Connexin 32 of gap junctions contains two cytoplasmic calmodulin-binding domains

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A fluorescent calmodulin derivative, 2-chloro-[4-(ϵ-amino-Lys^{75})]-[6-(4-diethylaminophenyl)-1,3,5-triazin-4-yl]-calmodulin (TA-calmodulin) [Török and Trentham (1994) Biochemistry **33**, 12807–12820], and equilibrium fluorescence methods were used to identify calmodulin-binding domains of connexin subunits of gap junctions. Synthetic peptides corresponding to six extramembrane regions of connexin 32, a major component of rat liver gap junctions, and peptides derived from connexin 43 and 26, were tested. Two cytoplasmically oriented peptides that correspond to an N-terminal 21-amino-acid sequence and a 15-amino-acid sequence at the C-terminal tail of connexin 32 bound TA-calmodulin in a Ca^{2+}-dependent manner. The dissociation constants (K_d) of TA-calmodulin binding to GAP 10 (MNWTGLYTLLSGVNRHSTAIG, residues 1–21) and GAP 8M (ACARRAQRRSNPPSR, residues 216–230) were 27 nM and 1.2 μM respectively at 150 mM ionic strength, 2 mM $MgCl_2$, 100 μM $CaCl_2$, pH 7.0 and 21 °C. Both halves of each peptide were required for calmodulin binding. Substitution of Trp^3 present in all connexins by Tyr increased K_d for TA-calmodulin by 40-fold. Liver gap junctions (whose connexons contain mainly connexin 32) and recombinant connexons constructed of connexin 26 expressed by baculovirus-infected insect cells exhibited weaker binding of TA-calmodulin with variable Ca^{2+}-dependence. These studies identify two calmodulin-binding amino-acid sequences in connexin 32, and provide independent evidence that calmodulin may function as an intracellular ligand, regulating Ca^{2+}-dependent intercellular communication across gap junctions.

INTRODUCTION

Gap junctions are specialized plasma-membrane regions that allow cells to communicate directly. They are constructed of connexins, a family of closely related channel-forming membrane proteins [1–3]. Models of gap-junction structure feature a hexameric association of the connexin subunits to form connexon hemichannels. The alignment and adhesion at the plasma membrane of connexons contributed by neighbouring cells provides a direct communication channel through which intercellular signalling occurs [4,5]. Topological studies of connexin 32 and 26 (the major proteins expressed by rodent liver) and connexin 43 (expressed by heart) show connexins to traverse the lipid bilayer four times, with the N- and C-termini located at the cytoplasmic face of the plasma membrane [6–12]. The third transmembrane sequence, by virtue of its pronounced amphipathic character, is postulated to contribute to the channel wall [5]. This arrangement of connexins in the membrane thus defines a single cytoplasmic loop and two extracellular (gap-located) loops linked by disulphide bonds [11] (Figure 1).

Intercellular communication across gap junctions is regulated by intracellular Ca^{2+} and H^+, voltage and phosphorylation of the connexin subunits [2]. Ca^{2+} induces a conformational change in isolated gap junctions, and models that account for the open/closed configuration of the junctional channel have been proposed [4,5]. A role for calmodulin, a Ca^{2+}-binding protein that mediates many Ca^{2+}-dependent processes *in vivo*, in regulating gap junctions has often been proposed. A number of observations suggest the involvement of calmodulin in regulating gap-junctional conductance, although it is not clear whether such effects are caused by direct interaction with the connexin subunits constituting the gap-junction channel. Gap-junction conductance is modified when calmodulin expression is suppressed [13]. Calmodulin inhibitors such as calmidazolium or phenothiazines, e.g. chlorpromazine, inhibit electrical uncoupling in *Xenopus* embryos [14,15] and in epidermal cells [16]. Calmodulin antagonists and antibodies inhibit cell proliferation [17], an effect which may be related to efficacy of gap-junction-mediated communication, since there is increasing evidence that there is a direct correlation between the functional expression of connexins and growth control in cells transfected with connexin genes [18]. Other more direct biochemical approaches have also shown that calmodulin binds to intact gap junctions and tryptic fragments of rat liver connexin 32 in a Ca^{2+}-independent manner [6,19]. Finally, studies involving a role for calmodulin in controlling junctional communication have addressed the binding of fluorescently labelled calmodulin to eye lens fibre junctions and in identifying calmodulin-binding motifs in MIP (main intrinsic polypeptide)-26 [20–22], the major intrinsic membrane protein of eye lens fibre junctions [23].

Functional regulation or modulation of gap-junction function by calmodulin may be exerted either by binding to calmodulin-binding domains and/or by phosphorylation by calmodulin-dependent kinases. Calmodulin binding to large tryptic fragments of connexin 32 has been shown by SDS/PAGE overlay assay [6,19]. However, neither a more precise definition of the calmod-

Abbreviations used: MLCK, myosin light chain kinase; TA-calmodulin, 2-chloro-[4-ϵ-amino-Lys^{75})]-[6-(4-diethylaminophenyl)-1,3,5-triazin-4-yl]-calmodulin.

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ulin-binding domain(s) nor a quantitative assessment of the interaction with calmodulin is possible by this methodology. In the present work, a fluorescence binding assay was employed to define and characterize the calmodulin-binding domain(s) of connexin 32. The binding studies were carried out in solution utilizing a panel of synthetic peptides that account for most of the extramembrane sequences of connexin 32. A Lys^{75}-labelled fluorescent derivative of calmodulin, 2-chloro-[4-(ϵ-amino-Lys^{75})]-[6-(4-diethylominophenyl)-1,3,5-triazin-4-yl]-calmodulin (TA-calmodulin) was developed for studies of the mechanism of regulation of target proteins by calmodulin. TA-calmodulin has been extensively characterized with respect to target-binding properties with the following results: TA-calmodulin is a competitive inhibitor of smooth-muscle myosin light chain kinase (MLCK) with a K_i/K_m ratio of 3.6 [24], TA-calmodulin activates phosphodiesterase with a 3.5-fold increased K_m compared with calmodulin (K. Török, D. J. Cowley, B. D. Brandmeier, S. Howell, A. Aitken and D. R. Trentham, unpublished work), and TA-calmodulin competes with calmodulin in peptide-binding assays, typically with a 2.5-fold higher K_d compared with calmodulin [24]. TA-calmodulin provides information both on the Ca^{2+}-dependence of target interaction and on target binding in the presence of Ca^{2+} [24].

MATERIALS AND METHODS

Peptides and proteins

Synthetic peptides corresponding to selected amino-acid sequences of rat connexin 32, 43 and 26 (Tables 1 and 2 below) were prepared by 9-fluorenylmethoxycarbonyl-polyamide solid-phase chemistry [11]. Peptides were cleaved from the resin and deprotected by treatment with aq. 95% trifluoroacetic acid. Purity and quality of the peptides were routinely assessed by HPLC and amino-acid analysis. Peptides were stored in water or PBS at -20 °C.

Gap junctions were prepared from rat liver plasma membranes using an alkaline extraction procedure [25]. Junctions synthesized by an insect cell-line containing a baculovirus vector incorporating the cDNA for connexin 26 [26] were detergent-extracted from cell homogenates and purified on sucrose gradients.

Calmodulin was purified from pig brain [27] and covalently modified by 2,4-dichloro-6-(4-*N*,*N*-diethylaminophenyl)-1,3,5-triazine (TA-Cl) at Lys^{75} [24]. The specifically labelled derivative was purified to homogeneity by ion-exchange FPLC. Fluorescence measurements were carried out in a Perkin–Elmer MPF-44A spectrofluorimeter. Binding data were obtained by continuous titration of Ca^{2+} or peptides or gap-junction plaques into a solution of TA-calmodulin in a stirred quartz cuvette. The data were corrected for dilution and fitted to the model of ligand binding with 1:1 stoichiometry using a non-linear least-squares minimizing algorithm (BINDPC; provided by Dr E. P. Morris Imperial College of Science, Technology and Medicine, London, UK). This method gives best-fit to both the end point of titration and the dissociation constant (K_d). The excitation and emission wavelengths were 365 nm and 415 nm respectively. Free Ca^{2+} concentrations were calculated using $K_d = 4.35 \times 10^7$ M for EGTA [28].

RESULTS AND DISCUSSION

Connexin peptides

The panel of synthetic peptides used in the present work was based on current topological models proposed for connexins 32, 26 and 43 (Figure 1). The peptide sequences are shown in Tables 1 and 2. Three peptides (GAP 10, 3 and 25) represented the cytoplasmically oriented N-terminus of connexin 32 and a peptide analogue (GAP 18M) corresponded to the N-terminal sequence of connexin 43. The intracellular loops of connexin 26, 32 and 43 were represented by a peptide each, DES 3, DES 1 and GAP 13 respectively. Various regions of the variable-length C-terminus of connexins 32 and 43 were represented by four peptides (GAP 8, 8M, 9 and 14). Two peptides (GAP 7M and 11) corresponded to the highly conserved amino acid sequences of the two extracellular loops of connexin 32 [1–3].

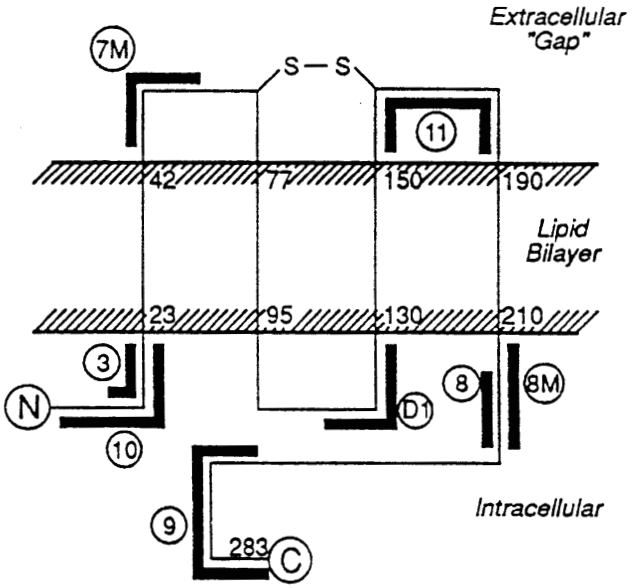

Figure 1 Topological model of rat liver connexin 32

The topological model of connexin 32 depicted here is based on data in [6–11]. The polypeptide chain is represented by the line that connects the N- and C-termini. The uncircled numbers indicate the approximate amino-acid-residue numbers at the points of entry into the lipid bilayers. The solid bars show the position in the sequence of eight peptides synthesized for the present work. D1 designates DES 1, the rest of the numbers in circles refer to peptides GAP 3, 10, 7M, 8, 8M, 9 and 11. The topological positions and sequences of all the peptides used in this study are shown in Tables 1 and 2.

Ca^{2+} binding to TA-calmodulin and TA-calmodulin–connexin peptide complexes

The fluorescence of TA-calmodulin was increased 5.5-fold by Ca^{2+} binding (Figure 2, record 1). The Ca^{2+}-induced fluorescence enhancement in TA-calmodulin reached half-maximum at 500 nM [Ca^{2+}] in 100 mM KCl and 2 mM $MgCl_2$ (Figure 2, record 1). This is significantly lower than the overall K_d of calmodulin for Ca^{2+} [29] and is similar to the half-maximum [Ca^{2+}] for Tyr^{99} fluorescence change in calmodulin [30]. Tyr^{99} fluorescence change in response to Ca^{2+} binding to calmodulin is attributed to the high-affinity sites and thus TA-calmodulin is also likely to be a reporter of the high-affinity sites. However, as little evidence of Ca^{2+} binding to low-affinity sites was observed, it may be that the hydrophobic nature of the TA-group causes all four Ca^{2+}-binding sites to have a high affinity. Given the sensitivity of TA-amine fluorescence to solvent polarity [31], it is likely that the 5.5-fold fluorescence enhancement is associated with an interaction between the fluorophore and one or other of the hydrophobic pockets that are presented when Ca^{2+} binds to calmodulin [32].

Table 1 Calmodulin-binding peptides and their analogues from connexins

Numbers in parentheses give the fluorescence increase; the value of 1 defines the fluorescence of Ca^{2+}-saturated TA-calmodulin.

Peptide	Connexin	Topological position [11]	Residue numbers	Sequence	$[Ca^{2+}]$ (nM) at half-maximum TA fluorescence*	K_d (M) (Ca^{2+}/TA-calmodulin)
GAP 10†	32	Intracellular N-terminal tail	1–21	MNWTGLYTLLSGVNRHSTAIG	220 (1.2)	2.7×10^{-8}
GAP 3†	32	Intracellular N-terminal tail	1–10	MNWTGLYTLL	500 (1.0)	n.d.
GAP 25	32	Intracellular N-terminal tail	7–21	YTLLSGVNRHSTAIG	n.d.‡	1.4×10^{-5}
GAP 18M	43	Intracellular N-terminal tail	1–16	MGDYSALGKLLDKVQA§	n.d.‡	1.2×10^{-6}
GAP 8M†	32	Intracellular C-terminal tail	216–230	ACARRAQRRSNPPSR	200 (1.6)	2.1×10^{-6}
GAP 8†	32	Intracellular C-terminal tail	219–230	RRAQRRSNPPSR	450 (1.1)	n.d.

* Half-maximum of TA-calmodulin fluorescence increase as a function of $[Ca^{2+}]$ in 50 mM Mes-K^+ adjusted to pH 7.0, 100 mM KCl and 2 mM $MgCl_2$ at 21 °C was at 500 nM (see Figure 2).

† The topological position of these peptides is illustrated in Figure 1.

‡ n.d., not determined.

§ The endogenous tryptophan residue was replaced by tyrosine in GAP 18M.

Table 2 Extramembranous connexin regions which did not shift the free $[Ca^{2+}]$ at the half-maximum TA-calmodulin fluorescence

Peptide	Connexin	Topological position [11]	Residue number	Sequence
GAP 9*	32	Intracellular C-terminal tail	264–283	RRSPGTGAGLAEKSDRCSAC
GAP 14	43	Intracellular C-terminal tail	314–325	SAEQNRMGQAGS
GAP 7M*	32	Extracellular loop 1	43–59	VWGDEKSSFICNTLQY
DES 1*	32	Intracellular loop	(102–112) + (116–124)	EKKMLRLEGHGHLEVVKRHK
DES 3	26	Intracellular loop	106–119	FMKGEIKNEFKDIEC
GAP 13	43	Intracellular loop	123–136	KVEMHLKQIEIKKFC
GAP 11*	32	Extracellular loop 2	151–187	YVFYLLYPGYAMVRLVKCEAFPCPNTVDCFVSRPTEK

* The topological position of these peptides is illustrated in Figure 1.

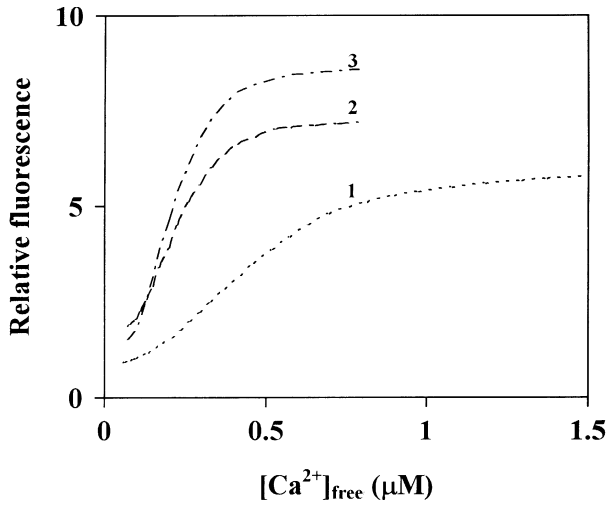

Figure 2 Fluorescence changes in TA-calmodulin upon Ca^{2+} and peptide binding

A solution of 10 mM $CaCl_2$ was injected into a continuously stirred cuvette containing 50 nM TA-calmodulin (record 1), 50 nM TA-calmodulin and 2.5 μM GAP 10 (record 2), or 50 nM TA-calmodulin and 24 μM GAP 8M (record 3). All solutions contained 50 mM Mes-K^+ adjusted to pH 7.0, 100 mM KCl, 2 mM $MgCl_2$ and mM EGTA at 21 °C. Fluorescence was monitored at $\lambda_{excitation} = 365$ nm and $\lambda_{emission} = 415$ nm. Free $[Ca^{2+}]$ was calculated from discrete points of the titration curve using $K_d = 4.35 \times 10^{-7}$ M for EGTA [28].

The affinity of calmodulin for target proteins or peptides increases substantially upon Ca^{2+} binding. Conversely, for thermodynamic reasons, a similar target-induced increase in affinity of Ca^{2+} for calmodulin must occur at some stage in the Ca^{2+}-binding process. The fluorescence of TA-calmodulin as a function of free $[Ca^{2+}]$ in the presence of saturating GAP 10 and GAP 8M increased 6.6-fold and 8.8-fold respectively (Figure 2, records 2 and 3). Titration of the GAP 10/TA-calmodulin (Figure 2, record 2) and the GAP 8M/TA-calmodulin (Figure 2, record 3) complexes showed half-maximum fluorescence changes at 220 nM and 200 nM free $[Ca^{2+}]$, respectively. Titration of all the other peptide/TA-calmodulin complexes (listed in Table 2) showed less than 10% effect on both the half-maximum fluorescence change and the fluorescence increase of TA-calmodulin alone. Smooth-muscle MLCK binding to calmodulin is enhanced 1000-fold by Ca^{2+} binding to calmodulin [33], yet the half-maximal $[Ca^{2+}]$ for TA-calmodulin binding to MLCK is only 6-fold decreased (K. Török and D. R. Trentham, unpublished work). Thus it appears that, in the case of MLCK, the affinity increase for Ca^{2+} affects the low-affinity sites to a large extent, and the TA group reports binding to the high-affinity binding sites which are only weakly affected by MLCK binding. The effects of GAP 10 and 8M binding on Ca^{2+} binding to TA-calmodulin, similarly to those of MLCK, were likely to report the increase in the Ca^{2+}-binding affinity of the high-affinity binding sites of TA-calmodulin.

Our data are consistent with the characteristics of calmodulin-

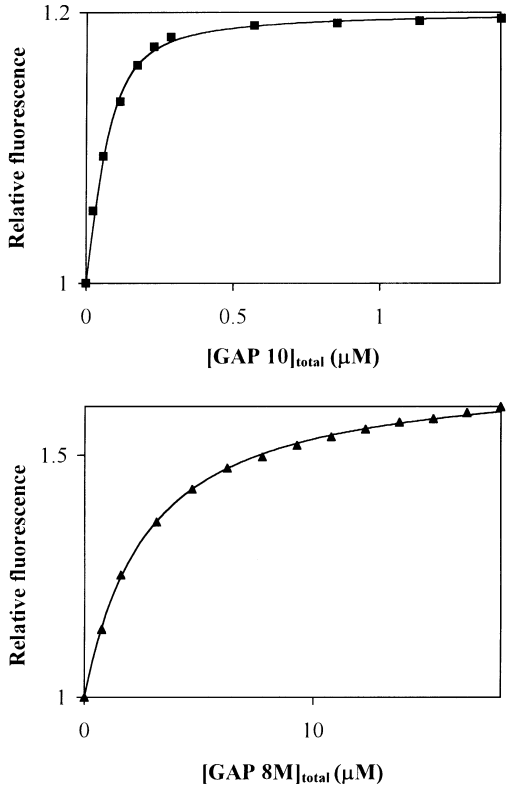

Figure 3 Equilibrium binding of peptides GAP 10 and GAP 8M to TA-calmodulin

In separate experiments, 89 μM GAP 10 (upper panel) and 488 μM GAP 8M (lower panel) were continuously titrated into 50 nM TA-calmodulin in 50 mM Mes-K^+ adjusted to pH 7.0, 100 mM KCl, 2 mM $MgCl_2$ and 100 μM $CaCl_2$ at 21 °C. The symbols (■ and ▲ for GAP 10 and GAP 8M respectively) represent discrete points from the experimental data, whereas the lines are fitted curves. K_d = 27 nM and 2.1 μM were determined for GAP 10 and GAP 8M respectively.

target peptide interactions identified in the atomic structures of such complexes. Atomic structures of calmodulin-target complexes show that each of the two hydrophobic pockets formed in Ca^{2+}-calmodulin requires an appropriate hydrophobic residue in the target peptide chain to bind to [35]. The number of amino acids between the two hydrophobic sites in the peptide can vary; the flexible linker between the two globular domains of calmodulin that expose the hydrophobic patches allows their distance to be adjustable [36].

Calmodulin-binding domains of connexin 32

The equilibrium dissociation constants (K_d) of GAP 10 and GAP 8M for TA-calmodulin were measured utilizing the fluorescence increases (1.3-fold and 1.6-fold respectively) that occurred at saturating, 100 μM, [Ca^{2+}], in addition to the Ca^{2+}-induced fluorescence enhancement. Figure 3 shows that the titration data gave a good fit, assuming 1:1 stoichiometry of binding. A K_d of 27 nM for GAP 10 and a K_d of 2.1 μM for GAP 8M were determined.

Analogues of GAP 8M and GAP 10 were used to identify important residues and to define the boundaries of the two putative calmodulin-binding domains of connexin 32. GAP 8 lacked the N-terminal ACA sequence of GAP 8M (see Table 1). In contrast with GAP 8M, Ca^{2+} binding to the mixture of 68 μM GAP 8 and 50 nM TA-calmodulin had less than 10% effect on the half-maximum fluorescence change and the fluorescence intensity of TA-calmodulin (see Table 1). Thus the ACA sequence is essential for calmodulin binding of GAP 8M. It is likely that one of the alanine residues (Ala^1 or Ala^3) is a determinant of binding, as it may be the crucial hydrophobic residue in the N-terminus of the peptide to associate with the C-terminal domain of calmodulin.

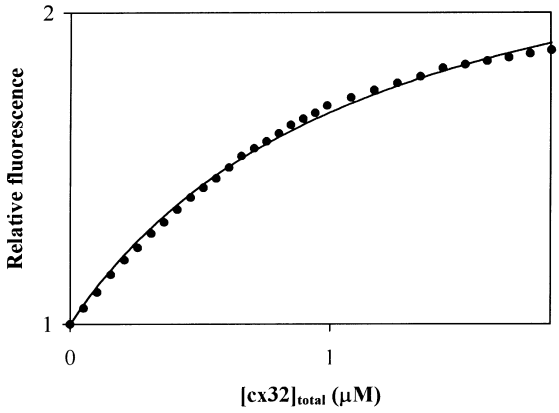

Figure 4 TA-calmodulin binding to purified rat liver gap junctions

A suspension of freshly prepared rat liver gap junctions [1 mg/ml connexin 32 (cx32)] was continuously titrated into 50 nM TA-calmodulin in 50 mM Mes-K^+ adjusted to pH 7.0, 100 mM KCl, 2 mM $MgCl_2$ and 100 μM $CaCl_2$ at 21 °C. Discrete points from the titration curve are overlayed with a curve fitted to 1:1 binding stoichimetry.

A similar approach was used to define important regions of the GAP 10 calmodulin-binding domain. Omission of the N-terminal six residues of GAP 10 in GAP 25 resulted in a 500-fold weakening in TA-calmodulin binding, K_d of GAP 25 for TA-calmodulin being increased to 14 μM (see Table 1). GAP 3 contained the first ten residues of GAP 10 only. Ca^{2+} binding to the mixture of 35 μM GAP 3 and 50 nM TA-calmodulin had less than 10% effect on the half-maximum fluorescence change and the fluorescence intensity of TA-calmodulin (see Table 1). Thus GAP 3 and GAP 25 appear to represent one-half each of the N-terminal calmodulin-binding domain. Each of the two halves is likely to contain an essential hydrophobic residue. Tryptophan in position 3 or 4 is often an important determinant in calmodulin binding [34] and Trp^3 is highly conserved in the connexin family [1–3]. The contribution of Trp^4 to a high affinity for calmodulin was tested. In GAP 18M, replacement of Trp^4 by Tyr increased K_d to 1.2 μM (see Table 1). Compared with the affinity of GAP 10 for TA-calmodulin, the binding of GAP 18M was reduced 40-fold. A similar significant decrease of affinity for calmodulin has been found when the Trp residue was replaced by Tyr in a calmodulin-binding peptide derived from smooth-muscle MLCK [24]. Thus we conclude that, in GAP 10, Trp^3 is a major N-terminal calmodulin-binding residue contributing to high-affinity binding to calmodulin. In the C-terminal half of GAP 10, Val^{13} or Ile^{20} are the candidates most likely to be important for calmodulin binding.

TA-calmodulin binding to rat liver gap junctions

The binding of TA-calmodulin to gap-junction plaques prepared from rat liver homogenates and insect cells expressing recombinant gap-junction-like plaques constructed of connexon 26 was also studied. As shown in Figure 4, with both of these gap-

junction preparations, a relatively weak binding of TA-calmodulin to the plaques was observed (K_d 1–2 μM). Interaction with these gap-junction preparations did not result in a shift in the fluorescence Ca^{2+}-titration curve of TA-calmodulin, in contrast with peptides GAP 10 and GAP 8M.

Several possibilities may account for these differences. The assembly of connexins into connexon hemichannels, followed by their accretion into gap-junctional plaques, could render the calmodulin-binding sites less accessible, or conformational changes may have been induced during the preparation of gap-junctional plaques from liver plasma membrane or the baculovirus-infected insect cells. It is also possible that a further calmodulin-binding site that is Ca^{2+}-independent may mask the Ca^{2+}-dependent interactions of calmodulin with gap junctions, and additional factors may be required to expose the calmodulin-binding regions in gap junctions. A further explanation accounting for differences in Ca^{2+}-dependent binding observed between the binding of TA-calmodulin to the peptides and gap-junctional plaques constructed of connexin 26 or 32 may be that calmodulin functions primarily during oligomerization of connexins into connexon hemichannels, a process occurring in a pre-Golgi compartment ([37,38]; W. H. Evans, A. M. Jones, G. Carlisle, E. Kalapothakis and D. Becker, unpublished work). In summary, the present results identify two cytoplasmically oriented calmodulin-binding domains in connexin 32.

The physiological significance of the calmodulin-binding sites in connexin proteins is of importance. Calmodulin interaction with isolated junctions should ideally be studied using a preparation in which the functional integrity of the gap-junction channel is preserved. Such preparations are not yet available. Routinely, and as was also the case for the present study, the isolation of gap junctions involves stripping the surrounding membrane with NaOH. This process may cause conformational changes that make the calmodulin-binding sites less accessible. Thus the observed weak binding of calmodulin to such preparations may not reflect the real situation with functionally intact gap junctions. As a result of our finding, it will now be possible to determine whether direct binding of calmodulin to gap junctions affects gap-junction function by overexpression and functional studies in cells of mutant connexins in which calmodulin-binding sites are disabled. Preliminary data suggest that the integrity of the N-terminal calmodulin-binding domain of connexin 32 is required for the function of the gap-junction channel. Injection of mRNA encoding the mutant of connexin 32, in which Trp^3 has been replaced by Tyr, into *Xenopus* oocyte pairs resulted in abrogation of gap-junction channel conductance (L. C. Barrio and W. H. Evans, unpublished work).

The present approach to assess the interaction of calmodulin with synthetic peptides corresponding in sequence to connexins by using a fluorescently labelled derivative of calmodulin has allowed the identification of specific calmodulin-binding domains and determination of the dissociation constants. A fluorescence assay was chosen to enable work in solution. The use of short peptides allowed detergent-free conditions. Calmodulin-binding domains of several calmodulin-regulated proteins have usually been identified from mixtures of peptides obtained by proteolysis, using the criterion of specific binding to calmodulin [34,39]. TA-calmodulin is well-characterized [24]. In gap junctions, any calmodulin-binding amino acid sequences should be at the cytoplasmic face of the connexins. The present data show that this was the case, for no calmodulin binding was detected in peptides corresponding in sequence to the two external loops that are highly conserved in all connexins thus far identified [1–3]. Furthermore, the peptides, including those corresponding to amino-acid sequences in the intracellular loop region and a further three peptides corresponding to similarly located sequences in connexins 26 and 43, exhibited no binding to TA-calmodulin.

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