MINIREVIEW

Calcium Antagonists and Experimental Atherosclerosis

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During the last 10–15 years, calcium antagonists have been found beneficial in the treatment of a number of cardiovascular and circulatory disorders and safe in long-term human use. Calcium antagonists have also been reported to be effective in suppressing experimental atherosclerosis (1, 2). If calcium antagonists are effective in inhibiting the progression of human atherosclerosis, it is of major medical interest. The aim of this survey of the literature was therefore, to compile and review the present knowledge on the antiatherogenic properties of calcium antagonists.

Calcium Antagonists and Other Anticalcifying Compounds

Fleckenstein *et al.* (3) observed that verapamil uncoupled excitation-contraction coupling in myocardium and that this effect could be reversed by adding Ca^{2+}. Fleckenstein named this new class of drugs "calcium antagonists." Today, calcium antagonists comprise a heterogenous group of therapeutic agents. At present they are classified into four categories, but several new compounds are under development. *Type I*: Verapamil and diltiazem (diphenylalkylamines and benzothiazepine derivatives) prolong AV nodal conduction and refractoriness. *Type II*: Nifedipine and other dihydropyridines are potent peripheral vasodilators but have no electrophysiologic effects in usual doses. *Type III*: Flunarizine and other piperazine derivatives are also potent dilators of peripheral vessels but have no calcium-blocking actions in the heart, and *Type IV* is comprised of agents with a broader pharmacological profile, e.g., bepridil, perhexiline, and lidoflazine (4).

Calcium antagonists show a puzzling heterogeneity in chemical structure, but share the common property of "calcium antagonism." The explanation probably lies in the recent identification of different receptor or binding sites for the chemically distinct types of calcium antagonists. The tissue selectivity of the various types of calcium antagonists involves differences in receptor distribution, function of the slow calcium channels, and in the Ca^{2+} requirements of the various target tissues. Calcium channels are also blocked by a number of inorganic ions, e.g., Mg^{2+}, Mn^{2+}, and La^{3+}. Of these, the antiatherosclerotic effect of La^{3+} has been evaluated in several animal studies (5). Anticalcifying compounds used in animal models of atherosclerosis comprise diphosphonates, thiophene carboxylic acid derivatives, chelating agents (magnesium EDTA), cetaben (sodium *p*-hexadecylaminobenzoate), and the phosphodiesterase inhibitor, trimazosin (5). Guilland *et al.* (6) reported that diphosphonates inhibit Ca^{2+} transport across the mitochondrial membrane of rat kidney cells *in vitro* and Fleisch *et al.* (7) found an inhibitory effect of phosphonates on the formation of calcium phosphate crystals *in vitro* and on aortic and kidney calcification *in vivo*. A putative antiatherosclerotic effect of the anticalcifying agents is thought mainly to take place via a decreased calcium and phosphate concentration in plasma and a secondary decreased calcification of arterial tissues (8). Most of these anticalcifying compounds are rather toxic both *in vivo* and *in vitro* and have no therapeutic potential in humans.

Slow Ca^{2+} Channels

Ca^{2+} traverses the plasma membrane of cardiac and vascular smooth muscle cells through channels which are voltage sensitive, gated, and ion selective. Each "slow" channel allows approximately 30,000 Ca^{2+} to enter per second and there may be as many as 30 channels/μm^2 of cell surface. Some of the slow Ca^{2+} channels are controlled by a receptor-like mechanism which involves a cyclic AMP-dependent phosphorylation causing a conformational change in the channel protein. Recent investigations suggest that some Ca^{2+} channels are controlled by guanine nucleotide regulatory proteins, that inositol phospholipids are important in intracellular release and distribution of Ca^{2+}, and

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that certain inositol phospholipids also take part in the control of plasma membrane Ca^{2+} gates (9, 10).

The slow channels are the predominant sites of action of the calcium antagonists used in the treatment of cardiovascular disorders but may not be the site of action in animal models of atherosclerosis (2).

Receptors for Calcium Antagonists

Saturation and displacement techniques with labeled calcium antagonists have identified three chemically distinct types of stereospecific binding sites for Types I–III calcium antagonists. In the vascular system, both low and high affinity binding sites have been characterized within each group (11).

Regulation of Cellular Calcium Ion Concentrations

The distribution of Ca^{2+} between the intra- and extracellular spaces of vascular smooth muscle cells is asymmetrical, with a cytosolic free Ca^{2+} concentration $[Ca^{2+}]c$ maintained within the range of 10^{-8}–10^{-7} M, compared with about 10^{-3} M in the extracellular fluid. Ca^{2+} in the cytosol acts as a second messenger in a variety of cellular processes and its concentration is regulated by the membrane systems which bound the cytosol, e.g., the plasma membrane, the endoplasmic reticulum, or the mitochondrial inner membrane (12). Several Ca^{2+}-binding proteins exist within cells and in the extracellular environment. The mediation of cellular responses by Ca^{2+} is presumably brought about by interaction with specific calcium-binding proteins, thereby altering enzymatic activity and leading to cellular changes. One of these proteins, calmodulin, plays a pivotal role in many Ca^{2+}-dependent processes and cellular regulation (13).

Agents that activate Ca^{2+}-dependent processes call forth increases in $[Ca^{2+}]c$ either by enhancing Ca^{2+} influx from the extracellular space or by mobilizing intracellular Ca^{2+}, whereas agents that suppress Ca^{2+}-dependent processes call forth decreases in $[Ca^{2+}]c$ either by blocking Ca^{2+} influx and/or by inhibiting the release of intracellular Ca^{2+} (14). Since a hierarchy of Ca^{2+} sensitivity is present among Ca^{2+}-dependent processes, various compounds will affect some processes more than others (4).

Calcium and Atherogenesis

$[Ca^{2+}]c$ influences a number of important cellular functions involved in atherogenesis, such as control of membrane permeability, cell proliferation (15), cell migration (16), secretion of extracellular matrix proteins (17) as well as degeneration and necrosis (18–20). Considerations of how anticalcifying compounds may interfere with the initiation and development of atherosclerosis by antagonizing the biologic actions of Ca^{2+} are, of course, dependent on knowledge of the regulatory roles of Ca^{2+} in the function of endothelium, macrophages, vascular smooth muscle cells, and platelets. The following sections summarize the specific Ca^{2+}-associated mechanisms in the complex sequence of physiologic events connected to atherogenesis.

In Vitro Experiments

Platelets. There is good reason to assume that platelets either have no voltage-operated channels or that the number is so low that they cannot be detected in radioligand binding studies (21). However, although no specific search for low affinity binding sites for calcium channel blockers has been performed, several functional studies indicate the existence of such binding sites associated with a receptor-operated channel. Even if the site of action is unknown, there is evidence that calcium antagonists in micromolar concentrations have an antiaggregatory effect *in vitro* and thereby possibly influence atherogenesis (22–25). When evaluating the effect of calcium antagonists in experimental atherosclerosis, it should also be kept in mind that calcium channel blockers possess a number of effects unrelated to the calcium channel, e.g., calmodulin inhibition, cyclic AMP-phosphodiesterase inhibition, nucleotide uptake inhibition, Na^+, K^+, and Cl^- channel blockade, α and β receptor blockade, and inhibition of oxidative phosphorylation (26–28). In particular, cyclic AMP-phosphodiesterase, calmodulin, and nucleotide uptake inhibition may be significant in understanding the antiaggregatory effect of some calcium channel blockers.

Endothelium. Voltage- or receptor-operated channels have never been demonstrated in endothelial cells, but endothelium probably have stretch-activated mechanoreceptors which regulate cell volume and mechanoreception. Comparatively little is known about the mechanisms of Ca^{2+} influx in nonexcitable cells, i.e., passive diffusion, facilitated diffusion, and agonist-sensitive facilitated diffusion (29). None of these mechanisms, however, are influenced by calcium antagonists, whereas the ATPase Ca^{2+} outflow transporter is inhibited by lanthanum and activated by calmodulin (30). It should also be mentioned that verapamil and diltiazem interact with serotonin receptors in cultured arterial endothelial cells and aortic strips (31, 32).

Endothelial permeability through gap junctions is regulated by the contractile microfilaments of the cytoskeleton (33), and $[Ca^{2+}]c$ and calmodulin control the contractility of these elements (34). Calcium antagonists may therefore influence endothelial permeability and thereby the transfer of plasma lipoproteins into the arterial intima.

Monocytes/Macrophages. One of the earliest events in atherogenesis is attachment of monocytes to injured endothelium and subsequent invasion of the intima. The macrophages here accumulate lipids and phagocytize extracellular material, a process which probably represents a normal scavenger function (35). Macrophages also take part in the repair process by secreting growth factors for fibroblasts, smooth muscle cells, and endothelium (35). Recently, Schmitz *et al.*

(36) have demonstrated that micromolar concentrations of nifedipine *in vitro* increase the scavenger function of the macrophages, cause a more efficient intracellular processing of ingested cholesterol, and promote cholesterol efflux, mainly as membrane-associated cholesterol via the lysosomal route. Data in this interesting paper also indicate that nifedipine in macrophages influences nucleoside and nucleotide metabolism, leading to an increase in intracellular adenosine due to enhanced ATP hydrolysis and inhibition of the nucleoside transporter activity. The site of action on the plasma membrane seems to be either the adenosine receptor or the benzodiazepine receptor (36), whereas the principal site of action within the cells may be the lysosome (37). Studies by Marangos *et al.* (38) support the view that Type II, but not other calcium antagonists, influences nucleoside metabolism. These authors demonstrated that dihydropyridine channel blockers inhibit adenosine receptors in dog heart preparations in a way which suggests that the calcium channel and adenosine uptake site may be coupled to each other.

Vascular Smooth Muscle. In the atherogenic process smooth muscle cells migrate from the media into the intima, where they proliferate and synthesize and secrete matrix elements. The start of proliferation requires that the cells have passed through a modulation from contractile to synthetic phenotype and that they are stimulated by growth factors (35). In cell cultures, several mechanisms have been identified which may be relevant to the antiatherogenic potential of calcium antagonists. Thus, Nakao *et al.* (39) demonstrated that migration of aortic smooth muscle cells *in vitro* induced by the eicosanoid 12-HETE is a Ca^{2+}-dependent process which is inhibited by nicardipine at concentrations from 10^{-9} to 10^{-5} *M*. Trifluoperazine, a specific calmodulin inhibitor, had the same effect. Nilsson *et al.* (40) reported that micromolar concentrations of nifedipine slow down the modulation of vascular smooth muscle cells from a contractile to a synthetic phenotype and inhibit both initiation of DNA synthesis and cellular proliferation. Stein *et al.* (41) studied the effects of micromolar concentrations of verapamil on bovine aortic smooth muscle cells as well as aortic endothelial cells and human skin fibroblasts. After 24–48 hr verapamil increased ^{125}I-labeled low density protein uptake and degradation by 70 to 200%. Stein *et al.* (41) suggested that this effect of verapamil, at least partly, is due to an enhanced receptor recycling, a process supposed to be regulated by calmodulin. Etingin and Hajjar (42) investigated cholesteryl ester hydrolytic activity in rabbit smooth muscle cells and demonstrated that microgram concentrations of nifedipine double the activity of lysosomal and cytoplasmic cholesteryl ester hydrolase after 2 hr of incubation. Although no changes were seen in concentration of total cholesterol and cholesteryl ester in normal smooth muscle cells after 1 week of nifedipine treatment, a 50% decrease was seen in lipid-laden smooth muscle cells from the aorta of animals fed a cholesterol-enriched diet. In the same experiments intracellular cyclic AMP levels increased 2- to 3-fold after exposure to microgram concentrations of nifedipine and adenylate cyclase inhibitors blocked the enhanced activity of cholesteryl ester hydrolases. Cyclic nucleotides regulate cholesteryl ester metabolism in arterial smooth muscle cells (43) and cyclic AMP has also been shown to enhance the mobilization and excretion of cholesteryl ester from atherosclerotic arteries (44). These observations and the mechanisms proposed may therefore contribute to an antiatherogenic potential of calcium antagonists.

Recently, Nomoto *et al.* (45) examined the *in vitro* inhibition of various calcium antagonists on smooth muscle cells proliferation and found no effect, whereas all calcium antagonists tested inhibited the *in vitro* migration of smooth muscle cells dose dependently. Only nilvadipine was effective, however, at concentrations relevant in human therapy.

Extracellular Matrix Elements. The extracellular components of the arterial wall comprise fibers (collagen and elastin) and interstitial matrix substance consisting of carbohydrate-rich macromolecules (proteoglycans and glycosaminoglycans). These elements form a barrier, which in the opinion of some investigators is important in maintaining the integrity of the arterial wall (46). A characteristic finding in human atherosclerotic lesions is an accumulation of proteoglycans containing sulfated chondroitin and dermatan, produced by smooth muscle cells (47). The extracellular matrix components may act as a molecular sieve, which form insoluble complexes with LDL in the presence of Ca^{2+} (48). This accumulation of trapped lipid may act as a chemoattractant for macrophages and become a source of foam cell formation. Both anticalcifying agents (5) and micromolar concentrations of organic calcium antagonists (49) inhibit matrix protein synthesis by smooth muscle cells. The site of the matrix inhibitory activity of Type II calcium antagonists is unknown, but is independent of calcium channel blockade (49).

Animal Experiments

Anticalcifying Agents in Hyperlipidemic Animals. The first rabbit studies concerning anticalcifying agents and atherosclerosis were performed by Lacson *et al.* (50) and Wartman *et al.* (51) using magnesium EDTA and an atherogenic diet (Table I). In the first study plaque regression was evaluated in eight animals. Total lipids in dry aortic tissue were somewhat lower in the EDTA-treated group. The results were not statistically evaluated, but the difference was hardly significant.

Table I. Studies of the Influence of Anticalcifying Agents on Aortic Atherogenesis in Hyperlipidemic Animals (mean ± SD)

First author	Experimental design								Plasma cholesterol (m*M*)	Aorta	
	Ca antagonist	Daily dosage		Animal	Experimental model	Duration (weeks)	No. of animals	Cholesterol addition (%)		Lesioned area (%)	Significant reduction ($P < 0.05$)
		Oral (mg/kg)	sc (mg/kg)								0/+
Lacson, 1966 (50)	Magnesium EDTA	—	40–60	Rabbit	Progression/ regression	12/4–6	8	1	40→5.2[a]	23	0
	Controls		Placebo				8	1	40→7.0	34	
Wartman, 1967 (51)	Magnesium EDTA	—	60	Rabbit	Progression/ regression	11/5–7	10	1	28 ± 7.6	29 ± 6.8[b]	0
	Controls		Placebo				8	1	28 ± 6.2	34 ± 6.7	
Rosenblum, 1975 (52)	EHDP	—	0.25	Rabbit	Progression	12	7	0.25–0.5	23 ± 8.6	4.7 ± 6.0	0
		—	1.0				5	0.25–0.5	27 ± 8.6	9.2 ± 5.8	
		—	2.5				6	0.25–0.5	34 ± 8.6	9.7 ± 6.0	
	Controls						4	0.25–0.5	22 ± 8.6	5.2 ± 5.9	
Chan, 1978 (58)	TCA[c]	280	—	Rabbit	Progression	8	10	2	91 ± 12	12 ± 2.3[d]	0
	5MTCA	140	—				10	2	85 ± 18	1.7 ± 0.1	+
	5BTA	140	—				10	2	80 ± 14	4.5 ± 1.8	+
	Controls						10	2	103 ± 20	13 ± 2.7	
Kramsch, 1978 (54)	EHDP	20	—	Rabbit	Progression	8	8	2	88 ± 26	18 (1.1 ± 0.3)[e]	+
		40	—				8	2	89 ± 20	15 (1.3 ± 0.5)	+
	Controls						8	2	98 ± 18	65 (2.8 ± 0.5)	
Hollander, 1978 (57)	PHB	100	—	Cynomolgus monkeys	Progression	24	10	2.5	13 ± 2.8	49 ± 22	+
	CMDP	—	5				10	2.5	20 ± 4.0	72 ± 16	0
	Controls						10	2.5	21 ± 3.7	73 ± 16	
Hollander, 1979 (55)	EHDP	—	5	Rabbit	Progression/ regression	8	10	2	83→8.9[a]	16 ± 9.0	+
	Controls						10	2	83→9.4	35 ± 16	
Kramsch, 1980 (60)	$LaCl_3$	20	—	Rabbit	Progression	8	8	2	63 ± 12.9	16 (1.9 ± 1.0)	+
		30					8	2	52 ± 23.9	7 (1.4 ± 0.7)	+
		40					8	2	66 ± 17.4	3 (0.6 ± 0.1)	+
	Controls						8	2	66 ± 14.7	64 (3.5 ± 1.2)	
Overturf, 1980 (59)	5MTCA	140	—	Rabbit	Progression	8	12	2	51 ± 11	26 ± 8	0
	Controls						12	2	43 ± 17	23 ± 11	
Kramsch, 1981 (56)	$LaCl_3$	120–40	—	Cynomolgus monkeys	Progression	96	8	0.1	11 ± 3.2	21 ± 10	+
	EHDP	40	—			96	8	0.1	11 ± 3.2	14 ± 7	+
	AHDP	40	—			96	8	0.1	11 ± 3.5	12 ± 8	+
	APDP	40	—			96	8	0.1	13 ± 3.4	18 ± 11	+
	Controls					96	8	0.1	12 ± 3.5	75 ± 22	
Ginsburg, 1983 (61)	$LaCl_3$	40	—	Rabbit	Progression	10	12	2	47 ± 7.2	33 ± 19	+
	Controls						12	2	33 ± 16	52 ± 22	
Rouleau, 1983 (62)	$LaCl_3$	35	—	Rabbit	Progression	10	9	0.5	53 ± 11	59 ± 31	0
	Controls						9	0.5	43 ± 8.4	73 ± 24	

[a] Change in plasma cholesterol concentration during the regression period.
[b] Gram lipid/100 g dry wt.
[c] TCA, 2-thiophenecarboxylic acid; 5MTCA, 5-methyl-2-thiophenecarboxylic acid; 5BTA, 5-bromo-2-thiophenecarboxaldehyde; AHDP, azacycloheptane-2,2-diphosphate; APDP, amino-1-hydroxypropane-1, 1-diphosphate.
[d] mg cholesterol/whole aorta.
[e] mg cholesterol/whole aorta/kg body wt.

Also, there was no difference in the aortic content of total, sulfated mucopolysaccharides. Wartman *et al.* (51) from the same laboratory did not find an inhibiting effect of EDTA on aortic total lipid concentration, on aortic calcium and magnesium concentration, or on collagen and elastin content.

Rosenblum *et al.* (52) evaluated the influence of disodium ethane-1-hydroxy-1,1-diphosphonate (EHDP) on rabbit atherosclerosis induced by vitamin D, nicotine, and hypercholesterolemia. The rabbits were selected in such a way that only "high responders" were included in the study. Due to a high mortality rate only a few animals terminated the study. In aorta the plaque area showed a 2-fold increase in the EHDP-treated animals, while the amount of calcification was decreased. Another EHDP study using White Carneau pigeons was performed by Wagner *et al.* (53). Because of its complicated design it has not been included in Table I. The pigeons were fed cholesterol either continuously or intermittently during a 14-month period and

treated daily with intramusclular injections of either saline, or 0.5 or 2.5 mg EHDP/kg body wt. EHDP did not influence plasma cholesterol or calcium concentrations in plasma, but the results of the aortic cholesterol estimations were conflicting. In animals continuously fed cholesterol the aortic cholesterol concentrations at both EHDP levels were higher than those in controls, and in animals fed cholesterol intermittently they were lower than in controls at both EHDP levels. In 1978 Kramsch and Chan (54) treated rabbits with EHDP at two dosage levels. In both cases EHDP significantly decreased the aortic cholesterol and calcium content. If small doses of colcemid were added to the diet, an additive effect was observed, which almost completely suppressed all aspects of atherogenesis. The common denominator of the effect of these compounds was a reduction of elevated plasma and arterial calcium concentrations. The authors therefore suggest that deposition of calcium may play an important part in early atherogenesis. Hollander *et al.* (55) investigated the influence of EHDP injected subcutaneously on the regression of preestablished atherosclerosis in rabbits. At autopsy the aortas of the experimental animals had fewer gross lesions and contained significantly less calcium and elastin.

Contrary to the observations of Kramsch *et al.* (56), Hollander *et al.* (57) did not find an antiatherosclerotic effect of diphosphonate treatment (dichloromethylene diphosphate) in cynomolgus monkeys, except for preventing calcium deposition in the aortic lesions. In the same study, however, Hollander *et al.* (57) tested the antiatherogenic effect of the anticalcifying compound cetaben (sodium *p*-hexadecylaminobenzoate, PHB). Both the clinical and necropsy findings indicated that PHB was an effective antiatherosclerotic agent which reduced the extent and severity of the lesions not only in the aorta, but also protected against obstructive disease of the coronary and peripheral arteries. PHB exerted a significant hypocholesterolemic effect, which probably largely accounts for the antiatherosclerotic effect, although an inhibition of the calcium content in the lesions also was seen.

Chan *et al.* (58) investigated the effect of thiophene compounds on rabbit atherogenesis. Although 2-thiophenecarboxylic acid had no influence on the aortic total cholesterol content, the derivatives 5-methyl-2-thiophenecarboxylic acid and 5-bromo-2-thiophenecarboxaldehyde effectively suppressed atherogenesis and the aortic content of cholesterol, calcium, collagen, and elastin without changes in plasma cholesterol levels. In contrast to these findings Overturf *et al.* (59) were not able to demonstrate a protective effect of 5-methy-2-thiophene carboxaldehyde in rabbit atherosclerosis, although they attempted to precisely duplicate the materials and methodologies of the previous study.

The first study using the inorganic calcium antagonist lanthanum was performed by Kramsch *et al.* (60). With increasing lanthanum dosage given to cholesterol-fed rabbits, the degree of atherosclerotic changes progressively decreased and were completely abolished at the highest daily dose of 40 mg/kg body wt. In another study of lanthanum and various diphosphonates, Kramsch *et al.* (56) demonstrated an inhibitory effect of these compounds on diet-induced atherosclerosis in macaque monkeys, thus supporting the concept that inhibition of calcium flux and calcium deposition in the arterial wall may be essential to the prevention of atherosclerosis. Ginsburg *et al.* (61) investigated the influence of lanthanum on atherogenesis in rabbits and found that lanthanum suppressed aortic atherogenesis, but had no influence on the degree of atherosclerosis in the intramural coronary arteries. The authors give no explanation of this puzzling observation. Also, Rouleau *et al.* (62) treated rabbits fed an atherogenic diet with lanthanum and found a moderate, but insignificant reduction in the degree of aortic atherosclerosis.

Organic Calcium Antagonists in Hyperlipidemic Animals. Nifedipine was the first calcium antagonist reported to suppress atherogenesis in rabbits (63). The dosage, expressed in mg/kg body weight, used in this and most other studies in Table II is considerably higher than human therapeutic doses. An exception is the study by Overturf *et al.* (64). These authors wanted to determine whether nifedipine, over an 8-month study period at a dosage corresponding to an oral human therapeutic dosage, could reduce atherosclerosis in rabbits with a mild degree of hypercholesterolemia. Under these circumstances nifedipine did not suppress atherogenesis. However, the dihydropyridine derivative PN-200-110 given orally to rabbits in a dosage about twice as high as that used therapeutically in humans resulted in a significant reduction in aortic cholesterol concentration (65) Stender *et al.* (66) also investigated the influence of nifedipine on the atherogenesis in rabbits. This study was carefully designed to reproduce the same experimental conditions as those described by Henry and Bentley (63); for example, the capsules were force-fed into the pharynx as described by these authors. The mean plasma cholesterol concentrations also were very similar at the end of the two studies, but Stender *et al.* (66) neither found an effect of nifedipine on the aortic cholesterol accumulation nor on the aortic influx of cholesterol and albumin. Willis *et al.* (67), with a 2% cholesterol addition to the diet, had an approximately 50% lower final plasma cholesterol concentration and a significant suppression of aortic cholesterol content in the animals receiving nifedipine and nicardipine. An antiatherogenic effect of nifedipine was also found by Ishikawa *et al.* (68) in cholesterol-fed rabbits, whereas flordipine, a new dihydropyridine derivative, did not influence atherogenesis in cholesterol-fed rabbits at three different dosages (69).

Van Niekerk *et al.* (70), Tilton *et al.* (71), and Ishikawa *et al.* (68) investigated whether the calcium

Table II. Studies of the Influence of Calcium Antagonists in Clinical Use on Aortic Atherogenesis in Hypercholesterolemic Rabbits (mean ± SD)

First author	Experimental design						Plasma cholesterol (m*M*)	Aorta		
	Ca antagonist	Daily dosage (mg/kg)		Cholesterol addition (%)	Duration (weeks)	No. of rabbits		Lesioned area (%)	Cholesterol (μmol/mg protein)	Significant reduction ($P < 0.05$)
		Oral	Parenteral							0/+
Henry, 1981 (63)	Nifedipine	15(C)[a]	—	2	8	13	48 ± 11	17 ± 11	0.08 ± 0.02	+
	Controls	Placebo		2		13	49 ± 13	40 ± 18	0.12 ± 0.05	
Ginsburg, 1983 (61)	Diltiazem	103[b]	—	2		9	45 ± 16	33 ± 18	—	+
	Flunarizine	5.7[b]	—	2	10	10	29 ± 7	34 ± 25	—	0
	Controls			2		9	33 ± 16	52 ± 20	—	
Rouleau, 1983 (62)	Verapamil	8	0.5	0.5		8	51 ± 8[c]	25 ± 26	—	+
	Verapamil	8	—	0.5	10	8	45 ± 8[c]	51 ± 22	—	0
	Controls			0.5		9	43 ± 8[c]	73 ± 24	—	
Stender, 1984 (66)	Nifedipine	15(C)	—	2	8	17	45 ± 16	—	0.23 ± 0.16	0
	Controls	Placebo		2		15	47 ± 14	—	0.22 ± 0.16	
Niekerk, 1984 (70)	Nifedipine	15	—	0	26	9[d]	13 ± 4	59 ± 17	—	0
	Controls			0		9[d]	17 ± 3	62 ± 19	—	
Naito, 1984 (84)	Nicardipine	20	—	1	12	9	50 ± 14	19 ± 8	—	0
	Controls			1		9	46 ± 12	14 ± 13	—	
	Diltiazem	70	—	1	12	8	44 ± 18	7 ± 9	—	0
	Controls			1		8	45 ± 9	4 ± 5	—	
Blumlein, 1984 (72)	Verapamil	—	1.5	0.5		8	40 ± 10	68 ± 22	—	0
	Verapamil	20–200	—	0.5	10	17	64 ± 23	27 ± 14	—	+
	Controls					16	53 ± 14	51 ± 12	—	
Willis, 1985 (67)	Nicardipine	80	—	2		19	19 ± 7	19 ± 15	—	+
	Nifedipine	80	—	2	8	4(6)	26 ± 9	16 ± 18	—	+
	Controls	Placebo		2		16	24 ± 12	37 ± 17	—	
Tilton, 1985 (71)	Verapamil	46	0.5	0	24	12[d]	14 ± 3.5	65 ± 17	—	0
	Controls			0		11[d]	14 ± 3.6	61 ± 17	—	
Stender, 1986 (73)	Verapamil	16(C)	2.0	2		11	65 ± 15	—	0.20 ± 0.13	+
	Verapamil	8(C)	0.5	2	10	11	75 ± 25	—	0.33 ± 0.29	0
	Controls	Placebo		2		11	76 ± 25	—	0.32 ± 0.13	
Sugano, 1986 (74)	Diltiazem	—	25	1	10	5	20 ± 6	2 ± 2	—	+
	Controls	Placebo				8	40 ± 20	26 ± 17	—	
Habib, 1986 (65)	PN 200-110	0.25(C)	—	1	10	13	52 ± 11	36 ± 18	0.14 ± 0.03	+
	Controls	Placebo	—	1		14	55 ± 11	52 ± 19	0.19 ± 0.06	
Ishikawa, 1987 (68)	Nifedipine	15(C)	—	0	20	7[d]	—	33 ± 14	0.78 ± 0.34	0
	Controls	Placebo		0		8[d]	—	27 ± 12	0.62 ± 0.16	
	Nifedipine	15(C)	—	2	12	5	78 ± 33	26 ± 8	0.55 ± 0.15	+/0
	Controls	Placebo		2		6	76 ± 23	56 ± 23	0.76 ± 0.34	
Diccianni, 1987 (76)	Diltiazem	120(C)[e]	—	1	16	7	34 ± 14	48 ± 28	—	0
	Controls	Placebo		1		7	43 ± 27	43 ± 21	—	
Overturf, 1987 (64)	Nifedipine	0.285	—	0.1	32	12	11 ± 5.4	45 ± 22	—	0
	Controls			0.1		14	10 ± 6.2	56 ± 23	—	
Kritchevsky, 1988 (69)	Flordipine	5	—	1	10	10	89 ± 25	29 ± 22	—	0
		15	—	1	10	10	101 ± 25	43 ± 25	—	0
		45	—	1	10	10	118 ± 29	33 ± 31	—	0
	Controls	Placebo	—	1	10	10	94 ± 45	24 ± 25		

[a] (C), Capsules.
[b] Oral gavage.
[c] Mean values over the period.
[d] Watanabe-heritable hyperlipidemic rabbits.
[e] Sustained release capsules.

antagonists nifedipine and verapamil influenced the atherogenic process in Watanabe-heritable hyperlipidemic rabbits. All three groups reported a lack of effect.

Rouleau *et al.* (62) evaluated the antiatherogenic effect of verapamil in rabbits. An oral dose of 8 mg/kg did not reduce aortic atherosclerosis, whereas an additional parenteral dose of 0.5 mg/kg significantly reduced the percentage of lipid-stained aortic surface. In a later study from the same group (72), an antiatherogenic action of verapamil was found only in rabbits with detectable plasma levels of verapamil when verapamil was given orally, but not when it was given subcutaneously. The same combination of oral and subcutaneous verapamil as that used by Rouleau *et al.* (62) was used by Stender *et al.* (73), but these investigators were not able to confirm the findings of Rouleau *et al.* (62). In the same study, Stender *et al.* (73) also used a higher verapamil dosage (16 mg/kg orally plus

2 mg/kg subcutaneously). This dosage resulted in plasma concentrations of verapamil in the same range as the usual therapeutic levels in humans and resulted in a significantly lower aortic cholesterol concentration in the treated group than in the placebo group. The authors point out, however, that, although not statistically different, the high verapamil group had a somewhat lower mean plasma concentration of cholesterol than the placebo group. It cannot be excluded that this reduction contributed to the lower aortic cholesterol concentration.

Ginsburg *et al.* (61) tested the effect of fluanarizine and diltiazem on the development of atherosclerosis in cholesterol-fed rabbits. Aortic cholesterol concentration was not determined, but planimetry showed in the diltiazem group a significant reduction in lesion area of the thoracic aorta, but not in the abdominal aorta. In the flunarizine group a nonsignificant borderline value was seen in the thoracic aorta. Neither diltiazem nor flunarizine influenced the extent or distribution of lesions in the intramural coronary arteries. Sugano *et al.* (74), while reporting no plasma diltiazem concentrations, employed an experimental regimen of 25 mg/kg diltiazem intraperitoneally per day and found a dramatic beneficial effect of diltiazem on aortic atherosclerosis in all five test animals. These animals had a plasma cholesterol level which was half of the value in the control animals. Three other groups (Ginsburg *et al.* (61), Naito *et al.* (75), and Diccianni *et al.* (76)) were not able to demonstrate an antiatherogenic effect of diltiazem.

Organic Calcium Antagonists in Normolipidemic Animals. Handley *et al.* (77) examined the two experimental calcium channel blockers PN 200-110 and PY 108-068 for their ability to inhibit neointimal lesion development in the rat carotid model. Both compounds significantly reduced the balloon catheter-induced lesion areas. The authors attribute the effect to an inhibition of the mitogen response of smooth muscle cells in the arterial wall. In a similar study in normolipidemic rats and rabbits, Jackson *et al.* (78) reported that nifedipine given orally (10 mg/kg) caused a significant reduction of neointimal aortic lesions after balloon catheterization. The authors attributed the antiatherogenic action of nifedipine to an inhibition of the myoproliferative response to injury. Nomoto *et al.* (45) evaluated the effect of nilvadipine on cuff-induced intimal thickening of the rabbit carotid artery model and found that intramuscular injections of nilvadipine inhibited the cuff-induced intimal thickening in a dose-dependent manner. Verapamil had no effect on the development of intimal thickening and diltiazem only had an inhibitory effect in unphysiologically high doses.

Discussion

Several investigators have reported substantial increases in total aortic calcium content in cholesterol-fed rabbits (57, 63, 79). The mechanism is unknown, but it has been suggested that increasing incorporation of free cholesterol into the plasma membrane influences the Ca^{2+} influx (80). Once $[Ca^{2+}]c$ is increased, it would be expected to accelerate the cellular processes known to be calcium dependent and involved in atherogenesis into a perilous cycle.

Many risk factors for the development of atherosclerosis have been identified, but the resulting lesions appear to progress in the same way (81). Consequently, it has been suggested that these risk factors all act through a single common final pathway leading to the formation of atherosclerotic lesions. Because many of the processes that are involved in atherogenesis are controlled by $[Ca^{2+}]c$, the calcium hypothesis for atherogenesis predictst that changes in arterial cell calcium must be at least partially intracellular. This has led several investigators to suggest that the final pathway of atherogenesis is dependent not only on Ca^{2+} movement across the smooth muscle plasma membrane, but also on intracellular Ca^{2+} distribution.

***In Vitro* Experiments.** The calcium hypothesis has recently gained some support in the investigations of Strickberger *et al.* (82) on cellular Ca^{2+} metabolism in aortic segments and in the cell culture experiments with calcium antagonists reported by Stein *et al.* (41), Etingin and Hajjar (42), and Schmitz *et al.* (36). However, the unphysiologically high doses used in some of these studies, up to a 1000-fold higher than human therapeutic plasma concentrations, present problems regarding the relevance for human atherosclerosis. An exception is the study by Nakao *et al.* (39) reporting an inhibitory effect of nicardipine at nanomolar concentrations on the *in vitro* migration of smooth muscle cells. This finding is in good agreement with the reports of Handley *et al.* (77) and Jackson *et al.* (78) that various calcium antagonists reduce the size of myoproliferative lesions after balloon injury.

Animal Experiments. There seems to be a consensus that anticalcifying agents and organic calcium antagonists effectively inhibit the myoproliferative response of the artery to injury and also inhibit the synthesis of extracellular matrix elements by smooth muscle cells (49). The precise mechanism is unknown, but is not mediated by platelet-derived growth factor (83).

However, obviously no consensus can be formed from the conflicting results compiled in Tables I and II. If we as a criterion for reproducible antiatherogenicity of a given compound require that the effect has been observed by at least three independent groups using similar experimental designs, none of the anticalcifying agents mentioned in Table I and only nifedipine among the calcium antagonists mentioned in Table II have shown reproducible antiatherogenicity. Even if this criterion is fulfilled, the antiatherogenic effect is

still questionable because it does not take into account studies (published and unpublished) which failed to demonstrate the effect.

For nifedipine there are three studies which demonstrate an antiatherogenicity (63, 67, 68). There are, however, four other studies which do not find this effect (64, 66, 68, 70). One of these studies (66) had a design nearly identical to the design used in the first demonstration of the antiatherogenic effect of nifedipine (63). Two of the four studies which did not show an effect were conducted in Watanabe hereditary hyperlipidemic rabbits (68, 70) and one study used a relatively small dose of nifedipine for rabbits (64).

The disparate results obtained not only for nifedipine, but also for verapamil and the other calcium antagonists may be related to the different design of the studies, concerning such factors as drug dosage, route of administration, duration of the experimental period, and plasma cholesterol levels.

Rabbits have a high first-pass metabolism in the liver of at least some of the calcium antagonists. In order to reach the same plasma concentrations of verapamil in rabbits and humans, rabbits require a 5–10 times higher oral dose per kg body weight than humans (73).

Even in apparently similar experiments there are still a number of differences which may be of importanct for the demonstration of antiatherogenicity, such as the strain of rabbits, composition of basic diet, and how the cholesterol is added to the diet. Also the method for evaluation of the lesions may play a role. It might also be of importance that the responses of rabbits to atherogenic challenges appear in some strains to be influenced by a biologic rhythm changing in a seasonal manner (84).

There are certain parallels between transfer of plasma lipoproteins into the arterial wall of hypercholesterolemic rabbits and middle-aged humans (85). If a compound interferes with this entrance process in rabbits, it is likely to act the same way in humans. Many factors interfere with the fate of lipoproteins after they have entered the arterial wall. These factors may not be similar in hypercholesterolemic rabbits and humans. Antiatherogenicity or lack of antiatherogenicity demonstrated in rabbits can therefore only be suggestive for a similar effect in humans. The suggestion becomes more important if the effect observed in rabbits can be demonstrated also in other animal models for development of atherosclerosis. There is still an obvious lack of studies of calcium antagonists on atherogenesis in swine and nonhuman primates.

In spite of the doubtful evidences for antiatherogenicity of calcium antagonists in animal experiments, two major multicenter clinical trials are now in progress (86). It is neither economically feasible nor ethical to test in humans all new calcium antagonists for a possible antiatherogenic effect. Candidate drugs for such investigations have to be selected based on properties in cell cultures and in animal experiments.

From the animal studies summarized in Table I and II, it is only safe to conclude that these calcium antagonists do not promote atherogenesis in experimental animals. Nifedipine, verapamil, diltiazem, and some of the other calcium antagonists may have an antiatherogenic effect in cholesterol-fed rabbits. The effect apparently depends on factors not yet defined. The explanations for the lack of reproducibility within this area is a major challenge for the investigators.

Human Studies

Most important are, of course, studies in humans. Two major multicenter clinical trials are in progress to study the effects of nifedipine and nicardipine of the progression of coronary disease in high-risk patients (86).

In a recent review Weinstein and Heider (86) conclude that nifedipine may inhibit *early* events in lesion formation and proliferation, but may not inhibit proliferation of lesions at advanced development stages. Since the ongoing clinical trials are designed to test the protective effect of calcium antagonists against progressive disease in high-risk populations, it will be interesting to see whether calcium antagonists can inhibit the progression of established atherosclerotic lesions.

Conclusion

The role of organic calcium antagonists in prevention of atherosclerosis in animals is unsettled and appears to involve interaction with variables not yet defined. Interpretation should therefore be made with caution and is premature at this time. Conclusions concerning the effectiveness of calcium antagonists in suppressing atherosclerosis in humans must await the results of clinical trials with nifedipine and nicardipine (86).

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