardium [4, 5].

Inhibition of an inward Ca^{2+} current is a well-recognized property of Ca^{2+} antagonists [6], especially important in the heart muscle cell and in the pharmacologic management of cardiovascular disease [7]. However, the membrane effects of Ca^{2+} antagonists are not limited to blockade of the potential-dependent Ca^{2+} channel. Some Ca^{2+} antagonists, for instance, inhibit sodium- and potassium-dependent membrane processes [8] and ligand binding to muscarinic, adrenergic, and opiate membrane receptors [9]. Ca^{2+} antagonists "cytoprotect" the heart muscle cell and its membranes in occlusion-reperfusion animal models of myocardial ischemia, but the biochemical basis of the cytoprotection is ill-defined [10].

We report here investigations on the abilities of Ca^{2+} antagonists to exert membrane-protective effects as antiperoxidants. The agents studied include multiple representatives of the four major chemical classes of Ca^{2+} antagonists and the three (diltiazem, nifedipine, verapamil) currently approved for clinical use in the United States [11]. Our results indicate that certain Ca^{2+} antagonists possess antiperoxidant properties which may contribute to their pharmacologic profiles.

MATERIALS AND METHODS

Materials. Butylated hydroxytoluene (BHT), allopurinol, hypoxanthine, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), tris(hydroxymethyl)aminomethane (Tris), adenosine diphosphate (ADP), 2-thiobarbituric acid (TBA), tetramethoxypropane, and hydroxylamine hydrochloride were from Sigma (St Louis, MO). Dilaurylthiodipropionate (DLTDP) and 2,4,5-trihydroxybutyrophenone (THBP) were from Poly-Science (Niles, IL). α-Tocopherol was synthesized by Hoffmann-La Roche. XOD (analytical preparation from bovine milk; 1 Unit/mg) and superoxide dismutase (SOD; superoxide:superoxide oxidoreductase, EC 1.15.1.1; analytical preparation from bovine erythrocytes; 5000 Units/mg) were purchased from Boehringer-Mannheim (Indianapolis, IN). Desferrioxamine B (Desferal) was a gift from

* To whom correspondence should be addressed.

† Abbreviations: XOD, xanthine oxidase; O_2^-, superoxide anion radical; BHT, butylated hydroxytoluene; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; ADP, adeonosine diphosphate; TBA, 2-thiobarbituric acid; DLTDP, dilaurylthiodipropionate; THBP, 2,4,5-trihydroxybutyrophenone; SOD, superoxide dismutase; TCA, trichloroacetic acid; DMSO, dimethyl sulfoxide; and MDA, malondialdehyde.

Ciba A.G. (Basle, Switzerland). Calcium antagonists were obtained from their manufacturers: bepridil (Wallace Laboratories, Cranberry, NJ); cinnarizine and flunarizine (Janssen Pharmaceuticals, Piscataway, NJ); diltiazem (Marion Laboratories, St Louis, MO); gallopamil and verapamil (Knoll Pharmaceuticals, Whippany, NJ); nifedipine and nitrendipine (Bayer AG, Wuppertal, F.R.G.); and prenylamine (Hoechst AG, Frankfurt, F.R.G.). All solvents were of analytical grade (Burdick & Jackson, Muskegon, MI).

Isolation and purification of rat myocardial lipids. The procedure was carried out in a dehumidified cold-room (3°). Conscious male, Sprague–Dawley rats (~275 g; Charles River, Boston, MA) maintained on a normal rodent diet were decapitated. The hearts were rapidly excised and perfused via the aorta with 50.0 ml of ice-cold 10 mM HEPES buffer, pH 7.4. The great vessels and atria were removed, and the trimmed ventricular myocardium was minced and homogenized (100 mg tissue/ml of ice-cold HEPES) for 3 × 5 sec with a Tissumizer (Tekmar, Cincinnati, OH) at "maximal" setting. The homogenate was filtered through 4-ply cheesecloth, and the myocardial lipids were extracted and purified from the filtrate by a modified [12] Bligh–Dyer [13] procedure. The final chloroform phase containing the purified lipid represented quantitative recovery of myocardial lipid by the chemical criteria detailed [14]. The myocardial lipids were resolved into their constituent phospholipid and neutral lipid fractions by silica column chromatography on Sep-Pak cartridges (Waters, Milford, MA) [15]. All lipids were stored in chloroform under argon at −20° [16].

Lipid quantification. Lipid phosphate was determined microchemically [17] on perchloric acid digests with KH_2PO_4 as standard. The hydroxamate reaction [18] was used to measure lipid ester; L-α-phosphatidylcholine dipalmitate (Avanti Polar Lipids, Birmingham, AL) was the standard.

Cardiac liposome preparation. A known amount of cardiac lipid in chloroform was placed in a rotating glass flask and evaporated to dryness under nitrogen at room temperature to yield a thin lipid film. The lipid was taken up in 10 mM HEPES–0.145 M KCl, pH 7.4, and was resuspended by indirect anaerobic sonication for 15 min at room temperature. The liposome suspension was used immediately.

Peroxidation reaction system. Cardiac liposomes were subjected to $O_2^{\cdot-}$-dependent, iron-promoted peroxidation in glass tubes. Experiments involving α-tocopherol, nifedipine, and nitrendipine were conducted under amber light. Ninety minutes before the start of the reaction, a chelate was formed in 10 mM HEPES–0.145 M KCl buffer, pH 7.4, between Fe^{3+} (1.0 mM $FeCl_3$) and 10 mM ADP with continuous stirring at room temperature. The peroxidation reaction, in a final volume of 1.0 ml, contained these components at their specified final concentrations: 10 mM HEPES–0.145 M KCl, pH 7.4; 1.0 mM hypoxanthine; 0.1 mM Fe^{3+}–1.0 mM ADP chelate; 125 μg lipid (as liposomal suspension); and 10 mUnits XOD. The reaction was started upon adding the XOD, mixing, and incubating at 37° in a shaking water bath. Liposomes were also incubated in parallel but without free radical generator (i.e. without hypoxanthine, iron-ADP, and XOD). Peroxidation was terminated by adding 0.15 ml of 76% (w/v) trichloroacetic acid (TCA) in 2.3 N HCl and immersing the sample (final pH = 2.2) in an ice-water bath. Concentrated stock solutions of BHT, DLTDP, and THBP were made in dimethyl sulfoxide (DMSO); α-tocopherol and all Ca^{2+} antagonists except diltiazem were solubilized in ethanol; diltiazem, desferrioxamine, and allopurinol were solubilized in HEPES–KCl buffer. The final concentration of DMSO or ethanol in the peroxidation reaction never exceeded 0.005 vol% and was >1000-fold below that where a solvent effect on lipid peroxidation could be noted.

Quantification of lipid peroxidation. TBA-reactive material was measured as malondialdehyde (MDA)-equivalents by the following modification of published methods [19, 20]. The reaction mixture contained water:BHT (7.145 M in absolute ethanol):TBA (1.514 wt% in 0.2 M Tris, pH 7.0) in the volume ratio 1:1:5. To each 1.15 ml of acidified peroxidation reaction (above), 0.35 ml of reaction mixture was added. Upon thorough mixing, the samples (pH = 2.4) were incubated in an 80° shaking water bath for 30 min. After this time, the tubes were plunged into an ice-water bath, and the TBA test was stopped with 0.5 ml of ice-cold 90% (w/v) TCA followed by 2.0 ml $CHCl_3$. After centrifugation for 30 min at 2000 *g* (4°), the absorbance of the upper phase was taken at 532 nm. Various amounts (0.8 to 40.0 nmol) of MDA standard, prepared by acidification of 1,1,3,3-tetramethoxypropane with 76% TCA–2.3 N HCl, were subjected to the identical TBA test procedure as the basis for constructing a standard curve of TBA-reactivity as MDA-equivalents. Computer-assisted regression analysis of the standard curve was used to quantify the molar amounts of MDA-equivalents in the experimental samples. Net peroxidative production of MDA-equivalents was considered the difference in TBA-reactivity between liposomes peroxidized in the complete peroxidation reaction system and non-peroxidized samples that had been incubated in parallel without free radical generator. Under these conditions, none of the studied compounds influenced color development from MDA standard and evidenced no TBA-reactivity themselves. Dose–response curves were generated with the assistance of RS/1 software (BBN Corp., Cambridge, MA) on an IBM PC-AT (IBM Corp., Boca Raton, FL).

XOD assay. XOD was assayed by monitoring the conversion of xanthine to uric acid [21] using a Beckman DU-7 spectrophotometer in the kinetic mode (Beckman Instruments, Palo Alto, CA). None of the calcium antagonists affected the ultraviolet detection of uric acid *per se*. DMSO or ethanol did not influence enzyme activity at the final solvent concentrations used.

Protein determination. Protein was quantified with a dye-binding microassay [22].

RESULTS

Kinetics of myocardial phospholipid peroxidation. The total myocardial lipid of the rat heart was readily peroxidized upon exposure to $O_2^{\cdot-}$-driven, iron-

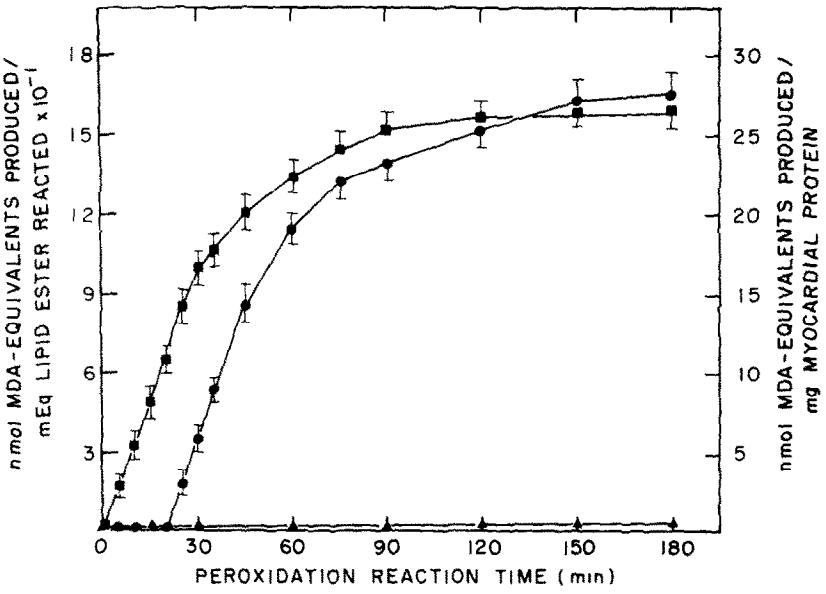

Fig. 1. Time–course of cardiac lipid peroxidation through $O_2^{\cdot-}$-dependent, iron-promoted Fenton chemistry. Total cardiac lipid (●) and the phospholipid (■) and neutral lipid (▲) subfractions therefrom were isolated and purified from rat myocardium and exposed, as liposomes, to (XOD + hypoxanthine + Fe^{3+}-ADP) free radical generator. Liposome samples were also incubated in parallel, but without radical generator. The net production of TBA-reactivity (as MDA-equivalents) was calculated over a 180-min period as the difference in TBA-reactivity between the peroxidized samples and their respective non-peroxidized controls. Data points are means ± SD ($N \geq 7$).

promoted Fenton chemistry with XOD + hypoxanthine + Fe^{3+}-ADP as free radical generator (Fig. 1). TBA-reactive substance, which we have demonstrated elsewhere [23] to be MDA, was produced after an ~20-min lag period. The linear accumulation of MDA-equivalents defined a propagation phase with a maximal peroxidation rate of 3.0 ± 0.2 nmol MDA-equivalents produced/mEq lipid ester reacted/min. As indicated by the greatly reduced development of TBA-reactivity after 90 min, myocardial lipid peroxidation was essentially complete by 2 hr.

Cellular membranes are the principal sites of free radical-induced tissue damage, for their fluid bilayers contain virtually all of the tissue phospholipid [24]. Consequently, we studied the peroxidation of myocardial phospholipid and neutral lipid resolved from the total myocardial lipid extract (Fig. 1). The myocardial neutral lipid, which represented ~19% of the total myocardial lipid complement, did not display a significant peroxidative response during incubation with XOD + hypoxanthine + Fe^{3+}-ADP. The myocardial phospholipid, however, was peroxidized with a maximal linear rate equal to the maximal rate of total cardiac lipid peroxidation. The shift of the lipid peroxidation progress curve to the left when phospholipid was separated from neutral lipid reflects removal thereby of endogenous membrane α-tocopherol [25]. The net formation of TBA-reactive substance during myocardial phospholipid peroxidation was equal to that produced from peroxidation of total myocardial lipid. These results demonstrate that myocardial membrane phospholipid, whether purified out of the total myocardial lipid extract or not, was the substrate for free radical attack.

Inhibition of myocardial phospholipid peroxidation. The antiperoxidant efficacies of compounds which can interdict at critical points in XOD-dependent, $O_2^{\cdot-}$-driven, iron-promoted Fenton chemistry were determined (Fig. 2). SOD, which specifically dismutates $O_2^{\cdot-}$ and thereby removes from the system a radical essential to Fenton chemistry [2, 26], prevented myocardial phospholipid peroxidation at nanomolar concentrations with an antiperoxidant IC_{50} of 8.0 ± 0.6 nM. Vitamin E (α-tocopherol), a lipophilic antioxidant [27], displayed an IC_{50} of ~1.0 μM. Desferrioxamine, a stoichiometric iron chelator [28], afforded significant protection only at a concentration at least as great as the iron concentration in the system (100 μM) so that iron substrate for the Fenton reaction was totally chelated and functionally removed from the peroxidation system. Allopurinol, a substrate-analog XOD inhibitor [29], acted as antiperoxidant at high micromolar concentrations (IC_{50} = 470 ± 32 μM) which approached the XOD substrate concentration (1.0 mM) in the reaction. "Radical-scavenger" antioxidants used as food additives [30] displayed antiperoxidant IC_{50} values in the 5–150 μM range: e.g. BHT (IC_{50} = 6.8 ± 0.3 μM), THBP (30 ± 2 μM), and DLTDP (100 ± 6 μM).

Effects of Ca^{2+} antagonists on myocardial phospholipid peroxidation. Ca^{2+} antagonists varied widely in their abilities to inhibit cardiac membrane phospholipid peroxidation resulting from a $O_2^{\cdot-}$-

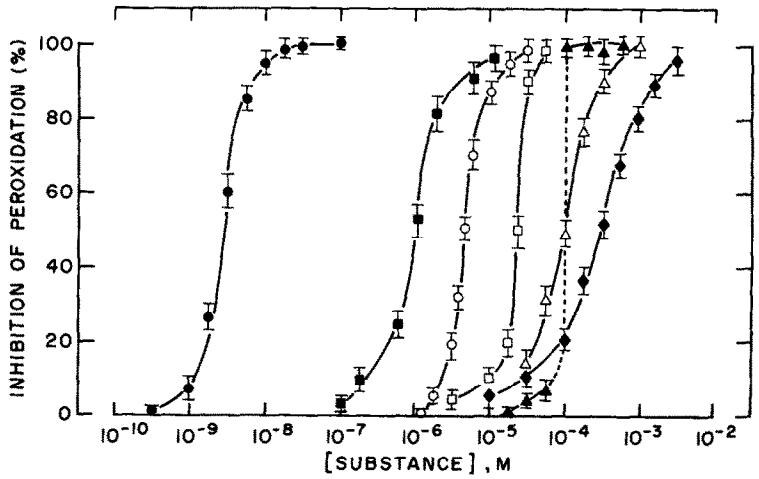

Fig. 2. Inhibition of myocardial phospholipid peroxidation by SOD (●), α-tocopherol (■), BHT (○), THBP (□), desferrioxamine (▲), DLTDP (△), and allopurinol (◆). Myocardial phospholipids were exposed to free radical generator (XOD + hypoxanthine + Fe^{3+}-ADP) for 60 min either in the absence or presence of each listed substance at various final concentrations. Inhibition of peroxidation (assessed as TBA-reactivity) is expressed relative to 0% inhibition of the reaction without test substance, and data are given as means ± SD (N > 5).

driven, iron-promoted free radical generator. The dihydropyridines nifedipine and nitrendipine did not protect the phospholipids from oxidative injury, even when present at a concentration (500 μM) approaching their solubility limit in the aqueous peroxidation system. The phenylalkylamines verapamil and gallopamil were weak antiperoxidants; although solubility limits prevented their being tested at a concentration above 500 μM, verapamil and gallopamil did inhibit peroxidation by, respectively, 30 and 40% at 500 μM. Diltiazem was also a weak antiperoxidant; the aqueous solubility of this benzothiazepine Ca^{2+} antagonist, however, allowed accurate determination of its antiperoxidant IC_{50} (510 ± 22 μM; Fig. 3). In contrast to the phenylalkylamines verapamil and gallopamil, the alkylamines bepridil and prenylamine very effectively protected cardiac membrane phospholipid from oxidative injury, with respective antiperoxidant IC_{50} values of 55 ± 3 and 75 ± 6 μM (Fig. 3). Both diphenylpiperazines tested, flunarizine (IC_{50} = 190 ± 11 μM) and cinnarizine (IC_{50} = 180 ± 10 μM), also demonstrated appreciable potency as antiperoxidants (Fig. 3). Bepridil, prenylamine, flunarizine, and cinnarizine were able to block completely myocardial lipid peroxidation.

Influence of Ca^{2+} *antagonist-antiperoxidants on myocardial phospholipid peroxidation kinetics.* All Ca^{2+} antagonists that displayed antiperoxidant activity had concentration-dependent effects not only upon the extent of myocardial phospholipid peroxidation, but also upon the kinetics and progression of peroxidative injury. For example, 75 μM bepridil inhibited peroxidation by ~50% at 60 min, delayed the initiation of peroxidation by some 20 min, suppressed the linear rate of propagation by ~50%, and slowed the progression of radical-induced phospholipid damage (Fig. 4). Higher concentrations of bepridil further delayed the initiation of peroxidation and attenuated both the linear propagation rate and the extent of peroxidation. Similar concentration-dependent, kinetic effects on the progression of myocardial lipid injury were observed with the other Ca^{2+} antagonist-antiperoxidants (data not shown) and with radical-trapping antiperoxidants such as α-tocopherol [25] and gossypol [31].

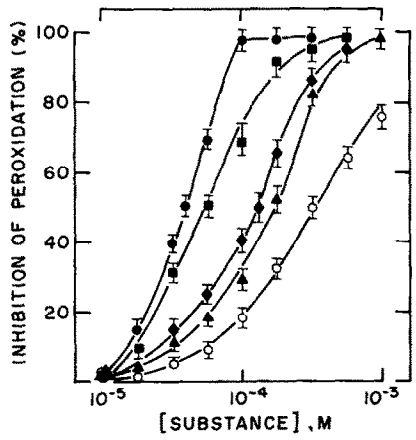

Fig. 3. Inhibition of myocardial phospholipid peroxidation by the Ca^{2+} channel antagonists bepridil (●), prenylamine (■), cinnarizine (◆), flunarizine (▲), and diltiazem (○). Myocardial phospholipids were exposed to free radical generator (XOD + hypoxanthine + Fe^{3+}-ADP) for 60 min either in the absence or presence of each listed Ca^{2+} antagonist at various final concentrations. Inhibition of peroxidation (assessed at TBA-reactivity) is expressed relative to 0% inhibition of the reaction without test substance. The data are given as means ± SD (N > 5).

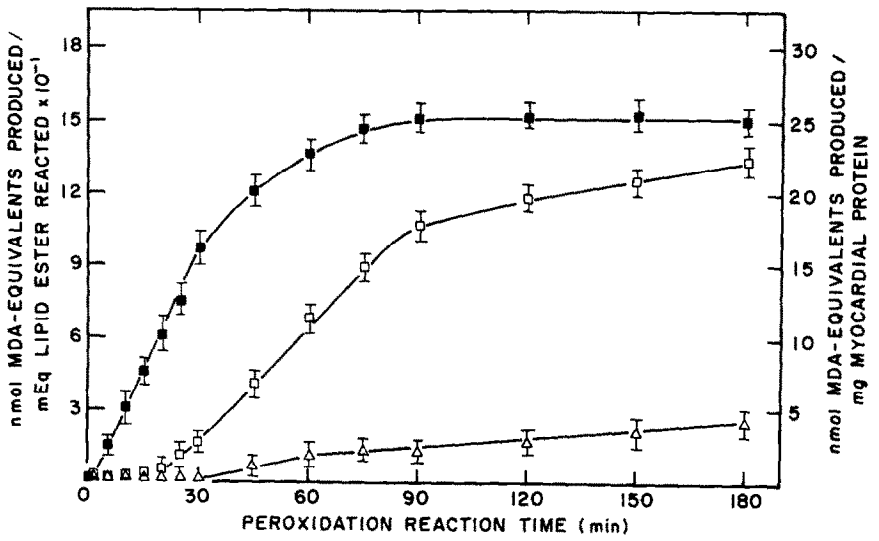

Fig. 4. Effect of bepridil on the kinetics of myocardial phospholipid peroxidation. Myocardial phospholipids were peroxidized through $O_2^{\cdot-}$-dependent, iron-promoted Fenton chemistry either in the absence (■) or presence [(□) 75 μM final concn; (△) 90 μM final concn] of bepridil. Net phospholipid peroxidative injury was assessed as in Fig. 1 and is expressed as mean MDA-equivalents produced ± SD (N > 5).

Ca^{2+} *antagonist-antiperoxidant effects on XOD.* The antiperoxidant efficacy of some Ca^{2+} antagonists in a XOD-dependent, iron-promoted oxidative injury system prompted us to test, directly and comparatively, their effects on XOD activity. Each Ca^{2+} antagonist-antiperoxidant was evaluated as a XOD inhibitor at its antiperoxidant IC_{50} and/or at a concentration that completely inhibited peroxidation. Those Ca^{2+} antagonists (i.e. nifedipine, nitrendipine, verapamil, and gallopamil) with limited solubility in the aqueous XOD assay system were tested at 500 μM, the highest concentration attainable. The data from these experiments demonstrated that concentrations of Ca^{2+} antagonist-antiperoxidants that significantly inhibited or prevented cardiac phospholipid damage through XOD-dependent, $O_2^{\cdot-}$-driven, iron-promoted oxygen chemistry did not inhibit XOD under conditions whereby concentrations of allopurinol (5–10 μM) ineffective as an antiperoxidant (Fig. 2) inhibited XOD by 50%. The dihydropyridine Ca^{2+} antagonists nifedipine and nitrendipine, which did not affect peroxidation at 500 μM, likewise did not influence XOD activity.

DISCUSSION

The efficacies of Ca^{2+} antagonists in reducing ischemic myocardial necrosis are clinically and experimentally documented [32, 33], as are their abilities to block current through potential-dependent Ca^{2+} channels [6]. Yet the limited quantitative information regarding Ca^{2+} antagonist effects other than inhibition of transmembrane Ca^{2+} flux [6, 7] and the multifactorial nature of ischemic and reperfusion injury [34] have not allowed direct attribution of the myocardial protective effects of Ca^{2+} antagonists to the reduction of myocyte Ca^{2+} influx through the potential-dependent Ca^{2+} channel. Consequently, the influence of Ca^{2+} antagonists on pathophysiological aspects of myocardial injury such as myocyte energy balance [35], oxygen demand [36], and activation of Ca^{2+}-dependent proteases and (phospho)-lipases [37] has received recent study. The present work extends this body of knowledge by demonstrating that certain Ca^{2+} antagonists can significantly inhibit and, in some cases, prevent cardiac membrane phospholipid peroxidation by $O_2^{\cdot-}$-dependent, iron-promoted Fenton chemistry of the type thought to be a causative factor in tissue injury [1], particularly during myocardial ischemia and reperfusion [5, 38].

Radical-mediated reactions, including membrane lipid peroxidation, are intimately related to heart muscle-cell Ca^{2+} homeostasis: lipid peroxidation in sarcolemma [39] and mitochondria [40] impairs Ca^{2+} regulation by these membrane systems. Sodium-Ca^{2+} exchange by cardiac sarcolemma is under redox control and can be directly modulated by combinations of oxidants/reductants and transition metals which are also well-recognized biological free radical generators [41]. The biophysical changes in membrane lipid packing and microviscosity brought about by peroxidation [42] alter the functionally significant Ca^{2+} pool bound to the myocyte sarcolemma [43]. Thus, some Ca^{2+} antagonists, through their antiperoxidant properties, may act as intracellular modulators of Ca^{2+} dynamics within the heart muscle cell independent of any inhibition of the slow inward Ca^{2+} current. Furthermore, the high polyunsaturated fatty-acyl content of biological membranes [24] would broaden the scope of the antiperoxidant protective effects of Ca^{2+} antagonist-antiperoxidants independent of the selectivity of these agents at the L-type Ca^{2+} channel.

The possible pharmacologic and therapeutic significance of the antiperoxidant activity of certain Ca^{2+} antagonists is enhanced by the fact that many Ca^{2+} antagonists are readily taken up by muscle cells and concentrate in cellular membranes [44, 45]. For example, the oil/water partition coefficients of verapamil and bepridil are >100, such that their local membrane concentrations could approach millimolar levels [45]. Direct autoradiographic study of [^{3}H]diltiazem distribution in the heart muscle cell has documented significant intramyocyte "binding" of diltiazem to mitochondria and sarcolemma [46]. Such interaction between membranes and Ca^{2+} antagonist-antiperoxidants would serve both to localize these agents at prime targets of cellular oxidative damage and to concentrate them there at effective antiperoxidant levels. However, since the more specific Ca^{2+} antagonists at the level of the Ca^{2+} channel (e.g. nifedipine, verapamil, diltiazem) were not as potent antiperoxidants as the less specific bepridil and prenylamine, antiperoxidative activity and Ca^{2+} antagonism need not parallel one another pharmacologically in the efficacy of these agents against ischemic myocardial necrosis.

Our direct, quantitative comparison of the lipid protective effects of Ca^{2+} antagonist-antiperoxidants with those of known antioxidants and iron chelators indicates that the antiperoxidant efficacy demonstrated by some Ca^{2+} antagonists rests with their ability to intercept free radicals and block the propagation of oxy- and/or lipid radical intermediates. The inability of these agents, at effective antiperoxidant concentrations, to inhibit XOD supports this conclusion. Further support is the recent demonstration by Robak and Duniec [47] that verapamil and diltiazem inhibit, in a dose-dependent fashion, ascorbic acid-induced rat hepatic membrane peroxidation with IC_{50} values of ~200 and ~500 μM respectively. While these reported IC_{50} values are somewhat lower than those reported here and may have been compromised by the non-specific methodology used to evaluate membrane peroxidation [20, 48], the membrane protection displayed by verapamil and diltiazem against a non-enzymatic free radical generator would support the conclusion made from our more comprehensive, detailed analysis that Ca^{2+} antagonist-antiperoxidants can interdict in the radical chemistry which is at the basis of lipid peroxidation.

The structural requirement(s) for the selectivity of antiperoxidative activity among the Ca^{2+} antagonists tested remains to be established. The fact that the most potent Ca^{2+} antagonist-antiperoxidants identified (bepridil, prenylamine, cinnarizine, and flunarizine) have at least one non-substituted phenyl ring, whereas the other tested Ca^{2+} antagonists have (often extensively) substituted phenyl moieties, makes it tempting to speculate that the electronic configuration of the unsubstituted phenyl groups may somehow be involved, as in the radical interactions between phenylhydrazine and hemoglobin [49]. Recent synthetic efforts aimed at producing analogs of known Ca^{2+} antagonists [e.g. Ref. 50] may provide the means with which to address this point experimentally.

Acknowledgements—We thank L. Klevans and A. C. Sullivan for support of this work, the Pharmacology Word Processing Unit (M. Johnson, P. Lotito, N. Manion, C. Muscara and L. Zielenski), B. Kinback, and J. Jusinski for secretarial assistance, and C. Burghardt and L. Rosenberger for comments.

REFERENCES

1. Halliwell B, Oxidants and human disease: Some new concepts. *FASEB J* **1**: 358–364, 1987.
2. Gutteridge JMC, The role of superoxide and hydroxyl radicals in phospholid peroxidation catalyzed by iron salts. *FEBS Lett* **150**: 454–458, 1982.
3. Simpson PJ and Lucchesi BR, Free radicals and myocardial ischemia and reperfusion injury. *J Lab Clin Med* **110**: 13–30, 1987.
4. Kingma JG, Denniss AR, Hearse DJ, Downey JM and Yellon DM, Limitation of infarct size for 24 hours by combined treatment with allopurinol plus verapamil during acute myocardial infarction in the dog. *Circulation* **75**: V25–V33, 1987.
5. Arroyo CM, Kramer JH, Dickens BF and Weglicki WB, Identification of free radicals in myocardial ischemia/reperfusion by spin trapping with nitrone DMPO. *FEBS Lett* **221**: 101–104, 1987.
6. Triggle DJ and Swamy VC, Calcium antagonists. Some chemical–pharmacologic aspects. *Circ Res* **52** (Suppl I): 17–28, 1983.
7. Nayler WG, Panagiotopoulos S, Elz JS and Sturrock WJ, Fundamental mechanisms of action of calcium antagonists in myocardial ischemia. *Am J Cardiol* **59**: 75B–83B, 1987.
8. Yatani A, Kunze DL and Brown AM, Effects of dihydropyridine calcium channel modulators on cardiac sodium channels. *Am J Physiol* **254**: H140–H147, 1988.
9. Fairhurst AS, Whittaker ML and Ehlert FJ, Interaction of D600 (methoxyverapamil) and local anesthetics with rat brain α-adrenergic and muscarinic receptors. *Biochem Pharmacol* **29**: 155–162, 1980.
10. Kloner RA and Braunwald E, Effects of calcium antagonists on infarcting myocardium. *Am J Cardiol* **59**: 84B–94B, 1987.
11. Godfraind T, Classification of calcium antagonists. *Am J Cardiol* **59**: 11B–23B, 1987.
12. Janero DR and Barrnett R, Cellular and thylakoid-membrane glycolipids of *Chlamydomonas reinhardtii* 137$^+$. *J Lipid Res* **22**: 1119–1125, 1981.
13. Bligh EG and Dyer WJ, A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37**: 911–917, 1959.
14. Janero DR and Barrnett R, Thylakoid membrane biogenesis in *Chlamydomonas reinhardtii* 137$^+$: Cell cycle variation in the synthesis and assembly of polar glycerolipid. *J Cell Biol* **91**: 126–134, 1981.
15. Juaneda P and Rocquelin G, Rapid and convenient separation of phospholipids and non-phosphorous lipids from rat heart using silica cartridges. *Lipids* **20**: 40–41, 1985.
16. Janero DR and Barrnett R, Analytical separation of green-plant and animal neutral lipids by thin-layer chromatography. *J Chromatogr* **216**: 417–422, 1981.
17. Letters R, The application of a two-dimensional paper chromatographic technique to the analysis of phospholipids. *Biochem J* **93**: 313–316, 1964.
18. Skidmore WD and Entenman C, The determination of esterified fatty acids in glycerides, cholesterol esters, and phosphatides. *J Lipid Res* **3**: 356–363, 1962.
19. Asakawa T and Matsushita S, Coloring conditions of the thiobarbituric acid test for detecting lipid hydroperoxides. *Lipids* **15**: 137–141, 1979.
20. Warso MA and Lands WEM, Pathophysiologic modu-

lation of arachidonate metabolism. *Clin Physiol Biochem* **2**: 70–77, 1984.
21. Cha S, Agurural RP and Parks RE, Non-steady state nature of inhibition of milk xanthine oxidase by allopurinol and alloxanthine and of human erythrocyte adenosine deaminase by coformycin. *Biochem Pharmacol* **24**: 2187–2197, 1975.
22. Fanger BO, Adaptation of the Bradford protein assay to membrane-bound proteins by solubilizing in glucopyranoside detergents. *Anal Biochem* **162**: 11–17, 1987.
23. Janero DR and Burghardt B, Isolation by HPLC of free malondialdehyde as a product of the xanthine oxidase dependent, iron-promoted peroxidation of cardiac membrane phospholipid. *J Am Oil Chem Soc* **63**: 476–477, 1986.
24. Dawidowicz EA, Dynamics of membrane lipid metabolism and turnover. *Ann Rev Biochem* **56**: 43–61, 1987.
25. Janero DR and Burghardt B, Influence of tissue tocopherol on cardiac lipid peroxidation through an iron-catalyzed Haber–Weiss reaction. *Fed Proc* **45**: 1808, 1986.
26. Gutteridge JMC, Superoxide dismutase inhibits the superoxide-driven Fenton reaction at two different levels. *FEBS Lett* **185**: 19–23, 1985.
27. McCay PB, Vitamin E: Interactions with free radicals and ascorbate. *Ann Rev Nutr* **5**: 323–340, 1985.
28. Lentz DJ, Henderson GH and Eyring EM, Kinetics of aqueous iron(III) complexation by desferrioxamine B. *Mol Pharmacol* **9**: 514–515, 1973.
29. Massey V, Komia H, Palmer G and Elion GB, On the mechanism of interaction of xanthine oxidase by allopurinol and other pyrazole[3,4-*d*]pyrimidines. *J Biol Chem* **245**: 2837–2844, 1970.
30. Sherwin ER, Antioxidants for food fats and oils. *J Am Oil Chem Soc* **49**: 468–472, 1972.
31. Burghardt B and Janero DR, Inhibition of free radical-induced phospholipid peroxidation by the antifertility drug gossypol. *Fed Proc* **45**: 214, 1986.
32. Gibson RS, Boden WE, Theroux P, Strauss HD, Pratt CM, Gheorghiade M, Capone RJ, Crawford MH, Schlant RC, Kleiger PR, Young PM, Schechtman K, Perryman MB and Roberts R, Diltiazem and reinfarction in patients with non-Q wave myocardial infarction. Results of a double-blind, randomized, multi-center trial. *N Engl J Med* **315**: 423–429, 1986.
33. Richard V and deLeiris J, Reduction of myocardial infarct size in rats under the effect of bepridil. *Adv Myocardiol* **6**: 563–572, 1985.
34. Ferrari R, Ceconi C, Curello S, Carnoni A, Agnoletti G, Boffa GM and Visioli O, Intracellular effects of myocardial ischemia and reperfusion: Role of calcium and oxygen. *Eur Heart J* **7** (Suppl A): 3–12, 1987.
35. Watts J and Maiorano L, Effects of diltiazem upon globally ischemic rat hearts. *Eur J Pharmacol* **138**: 335–342, 1987.
36. Schulte KL, Meyer-Sabellek WA, Haertenberger A, Thiede H-M, Roecher L, Distler A and Gotzen R, Antihypertensive and metabolic effects of diltiazem and nifedipine. *Hypertension* **8**: 859–765, 1986.
37. Kajiyama K, Pauly DF, Hughes H, Yoon SB, Entman ML and McMillin-Wood JB, Protection by verapamil of mitochondrial glutathione equilibrium and phospholipid changes during reperfusion of ischemic canine myocardium. *Circ Res* **61**: 301–310, 1987.
38. Zweier JL, Measurement of superoxide-derived free radicals in the reperfused heart. Evidence for a free radical mechanism of reperfusion injury. *J Biol Chem* **263**: 1353–1357, 1988.
39. Tones MA and Poole-Wilson PA, Alpha-adrenoreceptor stimulation, lysophosphoglycerides, and lipid peroxidation in reoxygenation induced calcium uptake in rabbit myocardium. *Cardiovasc Res* **19**: 228–236, 1985.
40. Rizzuto R, Pitton G and Azzone GF, Effect of Ca^{2+}, peroxides, SH reagents, phosphate, and aging on the permeability of mitochondrial membranes. *Eur J Biochem* **162**: 239–249, 1987.
41. Reeves JP, Barley CA and Hale CC, Redox modification of sodium–calcium exchange activity in cardiac sarcolemmal vesicles. *J Biol Chem* **261**: 4948–4955, 1986.
42. Ivanov II, A relay model of lipid peroxidation in biological membranes. *J Free Rad Biol Med* **1**: 247–253, 1985.
43. Langer GA, The effect of pH on cellular and membrane calcium binding and contraction of myocardium. A possible role for sarcolemmal phospholipid in EC coupling. *Circ Res* **57**: 374–382, 1985.
44. Pang DC and Sperelakis N, Nifedipine, diltiazem, bepridil, and verapamil uptakes into cardiac and smooth muscles. *Eur J Pharmacol* **87**: 199–207, 1983.
45. Pang DC and Sperelakis N, Uptake of calcium antagonistic drugs into muscles as related to their lipid solubilities. *Biochem Pharmacol* **33**: 821–826, 1984.
46. Taganu M, Nara Y, Kubota A, Sunaga T, Maezawa H, Horie R and Yamori Y, Electron microscopic autoradiographic study of the distribution of ^{3}H-diltiazem in myocardial cells. *Jpn Heart J* **26**: 823–832, 1985.
47. Robak J and Duniec Z, Membrane activity, antioxidant, antiaggregatory, and anti-hemolytic properties of four calcium channel blockers. *Pharmacol Res Commun* **18**: 1107–1117, 1986.
48. Janero DR and Burghardt B, Analysis of cardiac membrane phospholipid peroxidation kinetics as malondialdehyde (MDA): non-specifity of thiobarbituric acid (TBA)-reactivity. *Lipids* **23**: 452–458, 1988.
of superoxide anion generation by the interactions of phenylhydrazine with hemoglobin. *J Biol Chem* **251**: 3045–3051, 1976.
50. Fossheim R, Joslyn A, Solo AJ, Luchowski E, Rutledge A and Triggle DJ, Crystal structures and pharmacologic activities of 1,4-dihydropyridine calcium channel antagonists of the isobutyl methyl 2,6-dimethyl-4-(substituted phenyl)-1,4-dihydropyridine-3,5-dicarboxylate (nisoldipine) series. *J Med Chem* **31**: 300–305, 1988.