Biochemical Pharmacology, Vol. 38, No. 8, pp. 1263–1270, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

ACTIVATION OF PROTEIN KINASE C BY THE DIHYDROPYRIDINE CALCIUM CHANNEL BLOCKER, FELODIPINE

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(*Received* 28 *September* 1988; *accepted* 2 *November* 1988)

Abstract—Felodipine, a dihydropyridine Ca^{2+} channel blocker, appears to have intracellular sites of action in addition to its ability to attenuate voltage-dependent Ca^{2+} channels in smooth muscle cells. *In vitro*, felodipine inhibits several calmodulin-dependent enzymes such as myosin light chain kinase, cyclic nucleotide phosphodiesterase and caldesmon kinase [Walsh MP, Sutherland C and Scott-Woo GC, *Biochem Pharmacol* **37**: 1569–1580, 1988]. Such effects may partially explain the relaxant effects of felodipine and related dihydropyridines on vascular smooth muscle. We have examined the effects of felodipine on the activity of another important enzyme which has been implicated in the regulation of the contractile state of smooth muscle, protein kinase C. We chose to use a physiologically relevant substrate of protein kinase C for these studies, *viz.* platelet P47 protein, rather than the more commonly used lysine-rich histone which is probably not a physiologically important substrate. Protein kinase C and P47 were purified from human platelets and their important structural and functional properties were characterized. Felodipine and the *p*-chloro analogue of felodipine enhanced both the rate and extent of P47 phosphorylation by protein kinase C. Half-maximal activation was observed at 9.5 μM felodipine and 8.5 μM *p*-chloro analogue. Activation by felodipine was dependent upon the presence of phospholipid but did not require diacylglycerol. These observations suggest that the pharmacological actions of felodipine and related dihydropyridines may involve activation of protein kinase C in addition to their known effects on voltage-dependent Ca^{2+} channels and calmodulin-dependent enzymes.

Felodipine [4-(2,3-dichlorophenyl)-1,4-dihydropyridine-2,6-dimethyl 3,5-dicarboxylic 3-ethyl ester and 5-methyl ester], a dihydropyridine Ca^{2+} channel blocker [1], is a potent vasodilator with selectivity for arterial resistance vessels [2]. The primary site of action of dihydropyridines is the voltage-dependent Ca^{2+} channel [3]. However, additional (intracellular) sites of action of felodipine have been suggested [1] and felodipine has been shown to interact with calmodulin in a Ca^{2+}-dependent manner [4,5] and to inhibit various calmodulin-dependent enzymes which are involved in regulating the contractile state of smooth muscle [1, 6, 7].

The Ca^{2+}- and phospholipid-dependent protein kinase (protein kinase C) has also been implicated as a modulator of the contractile state of smooth muscle. A variety of extracellular signals (various peptide hormones, growth factors, muscarinic cholinergic agonists, α-adrenergic agonists, etc.) induce turnover of plasma membrane polyphosphoinositides generating the second-messengers diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP_3) [8]. DG activates protein kinase C and IP_3 mobilizes intracellular stored Ca^{2+}. Protein kinase C exhibits broad substrate specificity [9]. Among its *in vitro* substrates of relevance to smooth muscle contraction are myosin [10] and myosin light chain kinase (MLCK) [11]. Phosphorylation of smooth muscle heavy meromyosin by MLCK greatly enhances its actin-activated Mg-ATPase activity consistent with the requirement of Ca^{2+}/calmodulin-dependent myosin phosphorylation for force development [12]; subsequent phosphorylation by protein kinase C reduces this ATPase rate approximately 50% [13]. Phosphorylation of MLCK by protein kinase C reduces its affinity for calmodulin. Therefore, it has been suggested that protein kinase C-catalyzed phosphorylation of smooth muscle myosin and/or MLCK would favour the relaxed state of the muscle. One may predict, therefore, that activation of protein kinase C would enhance smooth muscle relaxation. Drugs which activate protein kinase C may, therefore, be suitable for the treatment of hypertension.

Based on its known vasodilatory effects, we decided to examine the effect of felodipine on protein kinase C. Protein kinase C is commonly assayed using a lysine-rich histone substrate, but this is probably an unphysiological phosphorylation. We have used, instead, the protein P47 (recently named pleckstrin [14]) isolated from human platelets since it is a true physiological substrate which is rapidly phosphorylated specifically by protein kinase C when platelets are stimulated with agonists such as thrombin [9, 15–17].

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP (10–40 Ci/mmol) and a monoclonal antibody (clone MC5) to bovine brain protein kinase C were purchased from Amersham (Oakville, Ontario, Canada). DEAE–Sephacel was

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purchased from Pharmacia (Mississauga, Ontario, Canada) and hydroxylapatite Biogel HTP, reagents for gel electrophoresis and molecular weight marker proteins from BioRad (Mississauga, Ontario, Canada). Outdated human platelets were donated by the Foothills Hospital Blood Bank (Calgary, Alberta, Canada). Phosphatidylserine (bovine brain) and 1,3-diolein were purchased from Serdary Research Laboratories (London, Ontario, Canada). Felodipine and the *p*-chloro analogue were supplied by Hässle Pharmaceutical (Mölndal, Sweden). General laboratory reagents used were of analytical grade or better and were purchased from Fisher Scientific Ltd. (Calgary, Alberta, Canada). Stock solutions (10 mM) of felodipine and the *p*-chloro analogue were freshly made at the start of each assay in absolute ethanol. The final concentration of ethanol in the assay mixtures was 0.56% (v/v); this ethanol concentration had no effect on protein kinase C activity.

Purification of protein kinase C and P47. Protein kinase C and P47 protein were purified from human platelets by a modification of the procedure for P47 purification described by Imaoka *et al.* [18] as follows. Human platelets (~20 units) were centrifuged at 600 *g* for 10 min at room temperature. All subsequent procedures were carried out at 4°. The supernatant was centrifuged at 16,000 *g* for 20 min and the resultant pellet resuspended in ~500 ml of wash solution (10 mM Tris–HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl) and centrifuged at 20,000 *g* for 10 min. The pellet was resuspended in ~25 ml of lysis solution (5 mM Tris–HCl (pH 7.5), 5 mM EDTA) and frozen at −80°. Combined lyzates obtained from ~300 units of platelets were thawed in a 20° water bath. PMSF was added to a final concentration of 0.1 mM and the lyzate was homogenized with a motor-driven Potter–Elvehjem homogenizer, diluted to 6 l. with 5 mM Tris–HCl (pH 7.5) and centrifuged at 14,000 *g* for 60 min. To the supernatant, solid ammonium sulfate was added to 58% saturation and allowed to stand for 20 min prior to centrifugation at 15,000 *g* for 20 min. Additional ammonium sulfate was added to saturate the supernatant, allowed to stand for 20 min and centrifuged at 15,000 *g* for 30 min. The pellet was redissolved in ~100 ml of 20 mM Tris–HCl (pH 7.5), 1 mM EGTA, 0.5 mM DTT (buffer A) and dialyzed overnight against two changes (10 l. each) of buffer A. The dialyzate was applied, at a flow rate of 50 ml/hr to a column (2.5 cm × 40 cm) of DEAE–Sephacel previously equilibrated with buffer A. Unbound proteins were washed through the column with buffer A until the A_{280} returned to baseline. Bound proteins were eluted with a linear NaCl gradient (0–0.35 M) generated from 600 ml each of buffer A and buffer A containing 0.35 M NaCl, collecting 4 ml fractions. Fractions were asayed for protein kinase C and P47 as described below and analyzed by SDS–PAGE. Protein kinase C-containing fractions were combined and stored at −80° in the presence of 10% (v/v) glycerol and 0.2 mg/ml soybean trypsin inhibitor. This preparation was found to be stable under these storage conditions for at least one year and could be thawed and re-frozen several times without loss of activity. P47-containing fractions eluted from the DEAE–Sephacel column were combined and further purified by hydroxylapatite column chromatography. The P47 pool was dialyzed against two changes (10 l. each) of buffer B [50 mM potassium phosphate (pH 7.0), 0.5 mM DTT, 0.1 mM PMSF], benzamidine was added to a final concentration of 1 mM and applied to a column (1.5 cm × 30 cm) of hydroxylapatite previously equilibrated with buffer B containing 1 mM benzamidine at a flow rate of 15 ml/hr. After washing the column with buffer B, bound proteins were eluted by stepwise addition of buffer B + 1 mM benzamidine containing 0.2 M potassium phosphate and 0.5 M potassium phosphate. Fractions (3 ml) were collected and analyzed for P47 content as described above. P47 eluted in the 0.5 M phosphate buffer. Combined P47-containing fractions were dialyzed overnight against two changes (10 l. each) of 20 mM Tris–HCl (pH 7.5), 0.5 mM DTT and stored at −80°.

Protein kinase C assay. The liposomal assay method for measuring protein kinase C activity was utilized [19]. Standard phosphorylation conditions were as follows: 20 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 0.5 mM $CaCl_2$ or 1 mM EGTA, 40 μg/ml phosphatidylserine, 0.8 μg/ml 1,3-diolein, 24 μg/ml P47, 40 μl/ml platelet protein kinase C and 10 μM [γ-^{32}P]ATP (~150 cpm/pmol). Phosphorylations were carried out at 30° and reactions quenched by addition to 0.5 ml of 25% (w/v) trichloroacetic acid, 2% (w/v) sodium pyrophosphate. Protein-bound [^{32}P]phosphate was quantified as described previously [20].

Phosphopeptide mapping and phosphoamino acid analysis. The site-specificity of P47 phosphorylation

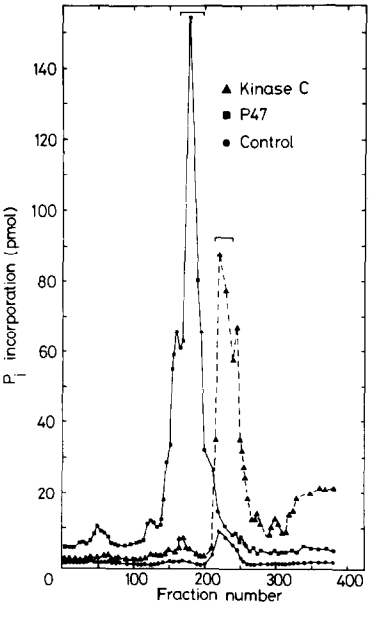

Fig. 1. Purification of human platelet protein kinase C and P47. Protein kinase C and P47 were separated by ion-exchange chromatography on a column of DEAE–Sephacel. Fractions were assayed for protein kinase C (▲---▲) and P47 (■——■). Controls (●——●) were incubated without added protein kinase C or histone substrate. Protein kinase C and P47 were separately pooled as indicated by the bars.

by protein kinase C was investigated by two-dimensional thin-layer electrophoresis/chromatography. P47 was phosphorylated by protein kinase C by incubation for 30 min at 30° under the following conditions: 20 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 0.5 mM $CaCl_2$ or 1 mM EGTA, 40 μg/ml phosphatidylserine, 0.8 μg/ml 1,3-diolein, 0.22 mg/ml P47, 40 μl/ml protein kinase C, 10 μM [γ-^{32}P]ATP (~150 cpm/pmol) in a reaction volume of 0.5 ml. Complete tryptic digestion was then achieved by incubation overnight at 30° with 44 μg/ml TPCK–trypsin [i.e. trypsin: P47 = 1:5 (w/w)]. The digests were then lyophilized and the residue dissolved at a concentration of 5 mg/ml in pyridine:acetic acid:H_2O (1:10:89). Samples (75 μg) of digests were spotted with basic fuchsin tracking dye on Eastman Chromagram cellulose thin-layer sheets and electrophoresed in the first dimension in pyridine:acetic acid:H_2O (1:10:89) at 500 V. After drying the plates, ascending chromatography in the second dimension was carried out in pyridine:*n*-butanol:acetic acid:H_2O [15:10:3:12 (v/v)]. Dried plates were covered with plastic wrap and autoradiographed. Phosphoamino acid analysis was carried out by two-dimensional thin-layer electrophoresis at pH 1.9 in the first dimension and pH 3.5 in the second dimension [21] following hydrolysis in 6 N HCl at 110° for 2 hr of P47 phosphorylated as described above. Dried thin-layer plates were sprayed with ninhydrin to visualize the phosphoamino acid standards added to the hydrolyzate and autoradiographed to localize the ^{32}P label.

Electrophoresis. Electrophoresis was performed in 7.5–20% polyacrylamide gradient slab gels (1.5 mm thick) with a 5% acrylamide stacking gel in the presence of 0.1% SDS at 36 mA using the discontinuous buffer system of Laemmli [22]. Autoradiographs were obtained using Kodak X-Omat AR film in Dupont Cronex cassettes fitted with Dupont Quanta III intensifying screens.

Amino acid analysis. Triplicate samples of P47 (70 pmol each) were hydrolyzed *in vacuo* at 110° in 6 N HCl containing 0.1% (w/v) phenol and 0.1% (v/v) thioglycolic acid for 24, 48 and 72 hr prior to amino acid analysis with a Beckman Model 121M amio acid analyzer using *o*-phthalaldehyde detection. Tryptophan was determined after hydrolysis *in vacuo* at 110° in 4 M methanesulfonic

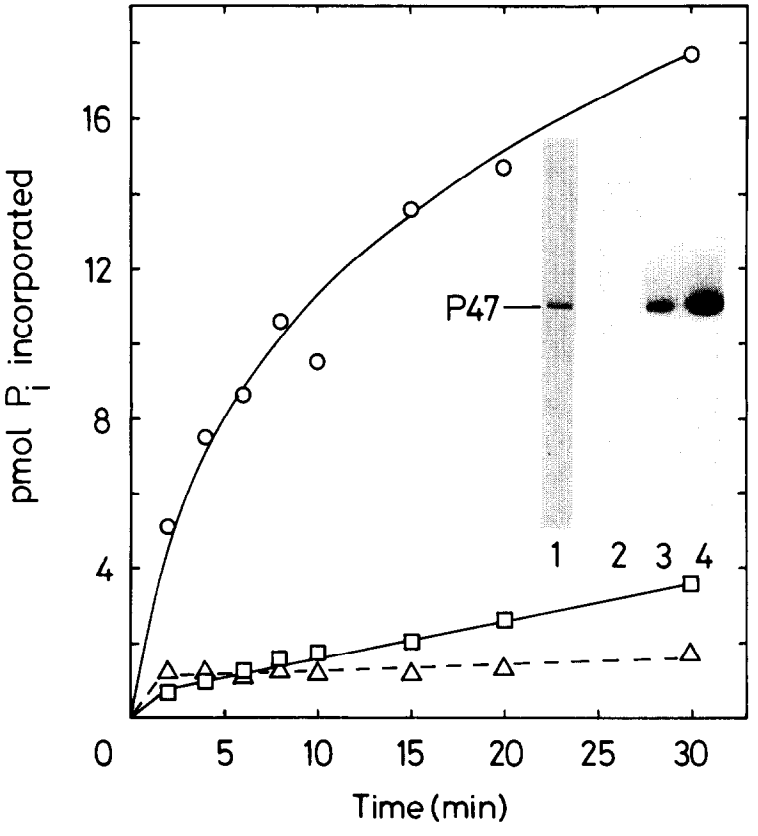

Fig. 2. Functional characterization of platelet protein kinase C and P47 preparations. Platelet protein kinase C (20 μl/ml) (△---△), P47 (72 μg/ml) (□——□) or both (○——○) were incubated at 30° in 20 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 40 μg/ml phosphatidylserine, 0.8 μg/ml 1,3-diolein, 10 μM [γ-^{32}P]ATP (147 cpm/pmol) in a reaction volume of 1.8 ml. Samples (0.2 ml) of reaction mixtures were withdrawn at the indicated times for quantification of protein-bound [^{32}P]P_i [20]. At the end of the reaction, 0.2 ml of SDS gel sample buffer was added to the remainder of each reaction mixture and boiled for SDS–PAGE (50 μl/lane) and autoradiography. Key to gel lanes: 1 = Coomassie Blue-stained gel of purified P47 (3.6 μg); 2–4 = autoradiograms of the phosphorylation reactions: protein kinase C alone (lane 2), P47 alone (lane 3), and both P47 and protein kinase C (lane 4).

acid containing 0.2% (w/v) tryptamine for 4 hr [23]. Cysteine was determined after performic acid oxidation as described by Hirs [24]. *N*-terminal sequence analysis was performed in an Applied Biosystems Model 470A gas-phase protein sequencer with methanolic–HCl conversion chemistry [25]. Phenylthiohydantoin-amino acids were identified by C18 reverse-phase HPLC [26, 27].

RESULTS

Purification of platelet protein kinase C and P47

Protein kinase C and P47 copurified through the early stages of purification and could be effectively separated by ion-exchange chromatography on a column of DEAE–Sephacel (Fig. 1). Selected column fractions were assayed for protein kinase C activity by incubation with histone III-S and for P47 by incubation with protein kinase C under standard phosphorylation conditions (see the Materials and Methods section). Controls were also carried out by incubating column fractions under identical conditions but without exogenous substrate or enzyme

Table 1. Amino acid composition of platelet P47

Amino acid	Mol residue/mol*
Lysine	34 (33)
Arginine	18 (20)
Histidine	6 (6)
Aspartic acid + asparagine	41 (38)
Threonine	15 (16)
Serine	25 (22)
Glutamic acid + glutamine	43 (40)
Proline	17 (14)
Glycine	27 (24)
Alanine	21 (20)
Cysteine	6 (7)
Valine	17 (17)
Methionine	6 (8)
Isoleucine	19 (22)
Leucine	28 (27)
Tyrosine	10 (10)
Phenylalanine	23 (20)
Tryptophan	3 (6)

* Determined using M_r = 40,087 derived from the deduced amino acid sequence [14]. Values in parentheses are taken from the published sequence of P47 [14].

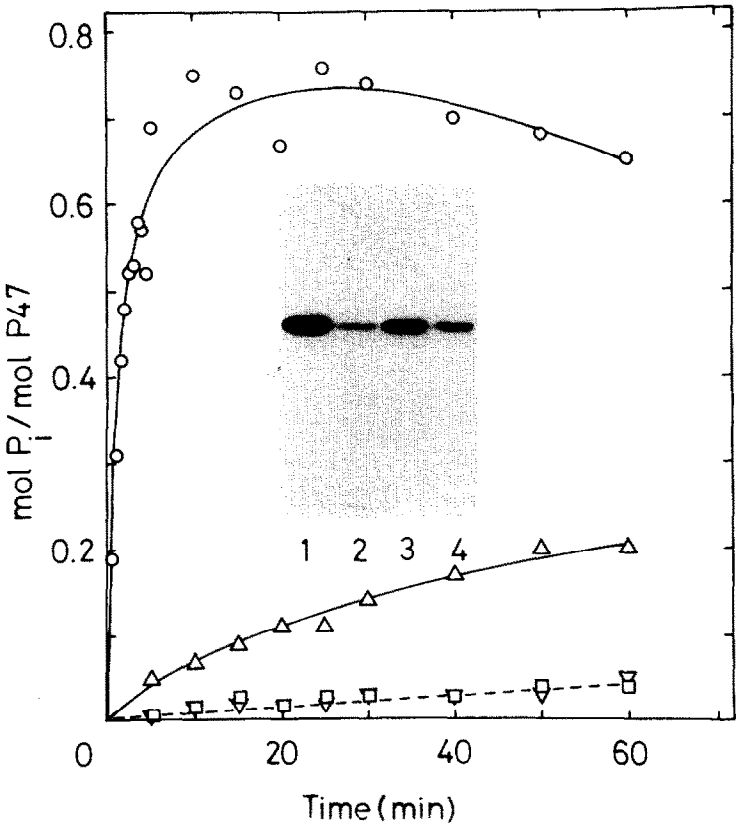

Fig. 3. Calcium- and phospholipid-dependence of platelet P47 phosphorylation by platelet protein kinase C. P47 (0.2 mg/ml) was incubated at 30° with protein kinase C (40 μl/ml) in 20 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 0.5 mM $CaCl_2$ (○, □) or 1 mM EGTA (△, ▽) and 10 μM [γ-^{32}P]ATP (187 cpm/pmol) in the presence (○, △) and absence (□, ▽) of 40 μg/ml phosphatidylserine and 0.8 μg/ml, 1,3-diolein. Samples (0.2 ml) of reaction mixtures were withdrawn at the indicated times for quantification of protein-bound [^{32}P]P_i [20]. At the end of the reaction, 0.2 ml of SDS gel sample buffer was added to the remainder of each reaction mixture and boiled for SDS–PAGE (50 μl/lane) and autoradiography. Gel lanes correspond to the following conditions: 1 = ○; 2 = □; 3 = △; 4 = ▽.

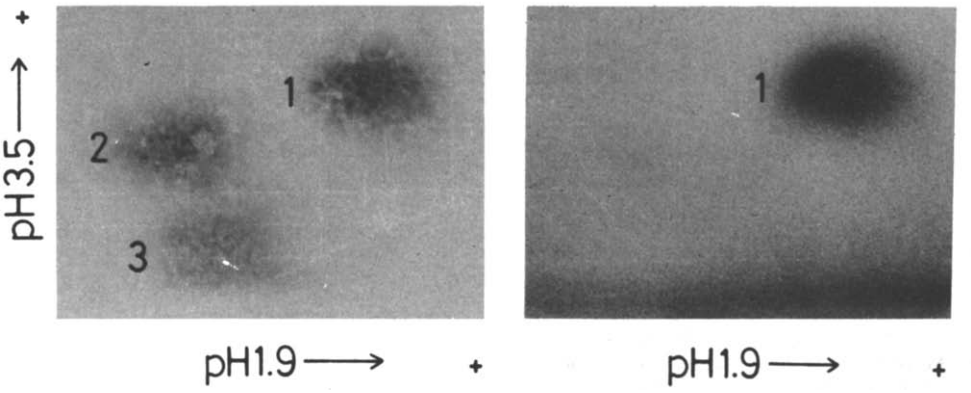

Fig. 4. Phosphoamino acid analysis of P47 phosphorylated by protein kinase C. P47 was maximally phosphorylated by protein kinase C as described in the Materials and Methods section and hydrolyzed to its constituent (phospho)amino acids which were separated by two-dimensional thin-layer electrophoresis. Phosphoamino acid standards (1 = phosphoserine, 2 = phosphothreonine, 3 = phosphotyrosine) added to the hydrolyzate were visualized by ninhydrin staining (left-hand panel) and radiolabeled phosphoamino acids identified by autoradiography (right-hand panel).

addition. SDS–PAGE analysis of column fractions confirmed that the protein kinase C substrate was indeed P47 (results not shown). Protein kinase C was used without further purification. It was found to have a molecular weight (80,000 daltons) identical to that of rat brain protein kinase C in immunoblotting experiments using a monoclonal antibody to the bovine brain enzyme (data not shown). P47 was further purified by hydroxylapatite column chromatography. The final product (Fig. 2, lane 1) was >92% pure as judged by laser densitometric scanning of a Coomassie Blue-stained SDS–polyacrylamide gel of the preparation and exhibited a molecular weight of 44,200 dalts by SDS–PAGE.

The data in Fig. 2 indicate that the protein kinase C preparation is devoid of P47 or any other substrate (△---△, lane 2) and that the P47 preparation is contaminated with a trace of protein kinase C (□——□, lane 3). Specific phosphorylation of P47 was achieved when the enzyme and substrate were combined (○——○, lane 4).

The identity of P47 was established by comparison

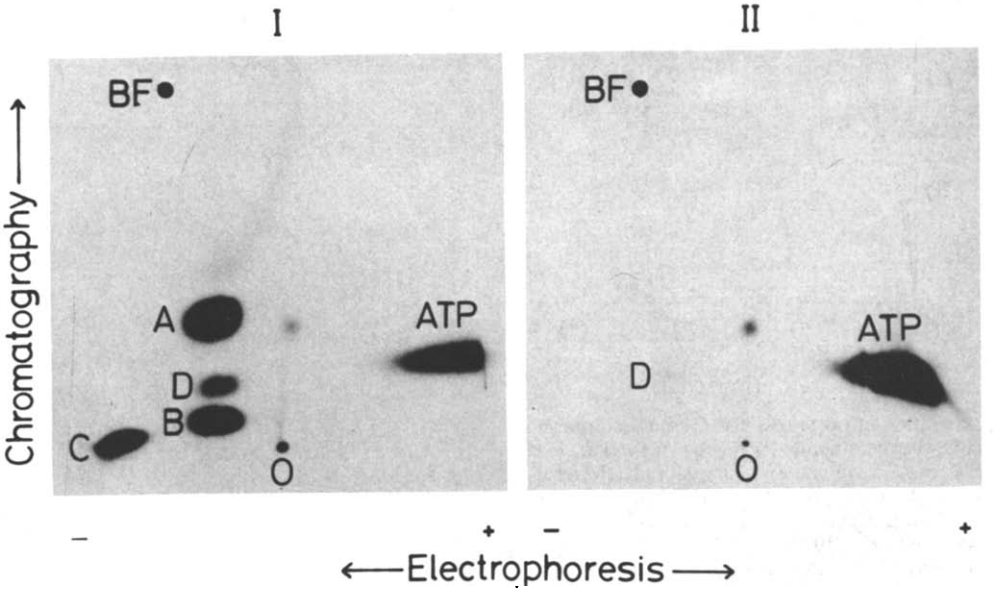

Fig. 5. Phosphopeptide mapping of P47 phosphorylated with protein kinase C. P47 was maximally phosphorylated by protein kinase C as described in the Materials and Methods section and completely digested with trypsin. Peptides were separated by thin-layer electrophoresis and ascending chromatography and phosphopeptides were identified by autoradiography. I = P47 phosphorylated in the presence of Ca^{2+}, phospholipid and diacylglycerol (0.75 mol P_i/mol); II = P47 phosphorylated in the absence of cofactors (0.0 mol P_i/mol). ATP was identified by two-dimensional electrophoresis/chromatography of radiolabeled ATP alone. Phosphopeptides are denoted A–D. O = origin; BF = basic fuchsin.

of the amino acid composition of the purified protein with that calculated from the published amino acid sequence deduced from the cDNA sequence [14] (Table 1). The minor differences observed are due to expected experimental error in the amino acid analysis (±5%) and minor contamination of the P47 preparation by other proteins (<8%). Amino acid sequencing indicated that the *N*-terminus of the isolated protein was blocked. P47 is known to have a blocked *N*-terminus [14].

Characterization of P47 phosphorylation

The Ca^{2+}- and phospholipid-dependence of P47 phosphorylation by protein kinase C is demonstrated in Fig. 3. Rapid and stoichiometric phosphorylation of P47 was observed in the presence of Ca^{2+}, phospholipid and diacylglycerol (○——○, lane 1). Elimination of phospholipid and diacylglycerol in the presence (□---□, lane 2) or in the absence (▽--▽, lane 4) of Ca^{2+} resulted in a very low rate of P47 phosphorylation. A significant rate of P47 phosphorylation was observed in the presence of phospholipid and diacylglycerol but absence of Ca^{2+} (△——△, lane 3). This may reflect the isoenzyme composition of the protein kinase C preparation: the type I isoenzyme is relatively less Ca^{2+}-dependent than types II and III [28].

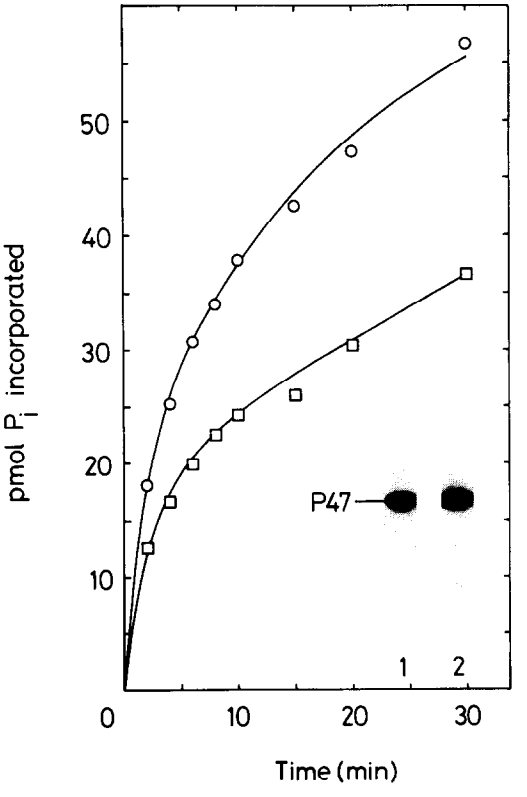

Fig. 6. Activation of protein kinase C-catalyzed phosphorylation of P47 by felodipine. P47 (72 μg/ml) was incubated at 30° in a reaction volume of 1.8 ml with platelet protein kinase C (40 μl/ml) in 20 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 40 μg/ml phosphatidylserine, 0.8 μg/ml 1,3-diolein, 10 μM [γ-^{32}P]ATP (110 cpm/pmol) in the presence (○) and absence (□) of 55.6 μM felodipine. Samples (0.2 ml) of reaction mixtures were withdrawn at the indicated times for quantification of protein-bound [^{32}P]P_i [20]. At the end of the reaction, 0.2 ml of SDS gel sample buffer was added to the remainder of each reaction mixture and boiled for SDS–PAGE and autoradiography (50 μl/lane). The autoradiogram is shown in the inset (1 = without felodipine; 2 = with felodipine). Values represent the means of triplicate determinations with standard deviations <2%.

P47 was maximally phosphorylated to the extent of 0.75 mol P_i/mol. All this phosphate was incorporated into serine residues as shown by phosphoamino acid analysis (Fig. 4). Tyers *et al.* [14] have identified 4 serine residues (40, 43, 113 and 117) as potential sites of phosphorylation by protein kinase C based on the known substrate specificity of the enzyme. Two-dimensional phosphopeptide mapping revealed that phosphate incorporation occurred at three major and one minor sites (Fig. 5): in the presence of Ca^{2+}, phospholipid and diacylglycerol phosphopepetides A–D were identified; in the absence of cofactors, only the minor phosphopeptide D was detectable. These observations (Figs 3–5) suggest that P47 was partially phosphorylated in the isolated form. From the amino acid composition (table 1) there are 33 lysine and 20 arginine residues/mol of P47. Complete tryptic digestion would, therefore, generate a total of 54 peptides.

Effects of felodipine on P47 phosphorylation

Figure 6 shows that the rate and extent of P47 phosphorylation by protein kinase C is significantly enhanced by felodipine. Analysis of the reaction products by SDS–PAGE and autoradiography (Fig. 6, inset) confirmed the enhanced phosphorylation of P47 in the presence of felodipine and established the specificity of the phosphorylation for this protein. The dose–response curve in Fig. 7 shows that half-maximal activation of protein kinase C occurred

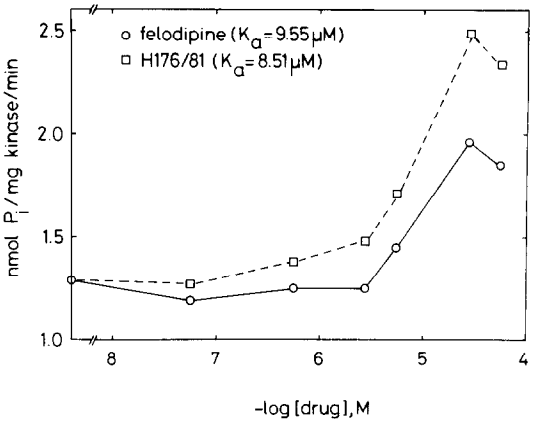

Fig. 7. Activation of protein kinase C-catalyzed phosphorylation of P47 by felodipine and the *p*-chloro analogue, H176/81. P47 phosphorylation was carried out as described in the legend to Fig. 6 in the presence of the indicated concentrations of felodipine (○——○) and the *p*-chloro analogue, H176/81 (□---□). Initial rates of phosphorylation were determined from the linear part of the phosphorylation time-course assays and are plotted as a function of drug concentration. Values represent the means of triplicate determinations with standard deviations <3%.

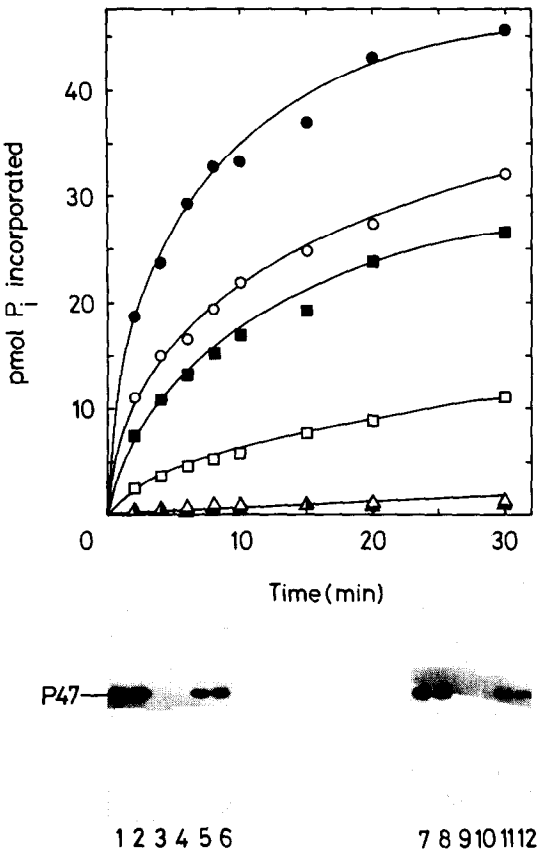

Fig. 8. Effect of felodipine on protein kinase C-catalyzed phosphorylation of P47 in the presence and absence of phospholipid and diacylglycerol. P47 phosphorylation was carried out in the presence of 0.1 mM $CaCl_2$ as described in the legend to Fig. 6 in the presence (●, ■, ▲) and absence (○, □, △) of 27.8 μM felodine under the following conditions: with phosphatidylserine and 1,3-diolein (○, ●), with phosphatidylserine, without 1,3-diolein (□, ■) or without either phosphatidylserine or 1,3-diolein (△, ▲). At the end of the reaction, 0.2 ml of SDS gel sample buffer was added to the remainder of each reaction mixture and boiled for SDS–PAGE (75 μl/lane) and autoradiography. The left-hand inset shows the autoradiogram in the presence of felodipine and on the right, in the absence of felodipine. Key to gel lanes: 1, 2 = ●; 3, 4 = ▲; 5, 6 = ■; 7, 8 = ○; 9, 10 = △; 11, 12 = □. Values represent the means of triplicate determinations with standard deviations <2%.

at ~9.5 μM felodipine. The *p*-chloro analogue of felodipine, H176/81, enhanced protein kinase C activity to a greater extent than did felodipine and half-maximal activation occurred at ~8.5 μM.

Finally, activation of protein kinase C-catalyzed phosphorylation by felodipine was dependent upon phospholipid but did not require diacylglycerol. As shown by the open symbols in Fig. 8, protein kinase C activity (○——○) was substantially reduced by the elimination of 1,3-diolein (□——□) and completely lost by the elimination of both phosphatidylserine and 1,3-diolein (△——△). Activation by felodipine was observed in the presence of both phosphatidylserine and 1,3-diolein (●——●) and phosphatidylserine alone (■——■) but not in the absence of both phosphatidylserine and 1,3-diolein (▲——▲). The quantitative data were confirmed by SDS–PAGE and autoradiography (Fig. 8, insets).

DISCUSSION

The primary site of action of felodipine appears to be the voltage-dependent Ca^{2+} channel of vascular smooth muscle [3] but intracellular sites of action have also been suggested [1]. For example, felodipine interacts with calmodulin in a Ca^{2+}-dependent manner [4, 5] and inhibits a variety of calmodulin-regulated enzymes (myosin light chain kinase, cyclic nucleotide phosphodiesterase and caldesmon kinase) [1, 6, 7]. Inhibition of these enzymes would favour the relaxed state of the muscle. Intracellular concentrations of felodipine comparable to concentrations required to inhibit these enzymes *in vitro* (~15 μM) are achieved (see Ref. 7 for discussion).

The Ca^{2+}- and phospholipid-dependent protein kinase (protein kinase C) may also play a role in modulating the contractile state of vascular smooth muscle through the phosphorylation of myosin [10] or myosin light chain kinase [11]. *In vitro* studies of these phosphorylations suggest that activation of protein kinase C would favour the relaxed state of the muscle. Therefore, we have investigated the effect of felodipine on the activity of this enzyme. Rather than using lysine-rich histone as the protein kinase C substrate in these studies, we have used the platelet P47 protein (pleckstrin) [14], which is known to be a specific and physiological substrate of this kinase. For this reason we also employed the platelet protein kinase C and conducted a rather complete characterization of the phosphorylation reaction prior to investigating the effects of felodipine.

We observed that felodipine enhanced both the rate and extent of protein kinase C-catalyzed phosphorylation of P47 with half-maximal activation at 9.5 μM, i.e. similar to the concentration required for inhibition of calmodulin-dependent enzymes [7]. Activation was also observed when the kinase was assayed in the absence of diacylglycerol, but not in the absence of phospholipid. An analogue of felodipine in which a chlorine atom is moved from an *ortho* to a *para* position, i.e. *p*-chloro felodipine, was approximately as potent as felodipine in activating protein kinase C (half-maximal activation at 8.5 μM). This analogue, however, is substantially less potent than felodipine in inhibiting contraction of the rat portal vein [7]. The vascular smooth muscle relaxant effects of felodipine may, therefore, be due primarily to its action on voltage-dependent calcium channels with secondary, and quantitatively less important, effects of inhibition of calmodulin-dependent enzymes [7] and activation of protein kinase C.

Acknowledgements—This work was supported by a grant from Hässle Pharmaceutical, Mölndal, Sweden. M.P.W. is a Medical Research Council of Canada Scientist and Alberta Heritage Foundation for Medical Research Scholar. The authors are very grateful to Marilyn Devlin for word-processing.

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