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Deterioration of the protein kinase C–K_{ATP} channel pathway in regulation of coronary flow in hypercholesterolaemic rabbits

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Abstract

We studied the effect of experimental hypercholesterolaemia/atherosclerosis on changes in coronary flow and cardiac function, induced by protein kinase C and ATP-sensitive K^+ (K_{ATP}) channel modulators in isolated Langendorff-perfused rabbit hearts. Both phorbol 12-myristate-13-acetate (PMA) and phorbol 12,13-dibutyrate (PDB, 0.1 μM each), activators of protein kinase C, decreased, whereas staurosporine, (0.1 μM), a protein kinase C inhibitor, increased coronary flow and left ventricular dP/dt, an index of ventricular contractility. Glyburide (5–50 μM), a K_{ATP} channel inhibitor, blocked the effect of staurosporine. The phorbol esters were without effect in the presence of pinacidil (5 μM), a K_{ATP} channel activator. Neither the protein kinase C modulators nor glyburide produced any effect on coronary flow and left ventricular contractility, when the hearts were prepared from animals either maintained on a cholesterol (1.5%)-enriched diet or treated with lovastatin (5 mg/kg/day per os). Treatment with farnesol (1 mg/kg twice a day for 7 days intravenously) restored the reactivity of hearts from either hypercholesterolaemic or lovastatin-treated animals to protein kinase C modulators. We conclude that non-cholesterol mevalonate products are necessary for the functional integrity of the protein kinase C–K_{ATP} channel pathway in the rabbit heart. © 2001 Published by Elsevier Science B.V.

Keywords: Protein kinase C; K_{ATP} channel; Hypercholesterolaemia; Farnesol; Heart, rabbit

1. Introduction

The opening and closing of ATP-sensitive K^+ (K_{ATP}) channels is involved in several cardiovascular adaptive responses (Light et al., 1996). For example, activation of these channels in cardiac myocytes has been found to underlie the anti-ischaemic effect of preconditioning, the most potent cardioprotective mechanism described to date (Gross and Auchampach, 1992; Auchampach et al., 1992). It is also widely accepted that opening of the K_{ATP} channels of vascular smooth muscle cells is responsible for hypoxic dilation of coronary arteries (Daut et al., 1990). Although the classical mechanism for activation of these channels is through a reduction in intracellular ATP concentration, K_{ATP} channel function also serves as a target for several post-receptor signalling pathways influencing either the contractility of cardiac myocytes or vascular tone (Noma, 1983; see for review, Coetzee, 1992). Some types of protection conferred by preconditioning, such as the reduction of infarct size or improvement of post-ischaemic contractile function, result from opening of these channels by adenosine A_1 receptor activation (Gross and Auchampach, 1992). In vascular smooth muscle cells, K_{ATP} channels are activated through stimulation of protein kinase A, whereas protein kinase C is considered to be inhibitory (see for reviews Edwards and Weston, 1993, 1994; Nelson and Quayle, 1995; Quast, 1996). Either activation or inhibition of K_{ATP} channel function by protein kinase C, however, requires the functional integrity of G-protein coupling (Flavahan, 1992).

We have shown that experimental hypercholesterolaemia/atherosclerosis blocks preconditioning (Szilvassy et

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al., 1995) and impairs gastrointestinal non-adrenergic, non-cholinergic (NANC) relaxation in both experimental animals and clinical patients (Szilvassy et al., 1996, 1997). Both mechanisms require the functional integrity of G protein coupling (Ferdinandy et al., 1998a,b; Szilvassy et al., 1998). Since G-protein coupling may be impaired in experimental hypercholesterolaemia, and under other conditions of a reduced synthesis of non-cholesterol mevalonate products, the aim of the present work was to study if dietary hypercholesterolaemia or treatment with lovastatin influenced the effect of protein kinase C activation on coronary flow and cardiac function, in isolated Langendorff-perfused rabbit hearts.

2. Methods

2.1. Ethics

The experiments performed in the present work conformed to the European Community Guiding principles for the care and use of laboratory animals. In addition, the experimental protocol applied was approved by the local ethics committee of the Medical University of Debrecen.

2.2. Experimental groups

The work was performed with male New Zealand white rabbits weighing 2.5–3.0 kg. The rabbits were fed on commercial laboratory chow with free access to water. They were kept in the Laboratory Centres of the Departments of Pharmacology the Medical Universities of Debrecen and Pecs, under pathogen-free conditions (12-h light/dark periods a day, temperature of 22–25°C, humidity of 50–70%). After a 2-week period of adaptation, the animals were randomised into two experimental groups. 'Group 1' animals continued to be given normal chow, whereas 'Group 2' animals were given laboratory chow enriched with 1.5% cholesterol over 8 weeks. The animals of both groups were further randomised into two subgroups after the 7th experimental week. Group 1/a rabbits were given 5 mg/kg lovastatin, an inhibitor of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase, p.os. (Mevacor, MSD, New Jersey, NY) once a day for 7 days, whereas 'Group 1/b' animals received the placebo for lovastatin for the same period. 'Group 2/a' animals were given 1 mg/kg farnesol (3,7,11-trimethyl-2,6,10 dodecatrien-1-ol mixed isomers, Sigma, St Louis, MO), a major non-cholesterol mevalonate product, twice a day for 7 days, whereas 'Group 2/b' animals received the placebo for farnesol in the same way. A third group of normal animals was used to study the effect of combined application of lovastatin and farnesol. The controls for this series of rabbits were those rabbits that were treated with the solvent for farnesol and the placebo for lovastatin.

2.3. Isolated heart preparations

The rabbits were premedicated with 0.5 ml of Hypnorm solution i.m. (10 mg fluanisone and 0.2 mg fentanyl per ml). Subsequently, anaesthesia was achieved by means of 25 mg/kg sodium pentobarbitone (Nembutal, Abbott, North Chicago IL). The trachea was exposed and the animals were mechanically ventilated with room air as described (Szilvássy et al., 1994). The heart was excised and mounted for Langendorff-perfusion with thermostatically controlled (37°C) oxygenized Krebs–Henseleit solution. A water-filled latex balloon with a catheter tip manometer (type MTC-HD, Drager Med. Electronics, Best, The Netherlands) was used to measure left ventricular pressure. Positive and negative dP/dt_{max} were obtained through electronic differentiation of the left ventricular pressure curve. Coronary flow and heart rate were continuously monitored with an electromagnetic flow meter (Nihon, Kohden, Japan) with the probe placed in series with the cannulated aorta. Myocardial temperature was controlled by means of a myocardial temperature probe (Shiley, Irvine, CA) inserted in the right ventricular wall.

2.4. Experimental protocol

The hearts from animals in each group underwent protocols as follows.

2.4.1. The effect of modulators of protein kinase C and K_{ATP} channels

In the first sets of experiments, after a 20-min equilibration period, the effects on cardiac function and coronary flow of phorbol 12-myristate-13-acetate (PMA) and phorbol 12,13-dibutyrate (PBD), protein kinase C activators, staurosporine, a protein kinase C inhibitor (0.1 μM for each), glyburide (5–50 μM), a K_{ATP} channel inhibitor, or pinacidil (5 μM), a K_{ATP} channel activator ($n = 6$ for each), were studied. The effects of each compound were monitored for 30 min.

2.4.2. Interaction between modulators of protein kinase C and K_{ATP} channels

After the stabilization period, the hearts were perfused with pinacidil (5 μM) and then PMA (0.1 μM), a protein kinase C activator, was added when the maximum increase in coronary flow, induced by the K_{ATP} channel opener, was reached. Similarly, staurosporine (0.1 μM), a protein kinase C inhibitor, was given after pre-perfusion with glyburide (5–50 μM).

2.5. Drugs and solutions

The drugs and chemicals used in the present work were purchased from Sigma. Glyburide, PMA, PBD, and staurosporine were dissolved in dimethylsulfoxide (DMSO:

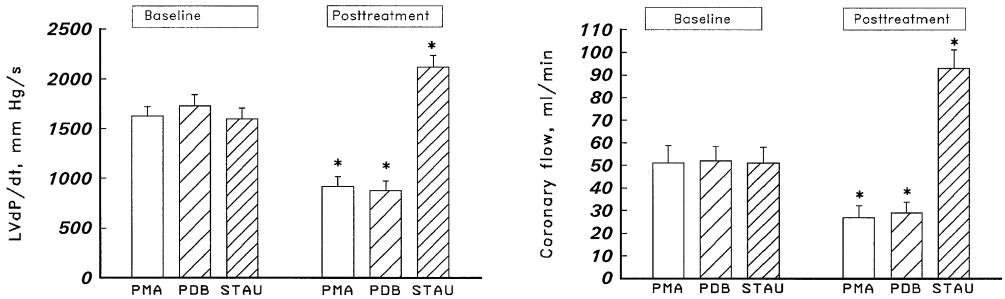

Fig. 1. Effect of protein kinase C modulators on coronary flow and cardiac function in isolated Langendorff-perfused rabbit heart. Left panel: changes in left ventricular dP/dt; right panel: Changes in coronary flow. The data are means ± S.D. obtained with six hearts in each group. (*) Baseline vs. post-treatment values at $P < 0.05$. Abbreviations: PMA = phorbol 12-myristate-13-acetate; PBD = phorbol 12,13-dibutyrate; STAU = staurosporine (0.1 μM).

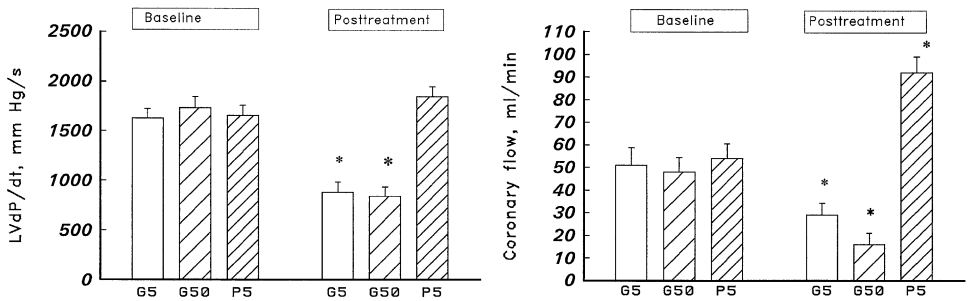

Fig. 2. Effect of ATP-sensitive potassium (K_{ATP}) channel modulators on coronary flow and cardiac function in isolated Langendorff-perfused rabbit heart. Left panel: changes in left ventricular dP/dt; right panel: changes in coronary flow. The data are means ± S.D. obtained with six hearts in each group. (*) Baseline vs. post-treatment values at $P < 0.05$. Abbreviations: G5 = glyburide, 5 μM; G50 = glyburide, 50 μM; P5: pinacidil, 5 μM.

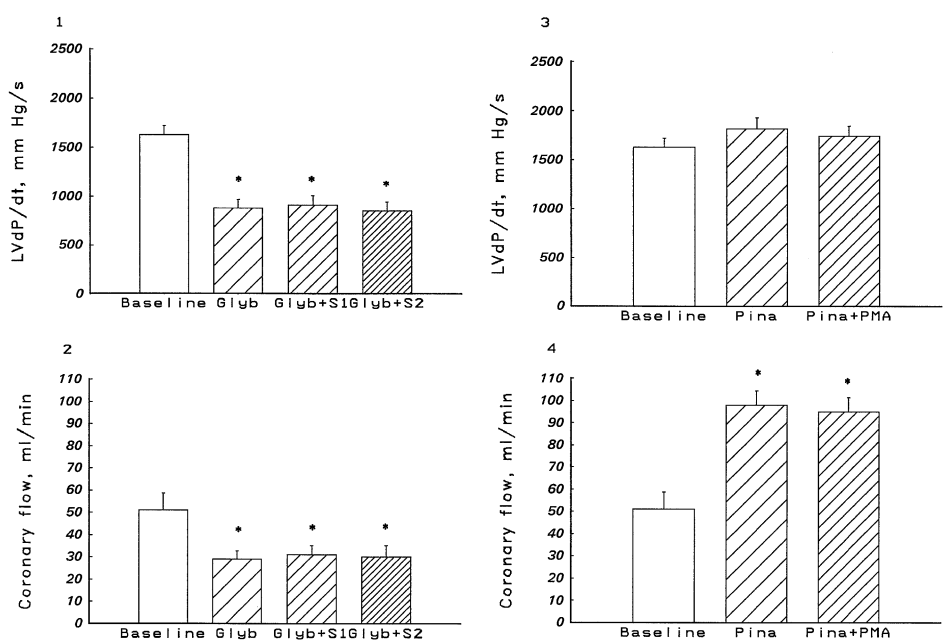

Fig. 3. Interaction between protein kinase C and K_{ATP} channel modulators on coronary flow and cardiac function in isolated Langendorff-perfused rabbit heart. The data are means ± S.D. obtained with six hearts in each group. (*) Significantly different from baseline at $P < 0.05$. Abbreviations: Glyburide; S1 = staurosporine, 0.01 μM; S2 = staurosporine, 0.1 μM; Pina: pinacidil; PMA: phorbol 12-myristate-13-acetate.

concentration in the perfusion fluid 0.001%). Pinacidil was dissolved in ethanol. To prevent photodegradation of phorbol esters, the equipment was covered with black photographic paper.

2.6. Statistics

The data expressed as means ± standard deviation (S.D.) were analysed with a one-way analysis of variance followed by a modified *t*-test according to Bonferroni's method (Wallenstein et al., 1980). Inter-group comparisons were made by the two-tailed *t*-test. Changes were considered significant at $P < 0.05$.

3. Results

3.1. Effect of protein kinase C and K_{ATP} channel modulators in hearts from animals fed on normal chow

Fig. 1 shows that PMA and PDB, activators of protein kinase C, decreased whereas staurosporine, a protein kinase C inhibitor, increased coronary flow and left ventricular dP/dt. Glyburide decreased both coronary flow and left ventricular dP/dt, whereas pinacidil increased coronary flow without having a significant effect on left ventricular dP/dt only (Fig. 2).

In the presence of 50 μM glyburide, staurosporine (0.01–0.1 μM), a protein kinase C inhibitor, did not increase either coronary flow or left ventricular dP/dt. Similarly, PMA, a protein kinase C activator, failed to influence these parameters in hearts pre-perfused with pinacidil (Fig. 3), a K_{ATP} channel opener.

None of the compounds studied produced any change in heart rate in isolated rabbit hearts (data not shown).

3.2. Interaction between experimental hypercholesterolaemia / atherosclerosis and the effects of protein kinase C or K_{ATP} channel modulators

Neither PMA or PDB, activators of protein kinase C, nor staurosporine, a protein kinase C inhibitor, influenced coronary flow or left ventricular dP/dt in hearts from Group 2/b animals. Glyburide decreased both parameters in hearts from Group 2/a animals but only tended to decrease these parameters in hearts from Group 2/b animals. Conversely, pinacidil increased coronary flow with a marginal increase in left ventricular dP/dt in hearts from Group 2/a rabbits (Fig. 4).

3.3. The effect of farnesol supplementation on the interaction between protein kinase C modulators and hypercholesterolaemia

In hypercholesterolaemic animals pre-treated with farnesol (Group 2/a), PMA and PDB, activators of protein

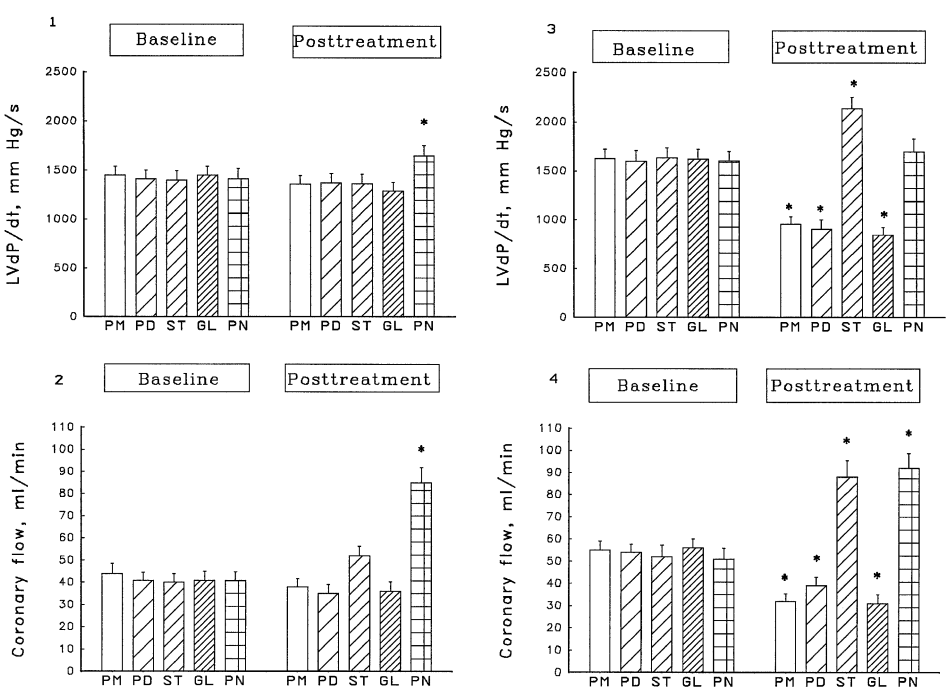

Fig. 4. Aftermath of farnesol supplementation on the effect of protein kinase C and K_{ATP} channel modulators on coronary flow and cardiac function in isolated Langendorff-perfused hearts from rabbits with dietary hypercholesterolaemia. Parts 1 and 2: hearts from hypercholesterolaemic rabbits (1.5% cholesterol for 8 weeks) treated with the solvent for farnesol; parts 3 and 4: hearts from hypercholesterolaemic rabbits treated with farnesol (1 mg/kg twice a day for 7 days). The data are means ± S.D. obtained with six hearts in each group. (*) Baseline vs. post-treatment at $P < 0.05$. Abbreviations: PM = phorbol 12-myristate-13-acetate; PD = phorbol 12,13-dibutyrate; ST = staurosporine; GL = glyburide; PN = pinacidil.

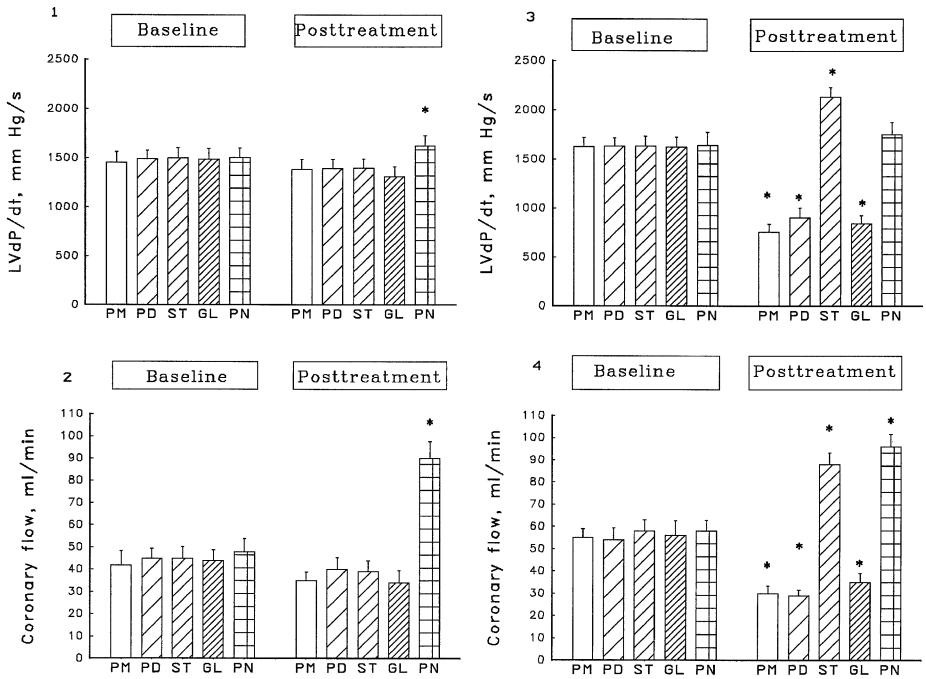

Fig. 5. Consequence of farnesol supplementation on the effect of protein kinase C and K_{ATP} channel modulators on coronary flow and cardiac function in isolated Langendorff-perfused hearts from rabbits treatedwith lovastatin (5 mg/kg once a day for 7 days). Parts 1 and 2: hearts from lovastatin-treated rabbits given the solvent for farnesol; parts 3 and 4: hearts from lovastatin-treated rabbits given a 7-day treatment with farnesol (1 mg/kg twice a day). The data are means ± S.D. obtained with six hearts in each group. (*) Baseline vs. post-treatment at $P < 0.05$. Abbreviations: PM = phorbol 12-myristate-13-acetate; PD = phorbol 12,13-dibutyrate; ST = staurosporine; GL = glyburide; PN = pinacidil.

kinase C, decreased, whereas staurosporine, an inhibitor of protein kinase C, increased coronary flow and left ventricular dP/dt to a degree similar to that found in hearts from Group 1/b rabbits (Fig. 4).

3.4. The effect of protein kinase C modulators on hearts from rabbits pre-treated with lovastatin

Neither PMA nor staurosporine produced any effect on coronary flow or left ventricular dP/dt in hearts from Group 1/a animals. However, when the hearts were isolated from animals treated with lovastatin and farnesol together for 1 week (Group 3), the protein kinase C modulators elicited similar responses to those found in hearts from 'Group 1/b' animals (Fig. 5).

4. Discussion

The results presented show that pharmacological activation of protein kinase C decreases coronary flow with a decline in cardiac function, whereas protein kinase C inhibition increases coronary flow with an improvement in cardiac function in isolated Langendorff-perfused rabbit hearts. The protein kinase C activators or blockers used in the present work seem to exert most of their effects on coronary flow and cardiac function through K_{ATP} channel modulation, since glyburide, an inhibitor of these channels, blocked the effect of staurosporine, and PMA failed to decrease either coronary flow or left ventricular dP/dt in the presence of pinacidil. The protein kinase C modulators were without effect on both variables in hearts from hypercholesterolaemic/atherosclerotic rabbits, which is the main original observation of the study. The effects of protein kinase C modulators, however, were restored in hearts from hypercholesterolaemic rabbits supplemented with farnesol during the last week of the atherogenic diet, similar to that seen in hearts from rabbits pre-treated with lovastatin, an HMG-CoA inhibitor.

Phorbol esters such as PDB and PMA have been shown to activate protein kinase C and to produce a sustained contraction in vascular smooth muscle cells (Miller et al., 1986; Rasmussen et al., 1987). However, besides a comparable activation of protein kinase C in isolated enzyme system (Castagna et al., 1982), PDB proved to be 150 times more potent in inhibiting P1075-induced $^{86}Rb^{+}$ efflux, an indicator of K^{+} channel inhibition, than PMA in rat aorta (Linde et al., 1987). The discrepancy was explained by a possible difference in membrane permeability of the two protein kinase C activators (Miller et al., 1986). Nevertheless, our present results revealed an equal effect of the two substances (at least at the concentration studied) in reducing coronary flow in isolated rabbit hearts. The discrepancy may be explained by the difference in species and the experimental protocol used.

Staurosporine, a protein kinase C inhibitor, at the concentration studied, produced a significant increase in coro-

nary flow, an effect which was completely blocked by either 5 or 50 μM glyburide. Assuming that staurosporine elicited its effect by inhibiting protein kinase C, it could mean that in the rabbit coronary arteries, the smooth muscle K_{ATP} channel is under considerable tonic inhibition by protein kinase C, at least in the crystalloid-perfused heart. In hearts from hypercholesterolaemic animals, however, neither activation nor inhibition of protein kinase C produced any change in either coronary flow or cardiac function.

In the vasculature, functional defects have long been identified in endothelial cells in hypercholesterolaemia/atherosclerosis. These defects are characterised by a deficiency in the release and effects of endothelium-derived substances, which require the integrity of several G protein effector systems (see for review: Flavahan, 1992). To fulfil their biological function, G proteins must undergo a post-translation modification with farnesyl or geranylgeranyl moieties that enable them to associate with the membrane. The availability of these moieties is reduced in dietary hypercholesterolaemia (Goldstein and Brown, 1990). The present results seem to support this assumption, since neither activators nor inhibitors of protein kinase C produced any significant effect on coronary flow and cardiac function, in hearts from hypercholesterolaemic animals. However, in hearts from healthy rabbits, substantial increases and decreases in these variables were seen with protein kinase C inhibitors and activators, respectively. Since the protein kinase C–K_{ATP} channel pathway requires the functional integrity of G-protein coupling, we consider that the defect appears at the level of G-protein function. This is further suggested by the finding that the effect of both protein kinase C activators and blockers was restored after a 7-day supplementation treatment with farnesol, a major polyprenyl product. Farnesyl analogues have been found to restore vascular tone adversely affected by either hypercholesterolaemia or inhibition of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase, a key enzyme in the mevalonate pathway, independent of plasma cholesterol levels (Roullet et al., 1993, 1995). Moreover, the rapid pacing-induced ischaemic preconditioning phenomenon of the isolated working rat heart, another G-protein-dependent process (Ferdinandy et al., 1998a), has been found to be similarly affected by both experimental hypercholesterolaemia and HMG-CoA reductase inhibitor treatment (Ferdinandy et al., 1998b). Based on the present results, it is not possible to define the precise mechanism of action of farnesol to restore the pharmacological responsiveness to protein kinase C modulators, in hearts from hypercholesterolaemic/atherosclerotic animals. Nevertheless, the importance of the availability of non-cholesterol mavalonate products in the control of protein kinase C action at the level of G-coupling is strongly suggested by the following parallelism: (1) nitrergic relaxant function is improved by farnesol supplementation in experimental hypercholesterolaemia (Szilvassy et al., 1998) ; (2) the ischaemic preconditioning phenomenon adversely affected by either hypercholesterolaemia or HMG-CoA reductase inhibition is restored by treatment with farnesol (Szilvássy et al., 1997; Ferdinandy et al., 1998b); (3) the effects of protein kinase C modulators are restored by farnesol supplementation in experimental hypercholesterolaemia, similar to that seen with the preconditioning response. Based on the present results, we interpret the effect of farnesol as that resulting from supplementation of an important endogenous substance that is essential for the chain of events in the signal transduction pathway, involving the protein kinase C–K_{ATP} channel mechanism. However, a recent study by Luft et al. (1999) suggested that farnesol was an endogenous Ca^{2+} channel blocker in vascular smooth muscle cells, having a hypotensive effect. Thus, theoretically, effects of farnesol other than simple supplementation cannot be excluded. Nevertheless, in the study by Luft et al., a farnesol dose of 500 mg/kg body weight was used in rats, which is 500 times higher than that used in the present work in rabbits. In addition, at a farnesol dose of 1 mg/kg, we have never seen any change in mean arterial blood pressure in rabbits. Finally, if, under the treatment schedule applied, farnesol had behaved as a slow Ca^{+} channel blocker in coronary smooth muscle cells, it would not have restored the decrease in coronary flow produced by protein kinase C activators.

Whatever the precise mechanism is, the results call attention to the potential clinical use of supplementary non-cholesterol mevalonate products in the treatment of hypercholesterolaemia.

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