UNIQUE CALCIUM-DEPENDENT HYDROPHOBIC BINDING PROTEINS: POSSIBLE INDEPENDENT MEDIATORS OF INTRACELLULAR CALCIUM DISTINCT FROM CALMODULIN

PAMELA B. MOORE*, NAOMI KRAUS-FRIEDMANN AND JOHN R. DEDMAN
Departments of Physiology and Cell Biology, and Internal Medicine (Endocrinology), University of Texas Medical School at Houston, P.O. Box 20708, Houston, TX 77025, U.S.A.

SUMMARY
Calcium-dependent regulation of cellular processes is mediated by specific intracellular proteins. A newly described set of proteins isolated from chicken gizzard with $M_r$ of $67 \times 10^3$, $35 \times 10^3$, $33 \times 10^3$ and $30 \times 10^3$ also express a hydrophobic site in the presence of calcium. These proteins are isolated from several other cellular tissues and are termed calcimedins. These proteins differ from calmodulin in isoelectric point, DEAE-cellulose binding characteristics and heat stability. The calcimedins do not activate calmodulin-dependent cyclic nucleotide phosphodiesterase but do activate a hepatic microsomal Ca$^{2+}$-ATPase system. Hence, the possibility is opened that calcium regulation of cellular processes is mediated by calcium-binding proteins in addition to calmodulin.

INTRODUCTION
Calcium has long been recognized as a 'second messenger' regulating diverse cellular events (Means & Dedman, 1980). This regulation is mediated by specific intracellular calcium-binding proteins (CaBPs). The first such recognized calcium mediator was troponin C (TnC), the calcium-binding subunit of muscle troponin complex. TnC is now known to belong to a family of related calcium-binding proteins that bind two to four Ca$^{2+}$ with high affinity. TnC shares 45% direct amino-acid homology with another protein in this family, calmodulin (CaM). This homology increases significantly within the Ca$^{2+}$-binding domains. CaM is currently thought to interact with and mediate the function of some dozen intracellular enzymes and cytoskeletal proteins. Both CaM and TnC undergo a conformational change upon calcium binding as shown by hydrogen ion titration, circular dichroism and fluorescence measurements (Tanaka & Hidaka, 1981; LaPorte, Wierman & Storm, 1980; Iida, 1979; Crouch & Klee, 1980). This shift both increases the alpha-helical content and induces a hydrophobic binding domain. This calcium-induced hydrophobic domain is now recognized as an important feature in calmodulin interaction with its acceptor proteins and the inhibitory compounds, the neuroleptic drugs.

* Present address: Bureau of Biological Research, Rutgers University, P.O. Box 1059, Piscataway, NJ 08854, U.S.A. Author to whom requests should be addressed.

Key words: Calmodulin, hydrophobic binding proteins, calcium mediation.
The calcium-dependent interaction with the latter class of compounds is the basis for development of several affinity matrices. Calmodulin purification can be simply accomplished by calcium-dependent chromatography on one of the drug-coupled Sepharose columns (Jamieson & Vanaman, 1979; Charbonneau & Cormier, 1979; Gopalkrishna & Anderson, 1982).

We have recently described a set of calcium-binding proteins that interact with a fluphenazine-Sepharose affinity matrix in a calcium-dependent manner. These proteins have apparent $M_r$ of $67 \times 10^3$, $35 \times 10^3$, $33 \times 10^3$, $30 \times 10^3$ and $17 \times 10^3$ (CaM) by sodium lauryl sulphate/polyacrylamide gel electrophoresis (SLS-PAGE). We will refer to the protein set devoid of CaM as calcimedins. This paper describes several properties of the calcimedins that are distinct from those of calmodulin. Since the hydrophobic domain probably plays an important role in the mode of action of calcium-binding proteins responding to the calcium signal, the calcimedins also have a potential role in mediating such calcium action. The role of the calcimedins, however, is likely to be separate from that of CaM and TnC.

**MATERIALS AND METHODS**

**Isolation of calcium-dependent fluphenazine-binding proteins**

Tissue extracts were prepared by homogenizing 100 g chicken gizzard in 50 ml 0.075 M-NaCl, 0.04 M-Tris-HCl (pH 7.3), containing 2 mM-EDTA, 0.1% NaN$_3$, leupeptin, 0.5 g aprotinin and $10^{-4}$ M-phenylmethylsulphonyl fluoride. The homogenate was centrifuged at 12 000 rev./min for 30 min. CaCl$_2$ was added to 1 mM above the level of EDTA. The supernatant was centrifuged at 18 000 rev./min for 20 min and filtered through layers of cheesecloth and chromatographed on a fluphenazine-Sepharose affinity matrix. The column was washed with the above buffer containing 2 mM-CaCl$_2$ in place of EDTA. This was followed by the same buffer containing 0.5 M-NaCl. Calcium-dependent binding proteins were then eluted by 0.075 M-NaCl, 0.04 M-Tris-HCl (pH 7.3), buffer containing 2 mM-EGTA and 0.1% NaN$_3$.

Calmodulin was separated from the calcimedin proteins by DEAE-cellulose chromatography. The protein fractions eluted by EGTA as above were dialysed into 0.01 M-imidazole, 1 mM-EDTA (pH 6-4) and bound to a DEAE-cellulose column. The calcimedins were eluted from the matrix with 0.01 M-imidazole, 0.1 M-NaCl, 1 mM-EDTA (pH 6-4). Calmodulin remained bound at this salt concentration and was subsequently eluted by washing the column with the same buffer containing 0.4 M-NaCl.

**Analytical procedures**

Protein concentrations were determined by the methods of Bradford (1976) using immunoglobulin as standard.

SDS/polyacrylamide gel electrophoresis was performed according to the disc-gel system of Laemmli (1970) using a slab-gel apparatus. For native gel conditions the SDS and 2-mercaptoethanol were omitted from the Laemmli buffers and the gel was subjected to electrophoresis at 4°C.

Two-dimensional gel electrophoresis followed the method outlined by Cabral et al. (1980) with the focusing range between pH 3 and pH 7. The second dimension was a SDS/10% polyacrylamide gel.

**Enzyme assays**

Cyclic-nucleotide-dependent phosphodiesterase (PDE) activity was measured by the spectrophotometric assay reported by Dedman & Means (1977). The total reaction volume was 1.0 ml and contained 50 μl PDE-enriched brain extract. The assay was initiated by the addition of cyclic AMP.
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The decrease in $A_{280}$ was followed for 5 min using a Gifford programmable model 2600 recording spectrophotometer. Ca$^{2+}$-activated ATPase activity was assayed in a reaction mixture containing $100 \text{mm-KCl}$, $20 \text{mm-HEPES}$, $1 \text{mm-NaNO}_3$, $1 \text{mm-MgCl}_2$ (pH 6.8). Protein added was 0.5–1.0 mg per assay. The final volume of the assay was adjusted to 1.0 ml. The reaction was initiated by adding K$^+$-ATP (1 mm-final concentration), and allowed to proceed for 30 min at 37 °C. The reaction was terminated by addition of 8% ice-cold trichloroacetic acid. P$_i$ released was measured according to Andia-Waltenbaugh, Lam, Hummel & Friedmann (1980).

RESULTS

Several proteins from the smooth muscle tissue of chicken gizzard have been identified as interacting with a fluphenazine-Sepharose matrix in a calcium-dependent manner (Moore & Dedman, 1982). Fig. 1A shows the $A_{280}$ profiles of gizzard extracts.
chromatographed in the presence of CaCl₂, MgCl₂ or CdCl₂. Maximal protein binding occurs in the presence of added Ca²⁺. Fig. 1B shows the SDS/PAGE profiles of the proteins eluted from the matrix by EGTA (Ca²⁺) or EDTA (Mg²⁺ or Ca²⁺). Gel 1 indicates that five polypeptides bound from Ca²⁺-containing extracts. These bands have apparent Mr corresponding to 67, 35, 33, 30 and 17 (×10³). The 17 × 10³ Mr band has been identified as calmodulin. Mg²⁺ is able to replace Ca²⁺ in many physiological reactions but cannot effectively substitute for Ca²⁺ in the induction of the trifluoperazine binding site on CaM (Levins & Weiss, 1979). Gel 2 shows that only a small quantity (less than 10 %) of the 67 × 10³ Mr protein interacts with the matrix when Ca²⁺ is replaced by Mg²⁺. No CAM is observed in the eluted protein peak. Ca²⁺ is one of the sulphydryl-binding transition metals. This cation has been suggested to compete for Ca²⁺ and hence disrupt excitation-contraction coupling in muscle (Bers & Langer, 1979). Gel 3 shows material eluted from the matrix with Cd²⁺ present in the extract. The major polypeptide comprising 78 % of the total protein shows a molecular mass of 44 × 10³. The minor band is CaM and is approximately 16 % of that seen to bind in the presence of Ca²⁺. Cd²⁺ has been shown to interact with CaM

Fig. 2. Chicken heart versus chicken gizzard fluphenazine-binding proteins. Gel 1, proteins isolated as described in Materials and Methods from chicken hearts by fluphenazine-Sepharose affinity chromatography; 30 µg protein. Gel 2, proteins from gel 1 were applied to a DEAE-cellulose column in 10 mM-imidazole. Protein eluted by 0.1 M-NaCl in 10 mM-imidazole were displayed by SDS/PAGE; 22 µg protein. Gel 3, chicken gizzard calcimedins, for comparison; 26 µg protein. Arrows point to calmodulin.

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(Andersson, Drakenberg, Forsen & Thulin, 1982) at the Ca\(^{2+}\)-binding sites.

Identical protein sets can be isolated from both chicken gizzard and heart tissues (Fig. 2). As previously shown for chicken gizzard, CaM can be separated from the heart calcimedins by DEAE-cellulose chromatography (lanes 1 and 2).

The relative amounts of the fluphenazine-binding proteins were estimated for a variety of tissues by extraction, calcium-dependent fluphenazine-Sepharose binding, followed by SDS/PAGE and densitometry of the stained bands. The quantities of the calcimedin proteins are shown relative to CaM in Table 1. The muscle tissues generally show lower levels of CaM. Of the non-muscle tissues, kidney appears to contain considerable quantities of the calcimedins. Since the kidney is a highly vascularized tissue, the increased levels may reflect the high content of smooth muscle in this tissue. In general, Table 1 indicates that different tissues have varying quantities of the calcimedins, possibly reflecting their physiological role. Note that the electric organ, known for extremely high levels of CaM, has relatively low levels of the 67 × 10\(^3\)M\(_r\) protein and undetectable amounts of the remaining calcimedins. Both brain tissue and 20 mosm-EDTA extracts of erythrocytes also have no detectable calcimedin proteins. Thus CAM is found in all tissues whereas the calcimedins are more variably distributed.

The four calcimedins can be separated from CaM by DEAE-cellulose chromatography. Like CaM they interact with the drug columns in a predominantly hydrophobic manner. These proteins interact with the matrix in phosphate, Tris • HCl and imidazole-based buffers ranging in pH from 6.2 to 8.0 and ionic strengths from 0.02 M.

Table 1. Relative percentage of protein in electrophoretic bands by densitometry

<table>
<thead>
<tr>
<th>Tissue</th>
<th>67 × 10(^3)</th>
<th>35/33 × 10(^3)</th>
<th>30 × 10(^3)</th>
<th>Calmodulin</th>
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<tbody>
<tr>
<td>Rabbit dorsi</td>
<td>11.0</td>
<td>7.3</td>
<td>5.4</td>
<td>76.3</td>
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<tr>
<td>Electrophax</td>
<td>7.4</td>
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<td>–</td>
<td>92.6</td>
</tr>
<tr>
<td>Eel muscle</td>
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<td>5.3</td>
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<td>36.0</td>
</tr>
<tr>
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<td>22.4</td>
<td>–</td>
<td>68.3</td>
</tr>
<tr>
<td>Bovine heart</td>
<td>8.7</td>
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<td>25.4</td>
<td>30.4</td>
</tr>
<tr>
<td>Chicken gizzard</td>
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<td>18.8</td>
<td>13.3</td>
<td>47.0</td>
</tr>
<tr>
<td>Hamster liver</td>
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<td>7.9</td>
<td>14.1</td>
<td>71.1</td>
</tr>
<tr>
<td>Rat brain</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>100.0</td>
</tr>
<tr>
<td>RBC lysate</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Relative abundance of the non-calmodulin fluphenazine-binding proteins determined by densitometry of Coomassie Brilliant Blue R250-stained SDS/polyacrylamide gels. Tissues were homogenized as described in Materials and Methods and the prepared extract was chromatographed on the fluphenazine-Sepharose column. Samples were electrophoresed and the areas under the curve for the 67, 35–33, 30 and 17 (×10\(^3\))M\(_r\) proteins were determined using a Gilford model 2600 recording spectrophotometer for disc gels or a BIOMED Instruments soft laser scanning densitometer for slab gels. Each peak is given as the percentage of the total for the four peaks.
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Fig. 3. Shaltiel alkylagarose chromatography of calcimedins (unbroken line) and calmodulin (broken line). Proteins were bound to 1 ml each of 4, 6, 8 and 10-carbon chain conjugated resins in the presence of calcium. After washing the column-bound proteins were eluted with 2 mM-EGTA to 0·6 M-NaCl. These proteins also bind the Shaltiel series of hydrophobic resins (Miles Research Laboratories) and can be eluted from them by EGTA (Fig. 3). Resins with chain lengths of 4, 6, 8 and 10 carbons bound CaM (broken line) while the calcimedins (unbroken line) mainly interacted with chain lengths of 6–8 carbons. In each case, conditions were such that the protein concentration was in a large excess over the column capacity. Gel analysis of the eluted proteins from the heptyl- and octyl-resins, showed the presence of all four calcimedin proteins.

The possible oligomeric nature of the calcimedin proteins was explored by electrophoresis on native gels and molecular-sieve chromatography in the presence of Ca$^{2+}$. These results comprise Figs 4–6. The calcimedins were electrophoresed in the
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Fig. 4. Fluphenazine-Sepharose calcium-dependent binding proteins analysis by electrophoresis. Lane 1, proteins separated by SDS/PAGE conditions; 20 μg. Lane 2, proteins separated under native conditions; 30 μg.

presence or absence of SDS and 2-mercaptoethanol (Fig. 4). No large oligomeric structure was observed under native conditions. CaM is known to shift its apparent Mr on SDS/polyacrylamide gels in the presence or absence of Ca²⁺. No shift in migration was seen for any of the calcimedins on SDS/PAGE. Under native conditions (Fig. 5), a single band appeared to alter its migration; however, no large oligomeric structure was seen again. A further test for the formation of such a high molecular weight structure was attempted by chromatography of these proteins on an AcA54 (LKB) gel permeation resin. Elution was characterized by the $K_w$ value versus the molecular mass (Fig. 6). The $K_w$ values were determined for the four calcimedins and are indicated by the arrows on the standard curve. Both the $67 \times 10^3 \, M_r$ protein and the $30 \times 10^3 \, M_r$ protein eluted in positions suggesting no interaction. The $35$ and $33 \times 10^3 \, M_r$ proteins, however, eluted with values that could represent some interaction or, alternatively, the hydrodynamic properties of these proteins are such that they vary from ideal globular proteins.

The calcimedins were also analysed by two-dimensional electrophoresis as shown in Fig. 7. The proteins were focused between pH 3 and 7 followed by electrophoresis through a SDS/10% polyacrylamide gel. A possible post-translational modification
Fig. 5. Calcium-induced migration shift. Two identical samples of DEAE-cellulose-isolated calcimedins were chromatographed under native conditions. Gel 1, calcimedins plus 5 mM-EDTA; 25 μg protein. Gel 2, calcimedins plus 5 mM-CaCl₂; 25 μg protein.

could be implied by the multiple spots for the 67 × 10³ Mᵣ protein. The 35/33 (× 10³ Mᵣ) doublet did not resolve well under the conditions used, while the 30 × 10³ Mᵣ protein may be composed of two distinct proteins, one minor in quantity. However, it is clear that these proteins all have isoelectric points significantly different from that previously observed for CaM (Dedman et al. 1977).

The calcimedins were tested for their ability to activate two enzymes that are thought to be physiologically regulated by CaM. Table 2 is a study of the activation of brain cyclic nucleotide phosphodiesterase. A value of 5 μM-CaCl₂ was chosen for the majority of the assays since at this concentration CaM gave a stimulation sufficiently above background. Increasing concentrations of fluphenazine caused sequential reversal of the Ca²⁺/CaM activation. The calcimedins did not activate the enzyme when they were substituted for CaM. However, if the enzyme was pre-treated with the calcimedins before CaM addition, the stimulation by CaM was attenuated. Since the calcimedins were pre-saturated with CaCl₂, the effect cannot be due to Ca²⁺ availability. The calcimedins were also able to compete with CaM for
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Fig. 6. Gel permeation chromatography of the calcimedin proteins in the presence of 2 mM-CaCl$_2$. The $K_w$ value was determined for creatine kinase (81 X $10^3 M_0$), bovine serum albumin (67 X $10^3 M_0$), ovalbumin (43 X $10^3 M_0$) and DNase I (31 X $10^3 M_0$). The $K_w$ values were then determined for each of the four calcimedin proteins and plotted on the standard generated line.

Fig. 7. Two-dimensional electrophoretogram of the non-calmodulin fluphenazine-binding proteins. The proteins were focused in the first dimension in an electric field between pH 3 and pH 7. The focused proteins were then reacted with SDS and separated on a SDS/10% polyacrylamide slab gel. The gel was stained, dried and photographed.
Table 2. Calmodulin-dependent cyclic nucleotide phosphodiesterase assay

<table>
<thead>
<tr>
<th>CaCl₂ (µM)</th>
<th>Calmodulin (µg)</th>
<th>Calcimedins (µg)</th>
<th>Fluphenazine (µM)</th>
<th>nmol cAMP/ml per min</th>
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<tbody>
<tr>
<td>0.1</td>
<td>20</td>
<td>–</td>
<td>–</td>
<td>0.13</td>
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<td>0.4</td>
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<td>–</td>
<td>–</td>
<td>3.13</td>
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<tr>
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<td>–</td>
<td>–</td>
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<td>8.0</td>
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<td>–</td>
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<td>50</td>
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<td>2.74</td>
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Calmodulin-dependent cyclic nucleotide phosphodiesterase activity. Phosphodiesterase activity was monitored according to Dedman & Means (1977). The change in 
$A_{254}$ was followed for 5 min after addition of cAMP. Preincubation of the enzyme with the calcimedins was for 15 min at 25°C.

fluphenazine and partially reverse the drug inhibition. Finally, as shown in Fig. 8, the calcimedins were able to stimulate the Ca$^{2+}$-ATPase activity of isolated liver microsomes. This stimulation could be inhibited by micromolar concentrations of trifluoperazine. Taken together, these results suggest that the calcimedins express a hydrophobic domain in the presence of calcium, which can interact with intracellular proteins in a manner completely analogous to that of CaM.

**DISCUSSION**

The proteins described interact with hydrophobic moieties in a calcium-dependent manner. This type of interaction is postulated to be the mode of CaM activation of several intracellular enzymes including cyclic nucleotide phosphodiesterase, erythrocyte Ca$^{2+}$-ATPase, myosin light-chain kinase and adenylate cyclase. The calcimedins do not activate cyclic nucleotide phosphodiesterase (Table 2) nor did they activate uterine myosin light-chain kinase nor adenylate cyclase (data not shown). The calcimedins, however, were able to interact with cyclic nucleotide phosphodiesterase, since they prevented activation by CaM. Their ability to activate the microsomal Ca$^{2+}$-ATPase activity also suggests the calcium-induced hydrophobic domain can
function in the physiological regulation of an intracellular enzyme system. These proteins then share a common property with CaM and the CaM-related proteins (e.g. TnC), in that binding of calcium results in a conformational shift. The shift exposes a previously cryptic hydrophobic domain, which is a key element in mediation of the calcium signal.

A phospholipid-dependent protein kinase that functions at micromolar calcium concentration has been recently described (Takai, Kishimoto, Inoue & Nishizuka, 1977). The enzyme is diversely localized in tissues, being high in brain and spleen but low in skeletal and heart muscle (Kuo et al. 1980). This kinase also interacts with the neuroleptic drugs, trifluoperazine, chlorpromazine and fluphenazine (Schatzman, Wise & Kuo, 1981). However, this interaction is apparently not directly with the kinase proper, but is instead a competitive inhibition of enzyme activation through the phospholipid component. None of the non-calmodulin calcimedins appears to be related to the kinase or its proenzyme, on the basis of apparent molecular weights estimated from SDS/polyacrylamide gels or their binding characteristics to DEAE-cellulose. We previously showed that brain tissue extracts and erythrocyte lysates did not contain fluphenazine-binding proteins similar to those isolated from the muscle tissues (Moore & Dedman, 1982). This observation is also consistent with these proteins not being identical to the phospholipid-dependent protein kinase. The calcimedins failed to activate brain cyclic nucleotide phosphodiesterase, although they could prevent calmodulin activation when pre-incubated
with the enzyme; the binding constant is much lower than that of calmodulin.

Most of the tissues examined appeared to contain a protein of apparent molecular mass of $67 \times 10^3$. Densitometric scans of cylindrical gels did not completely resolve the $35 \times 10^3$ and $33 \times 10^3 M_r$ bands and therefore were treated as a single peak. However, variations between tissues and species were generally reflected in the $33 \times 10^3$ and $30 \times 10^3$ bands. The molecular weight varied by $\pm 1000$ in some samples, and as it is unclear whether these are true differences or artifacts of the isolation procedure, the peaks are listed under the molecular weights determined for the chicken gizzard tissue. It is clear, however, that all tissues had major resin-binding proteins of these molecular weights, in addition to calmodulin.

The calcimedin proteins appear to be unrelated to the CaM family of proteins. The proteins are labile to the high-temperature treatment that is successful in CaM isolation (Moore & Dedman, 1982). All of the calcimedins are eluted from DEAE-cellulose of $0.1 \text{ m-NaCl}$, while CaM remains bound. The more acidic nature of CaM is also shown by the isoelectric point data. The calcimedins have isoelectric points ranging from pH 5.0 to 6.0, whereas CaM and TnC are more acidic (pH 3.9 and 4.1, respectively). The calcimedins do not appear to contain trimethyl-lysine, a modified amino acid that appears to be unique in CaM.

These proteins most probably represent an independent set of calcium regulatory proteins. Like calmodulin and the calmodulin-related high affinity calcium-binding proteins, troponin C and brain S-100 proteins, these proteins show induced hydrophobic interaction. It is becoming more evident that cells and tissues contain various calcium receptors, which do not possess an enzymic or structural function except in order to develop a hydrophobic domain. This induced site then interacts and regulates specific acceptor proteins and thus mediates the calcium signal. The expression of these calcium mediators varies from tissue to tissue (ratio, calmodulin : TnC : S-100 : $67 \times 10^3 : 35 \times 10^3 : 33 \times 10^3 : 30 \times 10^3 M_r$), apparently as a result of the individually differentiated physiological demands. Collectively, these observations are consistent with the fact that the intracellular calcium signal follows several independent pathways depending upon the degree of expression of individual calcimedins.

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