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Calmodulin Binds to Chick Lens Gap Junction Protein in a Calcium-Independent Manner

Abstract. *A biochemically active conjugate of calmodulin and tetramethylrhodamine isothiocyanate (CaM-RITC) was synthesized. When incubated with sections of chick lens, this conjugate bound to the surface membranes of lens fiber cells in the presence or absence of calcium. Incubation of lens sections with antibodies to gap junction protein of lens completely blocked the binding of the conjugate to cell membranes, whereas serum from nonimmunized animals or antibodies to other lens proteins reduced the binding only slightly. By means of a gel overlay procedure, ^{125}I-labeled calmodulin was found to bind to the gap junction protein of lens, also in a calcium-independent manner. These results support the concept that calmodulin may interact with and regulate gap junctions in living cells.*

Gap junctions are cell membrane structures that allow adjacent cells to communicate by permitting passage, directly from cell to cell, of molecules of molecular weight of approximately 1000 or less (*1, 2*). Gap junctions appear to be important in a number of significant functions of normal and abnormal cells and tissues (*3–11*). An increased understanding of the regulation of gap junctions would thus be relevant to a wide range of biological events.

Calmodulin is a ubiquitous calcium-binding protein that has been described as a mediator of calcium action in numerous events in eukaryotic cells (*12*). Gap junction permeability is believed to be regulated by calcium (*1, 2*), although recently *p*H has also been suggested as a potential regulatory factor (*4, 13*). Because the junctions appear to respond to calcium ion concentrations generally within the concentration range of calmodulin affinity for calcium (*1–4, 14, 15*), and because calmodulin affinity for calcium is also affected by *p*H (*15*), it seemed possible that calmodulin might regulate gap junctions by mediating the effects of calcium.

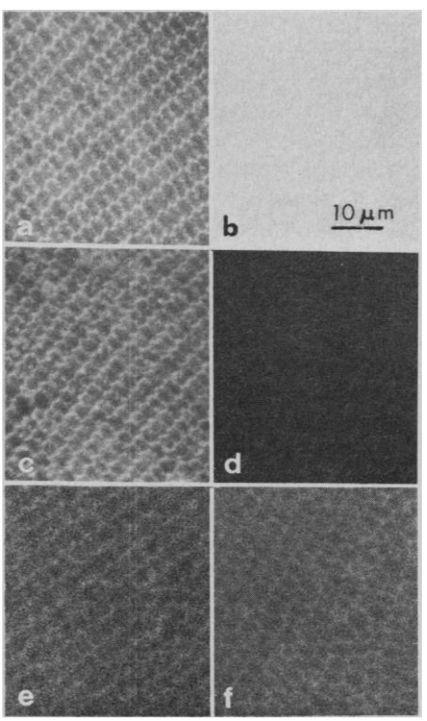

Fig. 1. The distribution of gap junction protein and CaM-RITC binding in chick lens. (a) The protein was located (*21*) on the fiber cell membranes. (b) Lens sections were incubated with CaM-RITC (*20*) in the presence of calcium, and the CaM-RITC bound to fiber cell cytoplasm and membranes. (c) In the presence of EGTA to chelate calcium, the CaM-RITC bound only to the fiber cell surface membranes. (d) Binding of CaM-RITC to the fiber cell membranes in the absence of calcium was blocked completely by incubation of lens sections with rabbit antibodies to lens gap junction protein. CaM-RITC binding was not blocked by (e) incubation with rabbit antibodies to water-soluble proteins of chick lens or (f) incubation with serum from nonimmunized rabbits. For (a and b), exposures were determined by the automatic camera on the microscope and were shorter than exposures for (c) to (f). Exposures for (c) to (f) were manually terminated after the same length of time. All prints were exposed and developed in the same manner.

To examine this possibility, we first determined the location of the calmodulin binding sites in a cell type rich in gap junctions, the lens fiber cell (*10*). Following the rationale of Pardue *et al.* (*16*) for identifying calmodulin binding sites in cells in vitro, we synthesized a conjugate of calmodulin and tetramethylrhodamine isothiocyanate (CaM-RITC) (*17*). The CaM-RITC was judged to be biochemically active because its migration pattern in sodium dodecyl sulfate (SDS)–polyacrylamide gels depended on the presence or absence of calcium; its mobility was identical to the native protein under the same conditions; and because it activated calmodulin-deficient bovine brain phosphodiesterase in a dose-dependent manner identical to that of the native protein (data not shown). The functional integrity of the CaM-RITC was further verified by microinjecting the conjugate into dividing cells and observing its rapid incorporation into the mitotic apparatus (*18*) in a pattern almost identical to that observed in cultured cells by indirect immunofluorescence (*19*). Calmodulin binding sites in chick lens were located as described (*20*). The distribution of the gap junction protein was then observed by indirect immunofluorescence (*21*).

Gap junction protein was located on the surface membranes of the lens fiber cells (Fig. 1a). When lens sections were incubated with CaM-RITC in the presence of 1 m*M* Ca^{2+}, the conjugate was found both in the fiber cell cytoplasm and at the cell surface membrane (Fig. 1b). However, when sections were incubated with CaM-RITC in the presence of 1 m*M* EGTA to chelate the Ca^{2+}, conjugate binding occurred only at the cell surfaces (Fig. 1c) in a pattern similar to the distribution of the gap junction protein as seen by indirect immunofluorescence. Incubation of the lens sections with rabbit antibodies to chick lens gap junction protein in the absence of Ca^{2+} (*22*) blocked CaM-RITC binding to the cell membranes (Fig. 1d). Control sections, in the absence of Ca^{2+}, were incubated with antibodies to water-soluble cytosol proteins of lens (Fig. 1e), with serum from nonimmunized rabbits (Fig. 1f), or with actin antibodies (not shown). These controls reduced but never eliminated CaM-RITC binding to the cell membranes. We believe that this reduction in CaM-RITC binding brought about by incubating the lens sections with antibodies to proteins other than gap junction protein indicates the presence of

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nonspecific binding sites for the conjugate. That complete blocking of the CaM-RITC binding to cell membranes occurs only when lens sections are incubated with antibodies to gap junction protein suggests that CaM-RITC binds specifically to the gap junction protein.

We also used a gel overlay procedure (*23*) to identify calmodulin-binding proteins. The Coomassie blue staining patterns for lens membrane and cytoskeletal protein fractions (*24*) are shown in Fig. 2, lanes 1 and 2. The binding of [^{125}I]calmodulin to these preparations in the presence of Ca^{2+} is shown in lanes 3 and 4, and in the absence of Ca^{2+} in lanes 5 and 6. In the presence of Ca^{2+}, [^{125}I]calmodulin bound to the 26,000-dalton gap junction protein of the fiber cell membrane fraction and, to a lesser extent, the 48,000-dalton δ-crystallin (*22*). In the absence of calcium, [^{125}I]calmodulin bound to the gap junction protein only. No protein in the cytoskeletal fraction bound the [^{125}I]calmodulin in the presence or absence of calcium. Trifluoperazine (50 μ*M*), a calmodulin inhibitor, reduced moderately but did not eliminate [^{125}I]calmodulin binding to the gap junction protein in the presence of calcium (results not shown). These results agree with the morphological results showing calcium-independent binding of CaM-RITC to lens fiber cell membranes in the vicinity of the gap junction protein. The calcium-dependent binding of calmodulin to the δ-crystallin, a major component of the cytosol of fiber cells (*25*), is consistent with the observation of calcium-dependent CaM-RITC binding to fiber cell cytoplasm in the lens sections.

A possible disadvantage of using the lens fiber cell for study of gap junction regulation is that it appears to be relatively nonresponsive to changes in Ca^{2+} (*9*). We used these cells for three reasons. First, the lens is ideally suited to morphological studies because the shape and arrangement of the fiber cells in the lens cortex permits viewing of the cell surface membranes on edge in frontal sections. This reinforces the fluorescent signal from membrane-bound fluorochromes. Second, the fiber cells possess the greatest density of gap junctions of any known tissue. Third, we believe the lens gap junction may, at least in part, be a valid model because the calmodulin inhibitor trifluoperazine affects lens junction structure, implying the possibility of a role for calmodulin in its function (*26*). We also now have evidence that calmodulin may bind to gap junction protein from tissues other than lens and that this binding also exhibits calcium independence (*27*).

Most calmodulin-regulated biochemical events previously studied have shared a mechanism involving the formation of a calcium-calmodulin complex followed by the binding of the complex to specific effector proteins (*12*). Thus the calcium-independent interaction of calmodulin with lens gap junction protein is of particular interest. Such calcium-independent binding is consistent with the possibility of calcium-dependent regulation of gap junctions by calmodulin; that is, calmodulin could induce calcium sensitivity in gap junctions but remain bound to the junction protein in the absence of calcium. Indeed, Glenny and Weber (*28*), using the gel overlay method, described a 110,000-dalton protein from the intestinal brush border that binds calmodulin in a calcium-independent manner. Other proteins can also bind calmodulin in the absence of calcium, and yet the calmodulin can induce them to become calcium-sensitive. The delta subunit of phosphorylase kinase is calmodulin and is associated with the holoenzyme whether or not calcium is present (*29*). Through the use of the gel overlay procedure proteins may be discovered that bind calmodulin only in the absence of calcium.

Trifluoperazine and chlorpromazine interfere with electrical coupling between cells as well as alter the structure of gap junctions as seen by freeze fracture (*26, 30*). Since these compounds are inhibitors of calmodulin function, such data also implicate calmodulin as a regulator of gap junction structure and function.

Our results, derived from morphological and biochemical experiments in vitro, show that the binding of calmodulin to lens gap junction protein does not necessarily depend on calcium. It would thus be interesting to determine the possible role of calmodulin in the regulation of gap junctions in vivo.

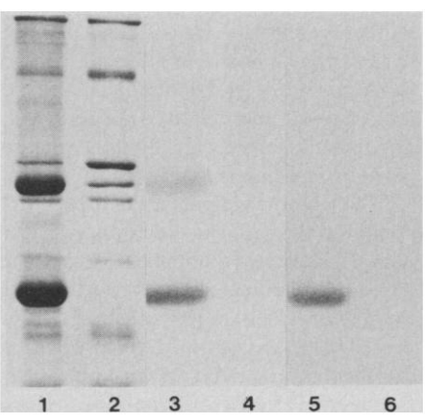

Fig. 2. Binding of [^{125}I]calmodulin to chick lens membrane and cytoskeletal fractions. The fractions were separated (*24*) on 12.5 percent SDS–polyacrylamide slab gels. Lanes 1 and 2: Coomassie blue staining pattern of (lane 1) membrane fractions and (lane 2) cytoskeleton fractions. The two major proteins in lane 1 are the 26,000-dalton gap junction protein and the 48,000-dalton δ-crystallin (*25*). Lanes 3 and 4: binding of [^{125}I]calmodulin to (lane 3) membrane and (lane 4) cytoskeleton proteins in the presence of calcium, revealed by autoradiography. Lanes 5 and 6: binding of [^{125}I]calmodulin to (lane 5) membrane and (lane 6) cytoskeletal proteins in the absence of calcium. The [^{125}I]calmodulin bound to the 26,000-dalton gap junction protein in the membrane fraction in the presence and absence of calcium and, to a small extent, to the δ-crystallin in the presence of calcium.

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20. Chick lenses were fixed overnight in Carnoy's fixative at 4°C, embedded in paraffin, and sectioned. Sections on glass slides were rehydrated and placed in 0.1*M*, 1,4-piperazinediethanesulfonic acid (Pipes) buffer, *p*H 7.3, containing either 1 m*M* Ca^{2+} or 1 m*M* EGTA for 10 minutes, and then incubated in CaM-RITC (5 μ*M*) for 1 hour at 37°C in buffer containing Ca^{2+} or EGTA; they were then rinsed and mounted in Elvanol. Incubation with nonimmune serum or antiserum (5 mg per milliliter of protein), prior to incubation with CaM-RITC, was performed at 37°C for 1 hour, with the sections being rinsed several times afterwards in Pipes buffer. Sections were viewed and photographed by means of a Leitz Orthoplan microscope equipped for rhodamine epi-illumination fluorescence microscopy. Photographs were made on Kodak Tri-X pan film.
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Neonatal Thymectomy Prevents Spontaneous Diabetes Mellitus in the BB/W Rat

Abstract. *Complete neonatal thymectomy reduced the frequency of spontaneous diabetes mellitus in BioBreeding/Worcester rats from 27 to 3 percent. Incomplete thymectomy also significantly reduced the frequency of diabetes (to 9 percent). These findings strengthen the hypothesis that thymus-dependent, cell-mediated autoimmune destruction of pancreatic β cells is responsible for the pathogenesis of diabetes in this experimental animal.*

An acute diabetic syndrome resembling insulin-dependent type 1 diabetes in humans occurs spontaneously in approximately 30 percent of a colony of BioBreeding/Worcester (BB/W) rats. Salient features of the syndrome include genetic predisposition (*1*); abrupt onset of insulin-dependent, ketosis-prone diabetes between 60 and 120 days of age; lymphocytic insulitis with virtually complete destruction of insulin-synthesizing pancreatic β cells (*2*); and occurrence of the syndrome in animals raised in a gnotobiotic environment (*3*).

It has been hypothesized that the BB/W diabetic syndrome is the result of a cell-mediated, autoimmune destruction of pancreatic β cells (*4*). Support for an immune pathogenesis stems from the predominantly lymphocytic nature of the insular infiltrate and the observation that injections of rabbit antiserum to rat lymphocytes frequently normalize plasma glucose levels in acutely diabetic rats and prevent hyperglycemia in susceptible littermates (*4*). Another characteristic of BB/W rats which resembles certain human type I diabetics and lends support to the syndrome's immune pathogenesis is the presence of lymphocytic thyroiditis in approximately 10 to 25 percent of nondiabetic animals and 50 to 60 percent of diabetic animals (*5*).

In this report we present evidence that complete neonatal thymectomy prevents the occurrence of diabetes in virtually all susceptible animals. Inadvertent partial thymectomy also provides significant protection. In contrast, sham-operated rats become diabetic with the expected frequency. The effectiveness of thymectomy in preventing diabetes supports the hypothesis that a thymus-dependent, cell-mediated immune destruction of pancreatic β cells is responsible for diabetes in the BB/W rat (*6*).

We subjected BB/W rats (*7*) to thymectomy or sham surgery within 24 hours of birth (*8*). Surviving animals were tested for glycosuria three times weekly from 60 to 150 days of age. Rats were defined as diabetic if their urine glucose was shown to be 2+ or greater with Testape (Lilly) and if their plasma glucose concentration exceeded 200 mg/dl (*9*). Diabetic animals were killed immediately and nondiabetic rats were killed at 150 days of age. At autopsy, the thoraxes of thymectomized rats were explored for evidence of thymic remnants (*10*). Among sham-operated animals, the presence of an intact thymus was verified by gross or microscopic examination or both. Pancreases were fixed in Bouin's solution, sectioned, stained with hematoxylin and eosin, and examined for evidence of insulitis. Thymus and pancreas slides were studied (by A.A.L.) without knowledge of the operative procedure performed and the physiological status of the animal. The frequencies of diabetes and insulitis among groups were evaluated by chi-square analysis.

Two groups of rats were subjected to the experimental procedures. The first group was studied in the summer of 1980 when the incidence of diabetes in the colony was approximately 20 percent. The second group was studied from January to June 1981 when the frequency of diabetes had reached approximately 40 percent (*11*). The data for both experimental groups are combined for the sake of brevity. This does not alter the results or the statistical evaluation of the data.

Complete thymectomy proved difficult to achieve. It was verified microscopically in 63 of 296 (21 percent) of the animals in which it was attempted (Fig. 1).

As shown in Table 1, 27 percent of the sham-operated rats became diabetic while only 3 percent of the completely thymectomized and 9 percent of the incompletely thymectomized animals evidenced diabetes. The severity of diabetes and the age of onset, however, were similar among the three groups. Insulitis with normoglycemia was present in 15 percent of the sham-operated rats, 11 percent of the completely thymectomized rats, and 20 percent of the incompletely thymectomized animals. Although these results are not significantly different, the incidence of combined diabetes and normoglycemic insulitis was significantly greater among sham-operated rats than among thymectomized animals (Table 1).

Table 1. Incidence of diabetes and insulitis in BB/W rats given complete thymectomy, incomplete thymectomy, or sham surgery as neonates. Numbers in parentheses are percentages.

Treatment	Number of rats showing disease		
	Diabetes	Insulitis with normoglycemia	Diabetes and insulitis
Sham surgery	39 of 144 (27)	16 of 105 (15)	55 of 144 (38)
Complete thymectomy	2 of 63 (3)*	7 of 61 (11)	9 of 63 (14)†
Incomplete thymectomy	21 of 233 (9)*	42 of 212 (20)	63 of 233 (27)‡

*Significantly different from corresponding control value at $P < .0005$ (chi-square test). †Significantly different from control value at $P < .001$ and from incomplete thymectomy value at $P < .05$. ‡Significantly different from control value at $P < .025$.

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