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Phosphorylation of vascular smooth muscle caldesmon by endogenous kinase

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Caldesmon was phosphorylated up to 1.2 molP_i/mol using a partially purified endogenous kinase fraction. The phosphorylation site was within the C-terminal 99 amino acids. We were also able to phosphorylate caldesmon incorporated into native and synthetic smooth muscle thin filaments. Phosphorylation did not alter caldesmon binding to actin or inhibition of actomyosin ATPase. It also did not change Ca^{2+} sensitivity in native thin filaments. Phosphorylated caldesmon bound to myosin less than unphosphorylated caldesmon, especially when the myosin was also not phosphorylated. This work did not support the hypothesis that caldesmon function is modulated by phosphorylation.

Caldesmon; Phosphorylation; Smooth muscle; Regulation; Actin; Myosin

1. INTRODUCTION

Caldesmon is located in the thin filaments of smooth muscle and since it binds to actin, calmodulin, tropomyosin and myosin, and is a potent inhibitor of actin activation of myosin MgATPase, it has been considered as a candidate regulatory protein [1,2]. The inhibitory function is known to be regulated by Ca^{2+} and a calcium binding protein such as calmodulin.

Smooth muscle actomyosin ATPase is also regulated by Ca^{2+}-dependent phosphorylation of the regulatory chain in myosin, and in vivo the tension development correlates reasonably well with the level of myosin phosphorylation [3]. On the other hand, many smooth muscles maintain high tension even after [Ca^{2+}] has dropped, the myosin is dephosphorylated, and there is little or no contractile activity [3]. Caldesmon crosslinks thin and thick filaments [2,4] and this property may account for the prolonged tension in smooth muscle, independent of myosin phosphorylation.

Smooth muscle caldesmon can be phosphorylated in vivo, and the extent of phosphorylation has been shown to increase upon certain stimulations [5–7]. In vivo phosphorylation of non-muscle caldesmon has also been reported, and it happens in connection with the cell cycle [8–9]. It is possible that one or more of caldesmon's functional properties may be modulated by phosphorylation.

There are a number of reports that caldesmon phosphorylation in vitro by exogenous pure kinase is associated with reduction in binding to actin or myosin. The site of phosphorylation as well as the properties of the phosphorylated caldesmon depend on the kinase used [9–15]. Ca^{2+}-calmodulin-dependent protein kinase II, protein kinase C, $p34^{cdc2}$ kinase and casein kinase II have been tested, and only $p34^{cdc2}$ kinase was proved to phosphorylate caldesmon at the in vivo sites [6], suggesting that none of the other kinases can be the 'caldesmon kinase' which phosphorylates caldesmon in intact tissue [5].

In this study, a crude mixture containing endogenous 'caldesmon kinase' activity has been separated from sheep aorta. We used this preparation to phosphorylate caldesmon, and native and synthetic thin filaments in vitro. The sites of phosphorylation have been located and the effect of phosphorylation has been investigated.

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2. MATERIALS AND METHODS

2.1. *Protein preparations*

Caldesmon was prepared from sheep aorta or chicken gizzard as in [4] except that the crude caldesmon was dephosphorylated by incubation with alkali and acid phosphatases (5 μl of each per 5–10 ml caldesmon) for at least 15 h before chromatography on Q Sepharose. Chicken gizzard caldesmon fragments expressed in *E. coli* were generously provided by C. Redwood and Dr. P. Huber. Sheep aorta thin filaments, F-actin and tropomyosin were prepared as previously described [16].

Skeletal muscle myosin was extracted from rabbit muscles as in [17]. Sheep aorta myosin was prepared and thiophosphorylated as in our previous work [18].

2.2. *Endogenous kinase*

Contractile proteins were extracted in ATP/EGTA/0.8 M KCl buffer from a washed homogenate of sheep aorta as in [25], and pelleted by high speed centrifugation in 20% glycol. The supernatant was dialysed against 10 mM HEPES, pH 7.3, 5 mM $MgCl_2$ and 2.5

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mM DTT (buffer A) for 2–3 h to reduce the glycol content before $(NH_4)_2SO_4$ fractionation. The proteins precipitating between 60 and 80% $(NH_4)_2SO_4$ saturation were collected by centrifugation, and dissolved in buffer A. After dialysis in buffer A, the solution was clarified by centrifugation, and the solution was concentrated by dialysing it against 20% polyethylene glycol 10,000 in 25 mM HEPES, pH 7.3, 100 mM KCl, 5 mM $MgCl_2$ and 2.5 mM DTT (buffer B). Finally, the crude kinase was dialysed against buffer B. Protease inhibitors (2 μg/ml leupeptin, pepstatin and chymostatin) were included in all protein solutions during the preparation. The final product from 100 g aorta was about 1–2 ml crude kinase (3–4 mg/ml protein).

2.3. *Phosphorylation*

Purified dephosphorylated caldesmon (0.8–2.5 mg/ml, 10–30 μM), thin filaments (6–10 mg/ml containing 3–4 μM caldesmon) or synthetic thin filaments (4 μM caldesmon, 62 μM actin and 10 μM tropomyosin) were incubated with the kinase (40–50 μl kinase in 250 μl) at 25°C in buffer B (see kinase preparation) with 5 nM okadaic acid, 1 mM EGTA or 1 mM $CaCl_2$. The reaction was started by adding Mg-[γ-^{32}P]ATP (specific radioactivity 2–3×10^7 cpm/nmol) to a final concentration of 10 mM. A control reaction was also set up without any added caldesmon. The radioactivity of aliquots (20–30 μl) was determined by spotting them onto 2 × 2 cm phosphocellulose paper (Whatman P81), and the reaction was terminated by pipetting 100 μl silicotungstic acid/TCA onto the paper. The filter papers were washed extensively with 30% acetic acid. The radioactivity was measured by Cerenkov radiation.

When larger amounts of phosphorylated caldesmon were required for functional studies, the phosphorylation reaction was stopped by quick heating, and after sedimenting the denatured proteins, caldesmon was purified by chromatography as described earlier. The phosphorylation of thin filaments was terminated by diluting the reaction with thin filament extraction buffer (see in ref. [21]), and glycol (20%). The filaments were collected by high speed centrifugation. There was no loss of caldesmon when thin filaments were phosphorylated and repelleted and the Ca^{2+}-regulation was also retained.

2.4. *Other methods*

Protein concentrations were determined by the Lowry method. Molecular masses used for calculation of the protein concentrations are: myosin, 450,000; HMM, 340,000; caldesmon, 87,000; tropomyosin, 70,000; actin, 42,000; thin filaments, 68,000 per actin.

3. RESULTS

3.1. *Endogenous caldesmon phosphorylating activity*

Caldesmon phosphorylating activity was found in the ATP extract of sheep aorta. After pelleting thin filaments in the presence of 20% glycol, the phosphorylating activity remained in the supernatant. This fraction also contained a high level of phosphatase activity which was only partially inhibited by okadaic acid. A 60–80% saturated ammonium sulphate precipitate showed an increased 'caldesmon kinase' activity with a much reduced phosphatase activity. This fraction was used for subsequent experiments.

Fig. 1a shows SDS gel electrophoresis of the 'caldesmon kinase' fraction and the other proteins used in the experiments. The major component of the kinase fraction is tropomyosin and the kinase itself is probably only a small fraction of total protein.

In the presence of 1 mM EGTA, 1–2 mol phosphate were incorporated per mol of caldesmon (Fig. 2), the stoichiometry of incorporation was the same for sheep aorta and chicken gizzard caldesmon. In the presence of 1 mM Ca^{2+} the level of phosphorylation was reduced, which is probably caused by the activation of phosphatases.

Caldesmon phosphorylation did not seem to be altered by its incorporation into native or synthetic thin filaments, although other proteins, particularly actin

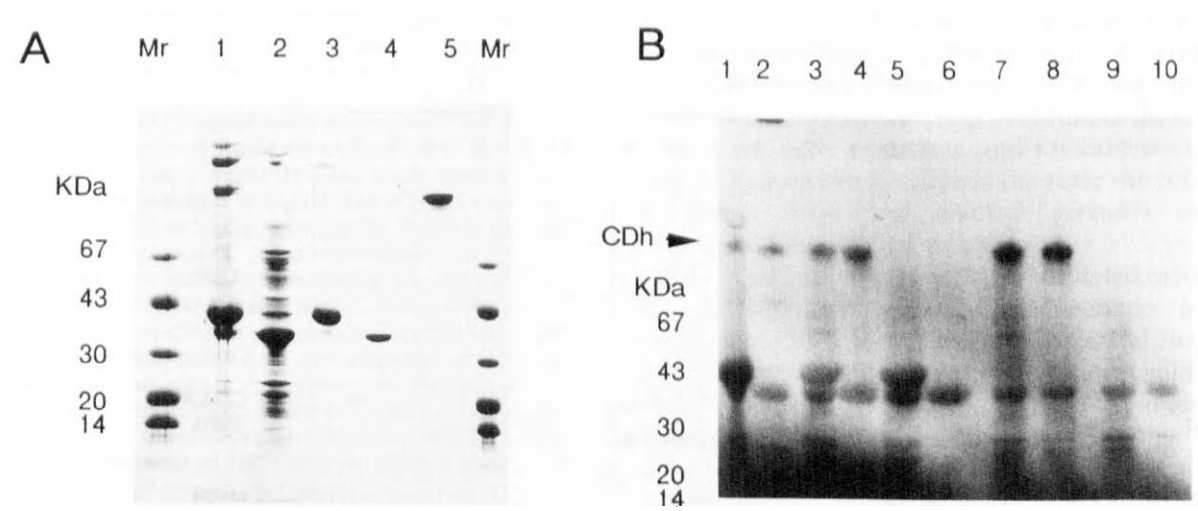

Fig. 1. The 'caldesmon kinase' and caldesmon phosphorylation. A: SDS gel electrophoresis stained with PAGE Blue 83 (BDH). Lane 1, thin filaments; lane 2, 'caldesmon kinase'; lane 3, actin; lane 4, tropomyosin; lane 5, caldesmon; Mr, molecular mass markers. All proteins are from sheep arteries. B: Autoradiography of SDS gel electrophoresis of phosphorylated proteins. Lane 1,2, thin filaments; lane 3,4, synthetic thin filaments (1:15:5 mol caldesmon/actin/tropomyosin); lane 5,6, control: actin + tropomyosin; lane 7,8, purified caldesmon; lane 9,10, control: contains only 'caldesmon kinase'. The position of the caldesmon band (CDh) and of the molecular weight markers is given on the left. Proteins were incubated for 60 min with caldesmon kinase and 10 mM Mg[γ-^{32}P]ATP. Reaction mixture was applied onto the gel directly (lanes with odd numbers) or the aliquots were heated at 95°C for 1–2 min and the denatured proteins were removed by centrifugation before mixing them with SDS sample buffer (lanes with even numbers). Caldesmon, tropomyosin and calmodulin are heat stable proteins.

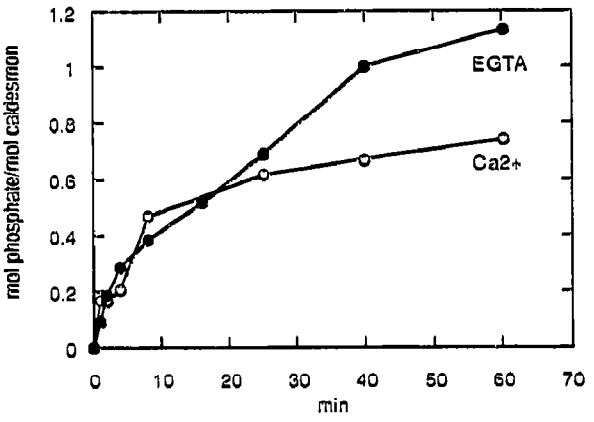

Fig. 2. The time course of the phosphorylation of sheep aorta caldesmon; (●) in the presence of 1 mM EGTA; (○) in the presence of 1 mM Ca^{2+}. The radioactivity incorporated into caldesmon was corrected by deducting the counts of the control reaction ('caldesmon kinase' only; see Fig. 1b).

and tropomyosin, also become phosphorylated (Fig. 1b).

3.2. *Location of phosphorylation sites*

In order to locate the phosphorylation sites, ^{32}P-phosphorylated chicken gizzard caldesmon was treated with 2-nitro-5-thiocyanobenzoic acid, which cleaves at cysteines 153 and 580 [2,16]. The radioactivity was confined to intact caldesmon and a 20K fragment, which could be either the N-terminal 1–153 or the C-terminal 580–756 fragment.

We have also made use of a series of caldesmon fragments expressed in *E. coli* in our laboratory. Three chicken gizzard fragments: N128 (amino acids 1–128), 'central helix' (amino acids 230–417) and 658C (amino acids 658–756 C terminus), and a human C-terminal fragment, CD33K (equivalent to amino acids 476–738 of gizzard) were incubated with 'caldesmon kinase'. Only 658C and CD33K were phosphorylated (Fig. 3), thus the phosphorylation site is in the C-terminal part of the molecule between amino acids 658 and 738.

3.3. *Functional effects of caldesmon phosphorylation*

Phosphorylated caldesmon inhibited actin-tropomyosin activation of myosin MgATPase and bound to actin. We did not observe any significant difference between phosphorylated and unphosphorylated caldesmon (Fig. 4). Even at 150 mM KCl, where caldesmon is partly dissociated from actin (so that changes in K_b would be readily detectable), no differences were observed.

The myosin binding ability of phosphorylated and unphosphorylated caldesmon was also quite similar, although there was more binding when both caldesmon and myosin were unphosphorylated (Fig. 5). This small effect was consistently observed in four separate preparations of caldesmon.

When native thin filaments were incubated with 'caldesmon kinase', several proteins, including caldesmon, were phosphorylated (Fig. 1b). We found no differences in activation of myosin MgATPase, Ca^{2+}-sensitivity, $[Ca^{2+}]$ for half maximal activation or caldesmon dissociation from phosphorylated or unphosphorylated thin filaments.

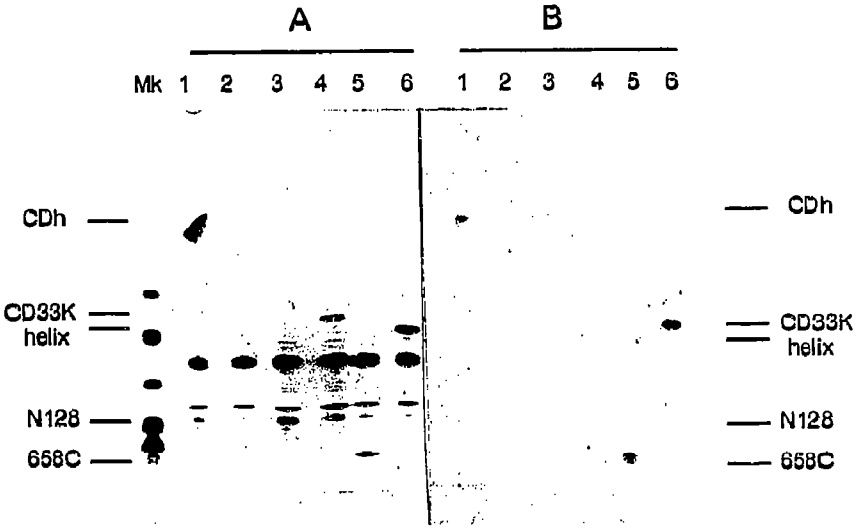

Fig. 3. Phosphorylation of caldesmon fragments. A: SDS gel electrophoresis stained with PAGE blue 83; B: autoradiography of the same gel. The proteins were phosphorylated for 60 min, then precipitated by 10% TCA and the pellet was dissolved in SDS sample buffer. Lane 1, chicken gizzard caldesmon; lane 2, phosphorylation control (the kinase alone); lane 3, N128 fragment; lane 4, 'central helix'; lane 5, 658C fragment; lane 6, CD33K fragment. M_k, molecular mass markers. The positions of the caldesmon fragments are indicated.

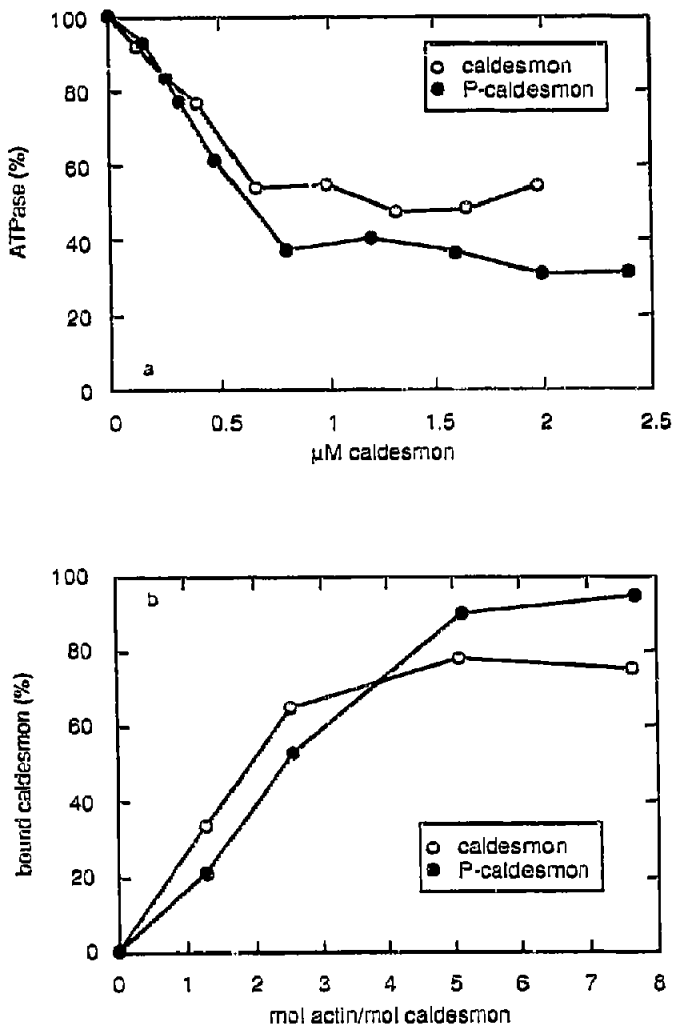

Fig. 4. The effect of caldesmon phosphorylation on caldesmon-actin interaction. A: Inhibition of actomyosin ATPase. (○) unphosphorylated caldesmon; (●) phosphorylated caldesmon. Mg-ATPase activity was measured at 25°C with 0.125 mg/ml skeletal muscle myosin, 0.5 mg/ml aorta actin, 0.125 mg/ml aorta tropomyosin and 0–0.13 mg/ml (0–1.5 μM) caldesmon in 5 mM PIPES, pH 7.1, 60 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM DTT and 2 mM MgATP. The released phosphate was measured as in [21]. The ATPase activity is expressed as percentage of the uninhibited activity. B: Actin binding. (○) unphosphorylated caldesmon; (●) phosphorylated caldesmon. Caldesmon (4–5 μM) was mixed with increasing amounts of actin (0–40 μM) in 5 mM PIPES, pH 7.1, 60 mM KCl, 2.5 mM $MgCl_2$ and 5 mM DTT. Incubation was 20 min at room temperature. Actin with the bound caldesmon was pelleted by centrifugation at 25°C at 150,000 × g for 20 min. Aliquots of the supernatants were mixed with SDS sample buffer and separated on 4–30% acrylamide gradient gels. The caldesmon bands on the gels were scanned by an LKB Ultrascan densitometer equipped with an HP 3390A recording integrator.

4. DISCUSSION

Our study specifically addressed the phosphorylation of caldesmon from vascular smooth muscle by an endogenous kinase since studies of caldesmon phosphorylation in vivo indicate that none of the pure kinases previously studied [11–15] phosphorylate isolated caldesmon at the sites phosphorylated in vivo [5,6,9].

We have found caldesmon phosphorylating activity in the ATP extract of sheep aorta (Figs. 1,2) which does not cosediment with the thin filaments as we demonstrated previously [18]. Using a partly purified kinase preparation we found that 1–2 phosphates were incorporated per caldesmon (Fig. 2) in the C-terminal part of the molecule between amino acids 658 and 756 (numbering according to Bryan et al. [27] (Fig. 3). This is in the same region as the site phosphorylated in vivo [6]; thus it seems likely that the 'caldesmon kinase' activity isolated from sheep arteries is the kinase which is active in vivo.

We are able to phosphorylate caldesmon when incorporated into native or synthetic thin filaments (Fig. 1b) even though the phosphorylation site is within the same C-terminal domain that binds to actin and inhibits actin activation [2,19]. Phosphorylation did not appear to affect the contact surface between caldesmon and actin, since binding and inhibition were unaltered by phosphorylation (Fig. 4). These results are not consistent with previous reports that phosphorylation of caldesmon near the C terminus by protein kinase C [13] or by $P34^{cdc2}$ kinase [9,10] reduced actin binding. $P34^{cdc2}$ kinase phosphorylates caldesmon in the same region as our endogenous 'caldesmon kinase' [6,10]; however, phosphorylation involving purified kinases occured at several sites, whereas phosphorylation by the endogenous 'caldesmon kinase' was only at a ratio of 1–2 mol phosphate per mol caldesmon (Fig. 2 [10]).

The effect of caldesmon phosphorylation on myosin binding, though small (Fig. 5) was rather unexpected since it is well documented that the S-2 portion of myosin binds to the N-terminal domain (amino acids 1–128) of caldesmon rather than the C-terminal one [12,21]. However, recent work has shown the presence of additional myosin binding potential in the C-terminal 288 amino acids of human and rat caldesmon [23,24], and indirect evidence suggests the same site is functional in sheep aorta and chicken gizzards caldesmon (Marston, unpublished result).

The stoichiometry of unphosphorylated myosin binding to unphosphorylated caldesmon in low ionic strength buffer was 10–20 CD/myosin and the effect of caldesmon and myosin phosphorylation could be to reduce this stoichiometry to around 4–5 rather than to weaken binding (Fig. 5). The origin of this high and variable stoichiometry is not known, but it could be a consequence of caldesmon self-aggregation, a process which may involve the C-terminal part of molecule [4,25].

In general, phosphorylation of caldesmon by the endogenous kinase did not change its properties very much. It has been found that those agonists which produced a slow sustained contraction of vascular smooth muscle, often without significant myosin light chain

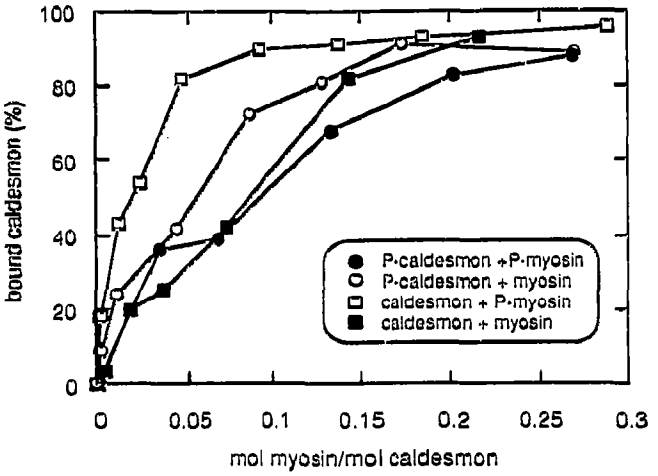

Fig. 5. The effect of caldesmon phosphorylation on the myosin binding. Binding conditions: 5 mM PIPES, pH 7.1, 5 mM $MgCl_2$, 0.3 mg/ml BSA, 3 mM Mg-ATP, 5 mM DTT and 0.1 mM $CaCl_2$ or 1 mM EGTA. Caldesmon was 4–5 μM, and the myosin varied between 0–2 μM. The proteins were incubated at room temperature for 20 min. The myosin with the bound caldesmon was pelleted at 40,000 × g for 10 min. Aliquots of the supernatant were analysed as described in Fig. 4.

phosphorylation or elevation of $[Ca^{2+}]$, also stimulated caldesmon phosphorylation in vivo [5–7,26]. The amount of phosphate incorporated into caldesmon was in the range 0.2 mol/mol caldesmon in resting muscle to 0.6 following stimulation by phorbol dibutyrate [5], which is somewhat less than the levels we observed in vitro, and was associated with the phosphorylation of several other proteins such as desmin and a 28K protein [7,26]. Thus, if caldesmon phosphorylation has any role in regulating force maintenance in vascular smooth muscle, it is clearly not the sole determinant and current evidence does not provide a possible mechanism.

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