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Evidence against the regulation of caldesmon inhibitory activity by p42/p44^{erk} mitogen-activated protein kinase in vitro and demonstration of another caldesmon kinase in intact gizzard smooth muscle

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Abstract The effect of direct phosphorylation by recombinant p44^{erk1} mitogen-activated protein kinase on the inhibitory activity of caldesmon and its C-terminal fragment H1 was studied in vitro. Neither inhibition of actin-tropomyosin activated ATPase of heavy meromyosin by caldesmon or H1, nor inhibition of the actin-tropomyosin motility over heavy meromyosin by H1 was significantly affected by the phosphorylation while only a moderate effect on the actin-activated component of heavy meromyosin ATPase inhibition was observed. Phosphopeptide mapping of caldesmon immunoprecipitated from [^{32}P]PO_4-labelled intact gizzard strips revealed that it is predominantly phosphorylated at mitogen-activated protein kinase sites in unstimulated tissue and that it is stimulated for 1 h with phorbol 12,13-dibutyrate. We find that phorbol 12,13-dibutyrate also induces a transitory phosphorylation of caldesmon peaking at 15 min after addition and this phosphorylation is not attributed to mitogen-activated protein kinase, protein kinase C, Ca^{2+}/calmodulin-dependent kinase II or casein kinase II. We suggest that a yet unidentified kinase, rather than mitogen-activated protein kinase, may be involved in regulation of the caldesmon function in vivo.

Key words: Caldesmon; Phosphorylation; Mitogen-activated protein kinase; Chicken gizzard

1. Introduction

Caldesmon is an actin-associated regulatory component of smooth muscle which provides a negative control of actomyosin-based contractility [1,2]. In vitro, it potently inhibits actotropomyosin-activated ATPase of smooth and skeletal muscle myosin and actin filament movement over myosin in the in vitro motility assay [3–5]. Exogenous caldesmon inhibits force production and promotes relaxation of skinned smooth muscles [6–8], yet, its functionally active fragments produce similar effects on skinned fibers [9] and isolated smooth muscle cells [10,11]. The modulatory function of caldesmon has recently been supported by its antisense knockout in smooth muscle in situ [12].

Since caldesmon was found as a phosphoprotein in smooth muscle, the phosphorylation has been suggested to regulate its functional activity (reviewed in [13]). The level of caldesmon phosphorylation was found to alter upon tissue stimulation with various contractile agonists [14–16] and attempts have been made to identify the kinases involved in caldesmon phosphorylation in vivo. On the basis of sequence identity of phosphopeptides of caldesmon isolated from arterial tissue to the sites phosphorylated in purified protein by p42/p44^{erk} mitogen-activated protein (MAP) kinases (hereafter referred to simply as MAP kinase), MAP kinase was identified as in vivo caldesmon kinase responsive to phorbol 12,13-dibutyrate (PDBu) stimulation [17,18]. Other stimulants as well as membrane depolarization were also shown to result in MAP kinase activation [19] and caldesmon phosphorylation at corresponding sites [14]. These studies led to a verdict that MAP kinase is the predominant, if not sole, caldesmon kinase in smooth muscle.

Although apparently physiologically relevant, the direct consequences of caldesmon phosphorylation by MAP kinase are rather uncertain. In vitro, it had moderate, if any, effects on binding abilities of caldesmon [20], while the inhibitory activity of the bacterially expressed caldesmon fragment was reduced after mutation of the MAP kinase phosphorylation site to mimic phosphorylation [21]. However, phosphorylation of endogenous caldesmon in skinned smooth muscle by exogenous MAP kinase neither induced contraction nor affected the Ca^{2+}-sensitivity [22] and had marginal effects in airway smooth muscle [23]. Specific inhibition of intracellular MAP kinase by PD098059 almost to a basal level failed to alter the vascular contractility [24], yet, the phosphorylation of caldesmon was only slightly decreased [25].

In the present study, we re-addressed this issue by demonstrating that MAP kinase does not exert a large regulatory effect on the caldesmon function in vitro and is not the only caldesmon kinase in smooth muscle. In view of the results obtained, we propose that kinase(s) other than MAP kinase are rather involved in regulation of the caldesmon function in vivo.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP and [^{32}P]trisodium orthophosphate were from the Institute of Physics and Energetics (Obninsk, Russia). PDBu was purchased from Sigma and protein G-agarose from Calbiochem. BL21 DE3 *Escherichia coli* strains were obtained from Gibco BRL. Okadaic acid was from LC Laboratories, biological buffers and chemicals were from Sigma or BDH.

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Abbreviations: CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CK2, casein kinase II; GST, glutathione *S*-transferase; HMM, heavy meromyosin; MAP kinase, p42/p44^{erk} mitogen-activated protein kinase; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C

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2.2. Tissue labelling and caldesmon phosphorylation in situ

Muscle slices (6×4×0.6 mm) were cut across the long axis of the larger chicken gizzard body, so that fiber bundles were aligned longitudinally. The strips were incubated in three changes of phosphate-free minimal essential medium (Gibco) for 1.5 h at 37°C with continuous carbogen administration. Then, medium was replaced for 4 h with HBSS containing 20 mM HEPES, pH 7.4, and 0.125–0.25 mCi/ml of [^{32}P]Na_3PO_4. After addition of 2 μM PDBu, individual strips were removed at appropriate times, rinsed with ice-cold HBSS and frozen in liquid nitrogen. The tissue was pulverized while frozen and homogenized in ice-cold 20 mM MOPS, pH 7.0, 1% Triton X-100, 0.35 M NaCl, 25 mM $MgCl_2$, 2 mM EGTA, 1 mM dithiothreitol, 1 mM *o*-vanadate and a protease inhibitor cocktail. Caldesmon was immunoprecipitated from clarified extracts by 15–25 μg of anti-chicken gizzard caldesmon polyclonal antibody [26] absorbed to protein G-agarose and subjected to SDS-PAGE in duplicate on separate gels. Following autoradiography, the relative level of caldesmon phosphorylation was calculated as a ratio of values obtained by scanning autoradiograms and Coomassie-stained gels, respectively. Gel pieces containing ^{32}P-labelled caldesmon were excised and extensively digested with TPCK-treated trypsin (Worthington Biochem. Corp.) [27]. The digest was lyophilized, dissolved in thin layer electrophoresis buffer (acetic acid/formic acid/H_2O, 15:5:80) and run on SilicaGel 60 20×20 cm plates (Merck) at 1000 V for 75 min at 10°C in the first dimension, followed by ascending chromatography in *n*-butanol/pyridine/acetic acid/H_2O (150:150:40:160) in the second dimension. Phosphopeptides were visualized by autoradiography on a Kodak Biomax film.

2.3. MAP kinase expression and activation

p44^{erkl} MAP kinase cDNA cloned into the pGEX-3X vector was a gift from Dr T. Voyno-Yasenetskaya (Chicago University, IL, USA). The kinase was expressed in BL21 DE3 *E. coli* cells and purified using the glutathione *S*-transferase (GST) purification module (Pharmacia Biotech). To activate GST-MAP kinase in vitro, serum-deprived subconfluent COS-7 cells grown on DMEM (Gibco) were stimulated for 10 min with 50 nM epidermal growth factor and activated MAP kinase (MEK) was immunoprecipitated within 3 h by anti-MEK 1&2 antibody (Transduction Laboratories) bound to protein G-agarose. GST-MAP kinase was activated by phosphorylation with the MEK immunocomplex and stored at −20°C in 50% glycerol.

2.4. Proteins and phosphorylation in vitro

Chicken gizzard caldesmon [28] and sheep aorta tropomyosin [29] were isolated as described. C-terminal caldesmon fragment H1 was expressed in BL21 DE3 cells and purified as in [29]. Rabbit skeletal muscle actin was purified from acetone powder according to [30] and HMM was prepared as in [31]. Casein kinase II (CK2) was isolated from rabbit liver [32], Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) was obtained from New England Biolabs and protein kinase C (PKC)-β1/β2 was a gift from D.L. Silver and Dr J.R. Sellers (NHLBI, NIH, USA). Phosphorylation by these kinases was performed as described [32]. Phosphorylation of 0.65 mg/ml caldesmon or H1 by 0.03 mg/ml GST-MAP kinase was carried out at 30°C for 2 h in the presence of 0.2 mM ATP and terminated by heating to 60–70°C to inactivate the kinase. Usually, around 1 mol of phosphate was incorporated per mol protein as determined with [γ-^{32}P]ATP by filter counting [32]. Comparative phosphorylation of a panel of recombinant caldesmon fragments revealed that the phosphorylation sites of caldesmon are conserved within H1 (data not shown). Protein concentrations were determined by either using the following optical extinction coefficients, caldesmon, $A_{280}^{1\%} = 3.03$, G-actin, $A_{280}^{1\%} = 0.63$, or by the Lowry method as in [3,4].

2.5. In vitro motility and ATPase assay

Actin-tropomyosin motility over heavy meromyosin (HMM) was assayed as in [4] at 27°C in the buffer containing 40 mM KCl, 25 mM imidazol-HCl, pH 7.4, 1 mM ATP, 4 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol, 0.5% methylcellulose, 3 mg/ml glucose, 0.1 mg/ml glucose oxidase and 0.02 mg/ml catalase. The number of motile filaments and their velocities were calculated as outlined [33]. ATPase of 2 μM skeletal muscle HMM was assayed with 10 μM skeletal muscle actin with or without 2.3 μM sheep aorta tropomyosin at 37°C in 5 mM Pipes/KOH (pH 7.1), 5 mM $MgCl_2$, 50 mM KCl, 3 mM MgATP and 0.1 mM dithiothreitol as described [3].

3. Results

3.1. Phosphorylation by MAP kinase does not affect inhibition of the actomyosin interaction by caldesmon

Caldesmon and its fully functional bacterially expressed C-terminal fragment H1 showed an equipotent inhibition of actin-tropomyosin activated HMM ATPase (Fig. 1). Phosphorylation by recombinant MAP kinase (~1 mol Pi/mol) had no effect on their inhibitory activity at low caldesmon or H1 ratios to actin suggesting that tropomyosin-dependent inhibition is unaffected by the phosphorylation. At higher ratios of caldesmon or H1 to actin, a little de-inhibition brought by the phosphorylation was observed (Fig. 1). Consistently, a somewhat stronger effect of phosphorylation on caldesmon or H1 inhibition of actin-activated ATPase was obtained in the absence of tropomyosin (Fig. 1, insert), which supports earlier findings [20] that phosphorylation by MAP kinase slightly affects the actin-binding affinity of caldesmon.

Because H1 retains the inhibitory and actin-binding capacity of caldesmon [29], it was used to inhibit the sliding of actin-tropomyosin filaments over HMM in in vitro motility assay. Using an automatic tracking programme described earlier [33], only a small reduction (up to 20%) in the filament velocity upon H1 addition was detected while this inhibition was not observed with phosphorylated H1 (data not shown). In agreement with our previous data, H1 strongly decreased the number of motile filaments [4], while phosphorylation of H1 by MAP kinase partially reversed this switching off the motility (Fig. 2). However, phosphorylation produced almost no difference in inhibition at low concentrations of H1 (5 μM) while the increasing amounts of phosphorylated H1 produced little further inhibition in contrast to the unphosphorylated fragment which inhibited in a clear dose-dependent manner. We suggest that, as in the ATPase assay, phosphorylation by MAP kinase relieves inhibition mediated by a low affinity

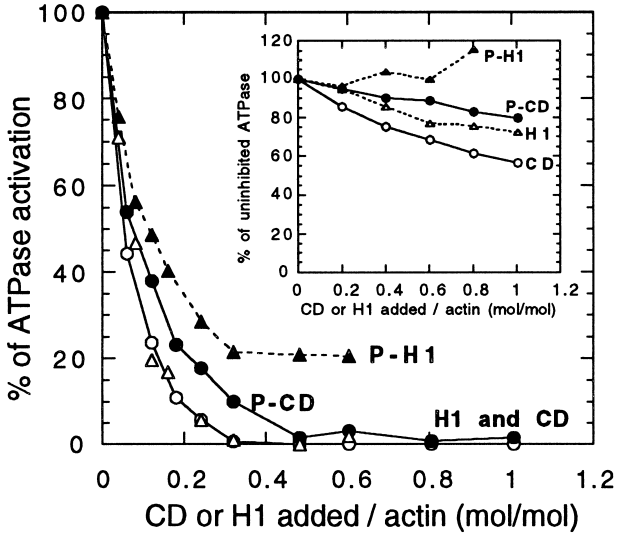

Fig. 1. The effect of caldesmon and H1 phosphorylation by MAP kinase on the inhibition of actin-tropomyosin or actin (the insert)-activated HMM ATPase. Inhibition by caldesmon (CD, open circles) and H1 (open triangles) was compared to that by phosphorylated caldesmon (P-CD, closed circles) or phosphorylated H1 (P-H1, closed triangles). The uninhibited acto-tropomyosin-HMM ATPase rate was 2.2 sec^{-1} and that for acto-HMM was 0.85 sec^{-1}.

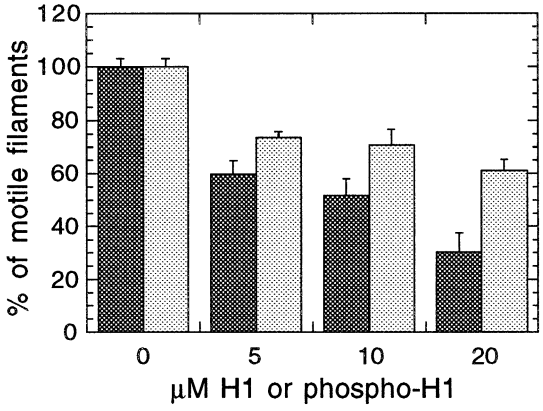

Fig. 2. Inhibition of the acto-tropomyosin filament motility by the C-terminal caldesmon fragment H1 (filled bars) or H1 phosphorylated by MAP kinase (dotted bars). Up to 800 filaments were automatically analysed in two experiments, each including 3–4 image processings. Bars indicate the S.D. values.

interaction of caldesmon or H1 with actin while the physiologically relevant, high affinity tropomyosin-dependent inhibition is rather unaffected.

3.2. *Caldesmon kinases stimulated by phorbol ester in intact gizzard smooth muscle*

To avoid non-specific stimulation of endogenous MAP kinase by mechanical stress [34], caldesmon phosphorylation was studied in unloaded strips of chicken gizzard metabolically labelled with [^{32}P]phosphate. Caldesmon was specifically immunoprecipitated from tissue extracts (Fig. 3A, left panel) by affinity-purified polyclonal antibody which equally recognizes both phosphorylated and unphosphorylated protein (data not shown). PDBu induced a rapid increase in caldesmon phosphorylation with the maximum at 10–25 min after addition. Then, the level of caldesmon phosphorylation declined and rose again after 60–90 min of continuous stimulation with PDBu (Fig. 3A, right panel). PDBu induced an about 2-fold maximum increase in caldesmon phosphorylation as assessed by scanning densitometry, in agreement with the data reported for other smooth muscles [14,16,23,35].

To investigate whether the biphasic nature of caldesmon phosphorylation is due to the distinct kinases involved, two dimensional phosphopeptide maps were generated from ^{32}P-labelled caldesmon immunoprecipitated from unstimulated

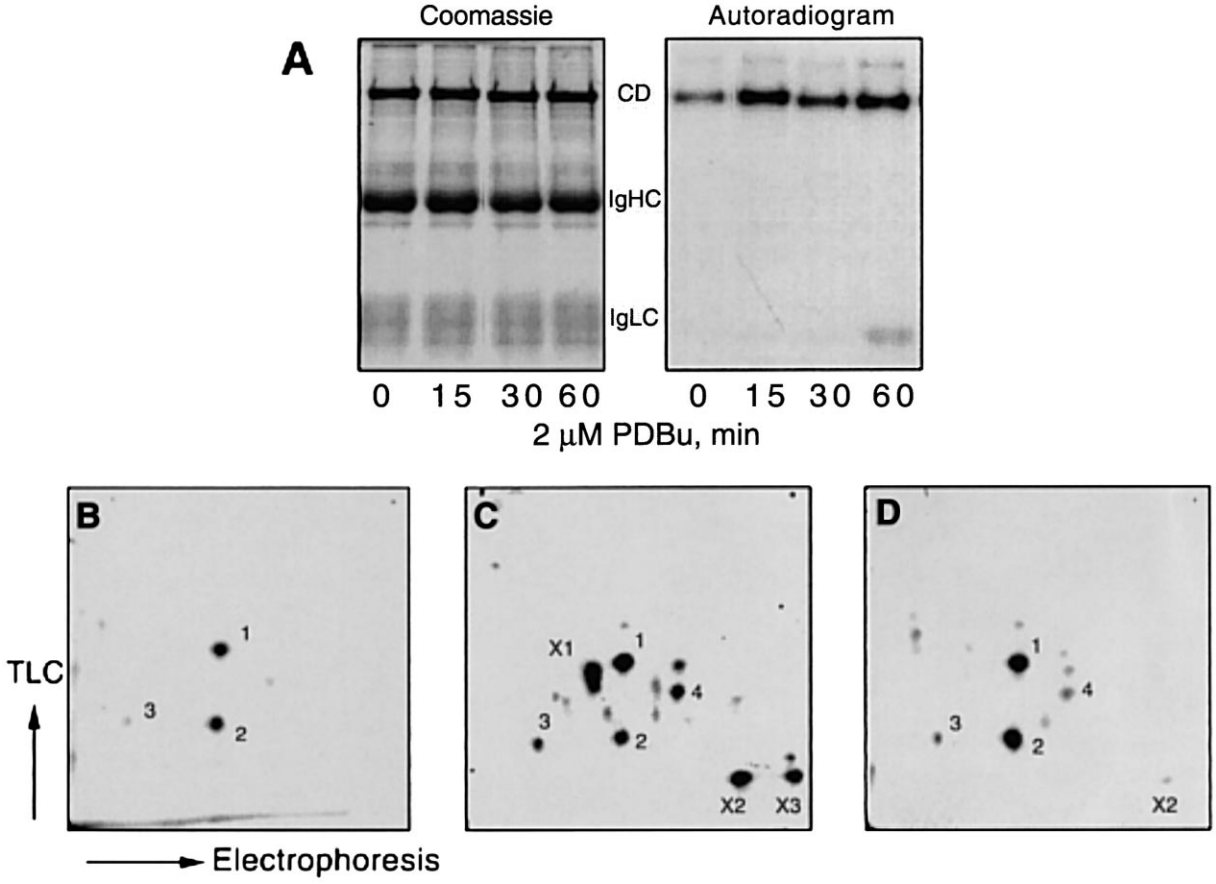

Fig. 3. Phosphorylation of caldesmon in PDBu-stimulated chicken gizzard strips. (A) Time course of caldesmon phosphorylation. Typical 10% SDS-PAGE of immunoprecipitates of caldesmon (CD) stained wtih Coomassie (left panel) and the corresponding autoradiogram (right panel) show a biphasic increase in caldesmon phosphorylation. IgHC and IgLC denote the immunoglobulin heavy and light chains, respectively. The appearance of an additional radioactive band at the bottom of the autoradiogram is due to caldesmon degradation. (B–D) Autoradiograms of two-dimensional tryptic phosphopeptide maps of caldesmon phosphorylated in situ in response to vehicle (B) or 2 μM PDBu stimulation for 15 min (C) and 60 min (D). The origin is at the left bottom corner.

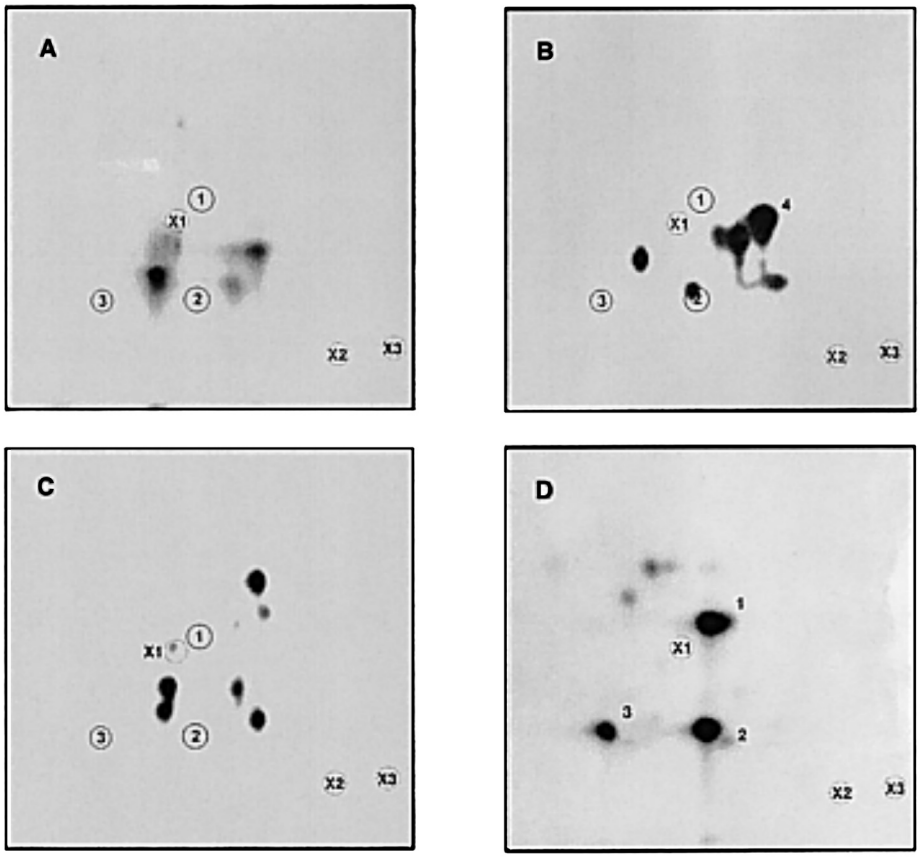

Fig. 4. Autoradiograms of two-dimensional phosphopeptide maps of caldesmon phosphorylated in vitro by CK2 (A), PKC-β (B), CaMKII (C) and MAP kinase (D). The stoichiometry of phosphorylation was 0.8, 1.2 and 2.4 mol of phosphate per mole of caldesmon, respectively. Numbers correspond to the positions of phosphopeptides shown in Fig. 3 and circles denote their positions as separately revealed by co-mapping (maps not shown).

gizzard and treated with 2 μM PDBu for 15 and 60 min (Fig. 3B–D, respectively). The major phosphopeptide spots, assigned as 1, 2 and 3, were characteristic for caldesmon from unstimulated muscle (Fig. 3B) and their intensity increased with the duration of PDBu treatment. In contrast, the intensities of the spots 4 and X1–X3 increased transiently showing a maximum at 15 min of PDBu stimulation and reaching 50% of the total phosphate associated with caldesmon at this time point. This suggests that phosphorylation of peptides 4 and X1–X3 is likely to account for the early response to PDBu observed in Fig. 3A.

To identify the in vivo caldesmon kinases, comparative phosphopeptide mapping analysis was performed for caldesmon immunoprecipitated from PDBu-stimulated gizzard and chicken gizzard caldesmon phosphorylated in vitro by CK2, PKCβ1/β2, CaMKII and MAP kinase (Fig. 4A–D, respectively). By co-mapping resulting phosphopeptides (data not shown), the phosphopeptides 1–3 of caldesmon were identified as containing MAP kinase phosphorylation sites. An alternative trypsin digestion and more than one phosphorylation site for MAP kinase in chicken gizzard caldesmon [20] may account for multiple generated phosphopeptides, similarly to that observed for platelet caldesmon [27]. Several radioactive spots were also detected for each other kinase tested, but none of them corresponded to phosphopeptides X1–X3 found on the 'in vivo' maps of caldesmon (Fig. 4). The phosphopeptide 4 contained the site phosphorylated by PKC (Fig. 4B), but the fraction of phosphate associated with this site in vivo was relatively small (Fig. 3).

4. Discussion

PDBu and agonists like endothelin, histamine, etc. have been found to induce a sustained contraction in vascular smooth muscle that is associated with considerably less or even no increase in the intracellular Ca^{2+} concentration compared to that elicited by KCl depolarization [36]. It has been suggested that such contractions are mediated by Ca^{2+}-hypersensitive or even Ca^{2+}-independent mechanisms which involve protein phosphorylation and caldesmon was implicated as one of the target components of the contractile machinery [19,35,37]. In a number of smooth muscles, the above-mentioned compounds induced an around 2-fold sustained increase in the level of caldesmon phosphorylation, similar to that obtained for gizzard tissue in this report. However, the time course of the phosphate incorporation into caldesmon has not been thoroughly investigated.

We demonstrated here that PDBu stimulates at least two caldesmon kinases in gizzard smooth muscle in a time-dependent manner (Fig. 3). The sustained phosphorylation is associated with modification of MAP kinase phosphorylation sites in caldesmon (Fig. 4), which confirms previously published data [14,16,17]. MAP kinase also appears to be the major caldesmon kinase both in unstimulated tissue and over-

stimulated (for 1 h) with PDBu (Fig. 3B, D). Hence, we searched for the consequences of this phosphorylation on the caldesmon functional activity and found that it fails to significantly reduce the capability of caldesmon to inhibit the physiologically relevant actin-tropomyosin-activated ATPase of the myosin subfragment (Fig. 1) and therefore weakly affects the inhibition of the actin filament motility by caldesmon in an in vitro motility assay (Fig. 2). Thus, a direct biochemical support is now provided for the growing physiological evidence that caldesmon phosphorylation by MAP kinase may not significantly alter the smooth muscle contractility [22,24].

For the first time, we demonstrate here that a kinase additional to MAP kinase is activated by PDBu and phosphorylates caldesmon in vivo (Fig. 3C). The phosphorylated sites are apparently under a strong phosphatase control which results in their dephosphorylation despite the continuous stimulation of tissue with PDBu. This yet unidentified kinase does not correspond to other well-described enzymes which can phosphorylate smooth muscle caldesmon in vitro, i.e. PKC, CaMKII and CK2 (Fig. 4). It neither may be a $p34^{cdc2}$ kinase, because it is unlikely to be active in highly differentiated smooth muscle. PKC was also found to phosphorylate caldesmon as it does in non-muscle cells [27], but it has minor impact in phosphorylation of caldesmon in gizzard (Fig. 3). It is possible, but seems unlikely, that other PDBu responsive forms, i.e. 'novel' PDCε, may phosphorylate other sites in caldesmon than the conventional PKCβ1/β2 isoform. p21-Activated kinase has recently been found to phosphorylate caldesmon in vitro and in skinned smooth muscle preparations [37]. Whether it accounts for the transitory phosphorylation of caldesmon in gizzard and can be stimulated by PDBu in vivo is currently under investigation.

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