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Localization of calmodulin in rat tissues

(Ca^{2+}/liver/muscle/corticotropin/glycogen)

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ABSTRACT **The localization of calmodulin, a calcium-dependent modulator of many enzymes, was studied in rat liver, skeletal muscle, and adrenal slices. Calmodulin is found in liver cytoplasm, nucleus, and plasma membrane. Much of the cytoplasmic calmodulin is associated with glycogen particles presumably bound to enzymes involved in glycogen metabolism. Skeletal muscle calmodulin is found on the I-band, also associated with glycogen particles. Intermyofibrillar staining that is not glycogen associated is also observed. Calmodulin is localized in the cytoplasm and nucleus of adrenal cortex cells. Injection of corticotropin leads to a greatly increased localization of calmodulin in nuclei of the adrenal cortex. These observations suggest that one role of calmodulin may be the regulation of hormone effects on nuclear processes.**

Calcium ions regulate many cellular processes that are usually mediated through their interaction with high-affinity calcium-binding proteins. One such protein, calmodulin, mediates the effect of calcium on a wide variety of cellular enzymes. Calmodulin was originally described as an activator of cyclic nucleotide phosphodiesterase (1, 2). Subsequently, calmodulin was found to regulate many other enzymes, including the Ca^{2+},Mg^{2+}-ATPase (or calcium transport) in several tissues (3–7), one class of brain adenylate cyclase (8–10), and the protein kinases that phosphorylate myosin light chain (11, 12), glycogen synthase (13, 14), and phosphorylase (15). Information concerning the intracellular locations of calmodulin may shed light on as yet unknown calmodulin functions. Indeed, Marcum *et al.* (16) used the immunofluorescent localization of calmodulin on spindle fibers of dividing cultured cells as a clue for an investigation of the role of calmodulin on microtubule disassembly.

This study details the immunofluorescent localization of calmodulin associated with glycogen and other cellular structures in several rat tissues. Calmodulin localization in adrenal cell nuclei can be altered by the actions of a hormone, suggesting a role for calmodulin in hormone action on nuclear processes.

METHODS AND MATERIALS

Antisera to dinitrophenylcalmodulin were raised in two rabbits (17). These sera were characterized by radioimmunoassay (ref. 17; unpublished results) and by their immunofluorescent staining of tissue slices. These techniques revealed several differences in the properties of these two antisera, although qualitative differences in their ability to stain tissue slices immunofluorescently were usually not apparent (unpublished results).

Tissue was usually obtained from male rats (150–300 g, Charles River CD) that had been killed by cervical dislocation. Rats providing skeletal muscle samples were injected with 50 mg of pentobarbital instead, and those providing adrenal tissue were injected with dexamethasone (0.5 mg intraperitoneally) 21, 16, and 11 hr prior to sacrifice. Some rats were also given 20 units of corticotropin (ACTH) respository gel subcutaneously 16, 11, and 1 hr before sacrifice. Liver or adrenal pieces were placed in optimal cutting temperature compound (OCT compound) prior to freezing in a dry ice/acetone bath; skeletal muscle was prepared differently (see below).

Immunofluorescence studies were performed with 4-μm-thick tissue slices by using an indirect "sandwich" technique (18). Briefly, tissue slices were washed twice for 5 min with phosphate-buffered saline and then incubated for 30 min with the Ig fraction of anticalmodulin antiserum. The antiserum was diluted 1:12 from the original serum volume with phosphate-buffered saline. This incubation was followed by a phosphate-buffered saline wash (three times at 3 min each), a 30-min incubation with fluorescein-conjugated goat anti-rabbit IgG (1:15), and a final wash in phosphate-buffered saline (three times at 3 min each), before the tissue was overlaid with 50% glycerol/phosphate-buffered saline and a coverslip was applied. The buffer contained neither added calcium nor chelator. In some experiments tissue slices were incubated with *Bacillus subtilis* α-amylase for 30 min prior to immunofluorescent staining in order to hydrolyze glycogen particles exposed within the slices.

Skeletal muscle (gastrocnemius) was prepared for immunocytochemistry by two methods, the choice of which depended upon the ultimate method of antibody localization. In both cases, rats were anesthesized with a barbiturate. Muscle destined for immunofluorescent studies was clamped against a razor blade *in situ*, frozen in liquid nitrogen, and then sliced frozen in optimal cutting temperature compound (18). The alternative staining method localized antibody-bound peroxidase in muscle tissue fixed with 2% paraformaldehyde. Staining was accomplished with anticalmodulin antiserum and then with peroxidase/antiperoxidase complex localized with diaminobenzadine. A densitometric trace of the staining was obtained after printing a photomicrograph onto Kodak medium-grain lantern slide plates. A recording of the density of the photographic emulsion through a thin zone parallel to the myofibril was obtained with a single pass of the densitometer.

The specificity of immunofluorescent staining for calmodulin was assessed in several ways. The dependence of fluorescein-

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Abbreviation: ACTH, corticotropin.

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conjugated second antiserum binding on the presence of prior specific first antiserum was tested by replacing the first (anticalmodulin) antiserum with either the dilution buffer or with serum from an unimmunized rabbit. Little or no staining was observed in any tissue. Alternatively, anticalmodulin antiserum could be absorbed (neutralized) with essentially pure calmodulin before application to the tissue. Absorption was achieved through incubation of antigen with antibody for 30 min at 37°C and then overnight at 4°C by using an empirically defined "optimal" ratio of antigen to antibody. The anticalmodulin antiserum was not absorbed with a similar concentration of purified troponin C, a Ca^{2+}-binding protein that has 70% sequence homology with calmodulin (19). Troponin C was also found not to interfere with the radioimmunoassay for calmodulin (17).

All photographs shown were obtained by using one of the anticalmodulin sera, but qualitatively identical results were obtained with the other antiserum (not shown). The photographic negatives and prints in each figure were exposed and developed under identical conditions to provide a semiquantitative record of immunofluorescent staining. Differences in light intensity observed in the microscope were thus recorded as differences of contrast in the final photographs. All results shown in the figures have been repeatable without contradiction.

Purified troponin C was a generous gift from T. C. Vanaman. Fluorescein-conjugated goat anti-rabbit IgG and optimal cutting temperature compound were purchased from Miles. ACTH gel is a product of Armour (Phoenix, AZ) and dexamethasone was obtained from Merck. *Bacillus subtilis* α-amylase (695 units/mg of protein) was obtained from Sigma.

RESULTS

The immunofluorescent localization of calmodulin has been performed in several rat tissues. Distinct patterns of localization have been obtained with each. Calmodulin-specific immunofluorescence of liver slices is presented in Fig. 1. Plasma membranes are usually well defined and brightly stained. Nuclear staining, seen as a reticular pattern of small irregularly shaped areas, is also apparent. The most striking feature, however, is the intensely stained and "dotted" cytoplasm. This granular pattern probably localizes calmodulin in glycogen particles, because the fluorescence intensity was diminished greatly by treatment of liver slices with α-amylase (Fig. 1*c*), a classical technique for demonstrating the disruption of glycogen particles (20). Depletion of glycogen accomplished physiologically in tissue slices prepared from fasted rats likewise decreased the granular cytoplasmic staining (Fig. 1*b*). Neither the staining in the plasma membrane nor that in the nucleus was changed by these treatments, suggesting that the calmodulin found associated with these sites has a function distinct from regulation of glycogen metabolism. Not all cytoplasmic calmodulin is associated with glycogen particles, because neither technique removed all cytoplasmic immunofluorescence. A control experiment with absorbed anticalmodulin antiserum showed that the staining observed in all areas was greatly decreased and was thus specific for the antigen calmodulin (Fig. 1*d*).

Skeletal muscle calmodulin is localized in a highly ordered pattern. At least two compartments are apparent in longitudinal sections of gastrocnemius muscle. Intense staining of one of the muscle bands is apparent, as well as faint longitudinal lines that

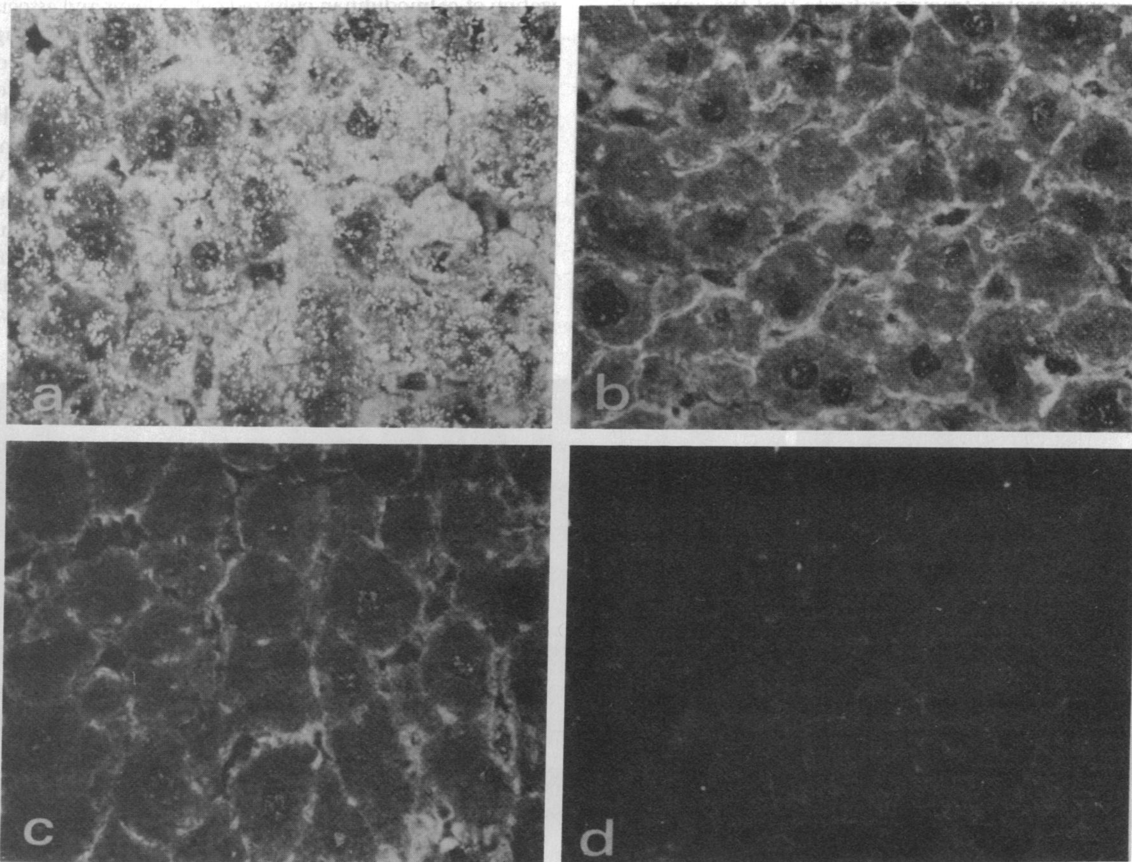

FIG. 1. Localization of calmodulin in rat liver. Rat liver slices (4 μm thick) were stained for calmodulin by using indirect immunofluorescence. (*a*) Liver taken from an untreated, fed animal. (*b*) Liver from an animal fasted overnight but given water ad lib. (*c*) Liver taken from a fed rat prior to staining the slice treated for 30 min with α-amylase (5 units in 50 μl). (*d*) Liver slice stained with antiserum absorbed with calmodulin. (×600.)

run the length of each fiber (Fig. 2). The latter apparently represents intermyofibrillar staining and is possibly due to calmodulin associated with sarcoplasmic reticulum, the plasma membrane, or mitochondria. Further characterization of the structures involved was not undertaken. Staining of each site was blocked specifically by absorption of the antiserum with purified calmodulin (Fig. 2*c*).

Experiments were conducted to define the molecular nature of the calmodulin localized in the sarcomere. Muscle preparations stained with peroxidase-labeled antiserum reveal under polarized light that the opaque, oxidized diaminobenzidine precipitate is localized on the isotropic (dark) band, because the anisotropic (light) bands are not obscured (not shown). This suggests that calmodulin is localized at the I-band. Other experiments, performed with fluorescein-labeled antisera, confirm the I-band localization. Tissues from the experiments can be photographed both with polarized light and with emitted fluorescence. Matching photographs of the same field show that the fluorescent label coincides with the I-band, as defined by the polarization micrograph (not shown).

Quantitation of the relative distribution of localized calmodulin on skeletal muscle bands can be achieved through the densitometric measurement of a photomicrograph obtained by using the immunoperoxidase staining technique. Fig. 3 shows the result of such an analysis. The densitometric tracing of calmodulin staining is aligned with the corresponding zone scanned (bound by arrows). The record of a 15-μm grid was used as a baseline; the spikes in the band pattern correspond to the arrow tips drawn onto the photograph. Calmodulin was found to be localized with a periodicity of 2.4 μm, consistent with the sarcomere length in resting skeletal muscle. The peaks found in the densitometric tracing indicate that the entire I-band, including the Z-line, stains for calmodulin. There is also some staining in the region of the A-band, seen in the graph as a slight peak above the baseline; the I-band accounts for at least 75% of total calmodulin staining.

Both glycogen and the terminal cisternae of the sarcoplasmic reticulum are known to be localized along the I-band (21, 22). The possibility that calmodulin is associated with glycogen in skeletal muscle as in the liver was tested by using the α-amylase technique. Muscle slices incubated with α-amylase showed greatly reduced staining for calmodulin along the I-band (Fig. 2*b*), supporting the notion that calmodulin is associated with glycogen particles. A rather high α-amylase concentration (500 units in 50 μl) is required to obtain this effect. However, the staining pattern with an antiserum specific for cyclic GMP was unaffected by this treatment (not shown). We have previously shown that cyclic GMP is located on the A-band (23). Furthermore, α-amylase digestion of the tissue slice did not alter the intermyofibrillar staining for calmodulin (Fig. 2*b*).

Specific localization of calmodulin can also be demonstrated in adrenal cortical cells (Fig. 4). Furthermore, an effect of ACTH on the immunofluorescence pattern can be demonstrated. All rats from which adrenals were to be removed were injected with dexamethasone prior to sacrifice. Adrenals from rats treated only with the glucocorticoid show little specific immunofluorescence for calmodulin, although faint nuclear and some cytoplasmic staining are apparent. The dark vacuoles in the cytoplasm probably represent cholesterol droplets.

The nuclear immunofluorescence for calmodulin varied with the endocrine state of the animals. Those rats that received ACTH for a total of 16 hr exhibit dramatic changes in the amount of staining. Intense immunofluorescence distributed in multiple sites was found in nuclei after hormone administration. There is specificity of this hormone effect, because it is seen only in cells responsive to ACTH. Cells from the adrenal medulla in the same preparations do not show any changes in immunofluorescence after ACTH injection (not shown). No immunofluorescence was observed in adrenal cortex when tissue slices were stained with serum from an unimmunized rabbit (Fig. 4*c*) or with absorbed antiserum (not shown).

DISCUSSION

Several studies have reported the immunofluorescent localization of calmodulin in cultured cells. Means and associates (24, 25) and Andersen *et al.* (26) found calmodulin staining associated with a stress cable (actinlike) pattern in quiescent cells, but no nuclear staining was observed. Dividing cultured cells, however, had certain areas of the mitotic apparatus that were heavily stained for calmodulin. These investigators may have used cell preparations for which the antisera did not have access to nuclear calmodulin during the interphase. Alternatively, their antisera, which were produced against native calmodulin, may recognize only those parts of the antigen that are bound to and thus screened by nuclear molecules. Wood *et al.* (27) have used an immunoperoxidase technique at the electron microscopic

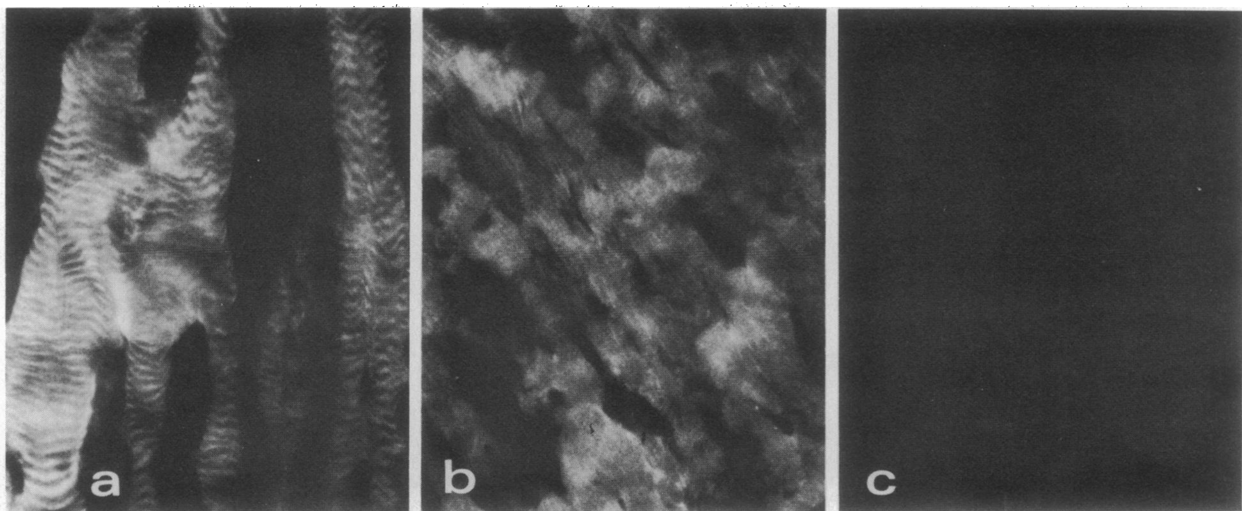

FIG. 2. Localization of calmodulin in rat skeletal muscle. Slices taken from the rat gastrocnemius muscle were stained for calmodulin. (*a*) Untreated slice. (*b*) Slice treated for 30 min with α-amylase (500 units in 50 μl). (*c*) Untreated tissue slice stained with antiserum absorbed with calmodulin prior to use. (×575.)

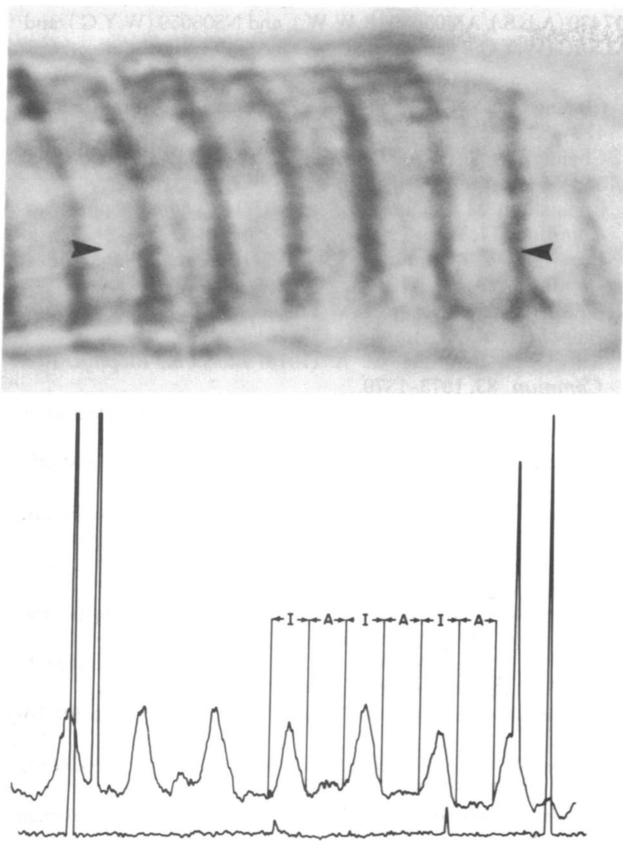

FIG. 3. Quantitation of staining on skeletal muscle. Skeletal muscle fixed at its resting length was stained for calmodulin by the immunoperoxidase technique. A densitometer trace of the muscle band pattern is shown below the corresponding photograph of the area analyzed. The baseline spikes are calibrated at 15 μm, and the spikes in the trace correspond to the points marked by the arrows in the photograph. The areas of I- and A-bands are shown. Band areas were defined by polarization microscopy. (×4000.)

level to demonstrate localization of calmodulin in the postsynaptic densities and dendritic microtubules in mouse brain.

Our results indicate that quiescent nuclei in slices of rat tissue do contain immunoreactive calmodulin. The specificity of the staining can be shown by using antisera previously absorbed with purified calmodulin. The functional significance of nuclear calmodulin in resting cells is unknown at this point. Calmodulin may only serve as a protein waiting to be utilized during cell division. This seems unlikely for several reasons. Cells in the liver and adrenal cortex seldom undergo division, although both cell types show staining in the nucleus. Furthermore, the administration of ACTH produces a large increase in nuclear calmodulin staining relative to dexamethasone-treated rats. This treatment, which would be expected to produce hypertrophy but not hyperplasia at the time studied, nevertheless increases the intensity of nuclear immunofluorescence. Calmodulin may be present as a regulator of RNA synthesis in response to ACTH. The bright staining of ACTH-stimulated adrenal cell nuclei occurs in a few discrete nuclear sites, a pattern consistent with a nucleolar localization of calmodulin.

In most cases we can only guess at the biochemical significance of calmodulin localized to any particular cellular compartment. The use of the specific enzyme α-amylase, however, affords us a relatively specific control. Disruption of glycogen particles, which are known to contain many enzymes responsible for glycogen metabolism (28), probably allows the cytoplasmic calmodulin localized in liver slices and on the skeletal muscle I-band to be washed from the tissue slice. The effect of α-amylase in both tissues is consistent with the specific removal of glycogen-associated enzymes, such as phosphorylase b kinase and glycogen synthase kinase, both of which are activated by the calcium–calmodulin complex (13–15).

Glycogen and enzymes involved in its metabolism in skeletal muscle are located along the I-band (21, 28). Jennissen *et al.* (29) have reported that the immunofluorescent localization of phosphorylase kinase shows faint staining along what may be the I-band as well as in sarcoplasmic reticulum. Although the amylase technique was not tested in these studies, the results are consistent with the notion that the enzymes are localized in glycogen particles.

It is not known whether the staining associated with glycogen in skeletal muscle is also associated with the sarcoplasmic reticulum. Two classes of skeletal muscle glycogen unit have been described, both of which contain enzymes for the metabolism of glycogen (30–32). One type of glycogen particle contains no marker enzymes of sarcoplasmic reticulum origin, whereas the other consists of sarcoplasmic reticulum vesicles that have

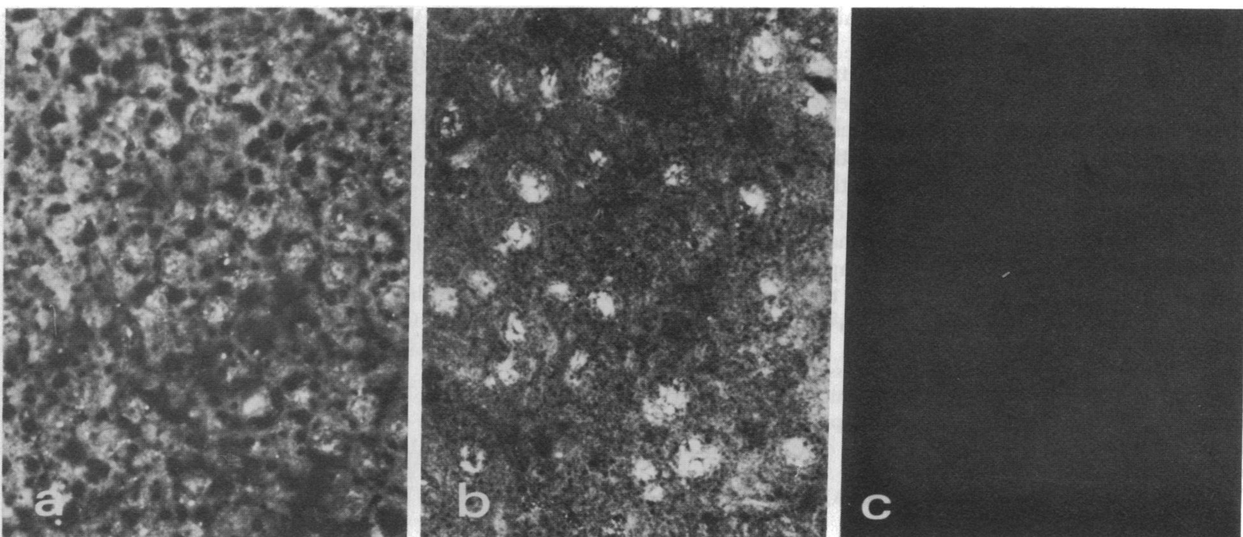

FIG. 4. Effect of ACTH on distribution of calmodulin in adrenal cortex. Rat adrenals were removed after drug injections. (*a*) Adrenal tissue removed from a dexamethasone-treated rat. (*b*) Tissue removed from a rat that had been treated with both dexamethasone and ACTH. (*c*) Tissue stained with serum from an unimmunized rabbit. (×575.)

glycogen particles specifically attached to their membranes. Amylase digestion of the glycogen in either particle releases the associated enzymes (30, 31). Calmodulin could be associated with one or both types of glycogen.

Pretreatment of tissue slices with α-amylase did not remove all immunofluorescence. The cellular functions of the calmodulin not removed by α-amylase are unknown. Calmodulin associated with membranes, especially skeletal muscle sarcoplasmic reticulum, may be present as a regulator of calcium transport. Indeed, calmodulin has been found to be an activator of the Ca^{2+},Mg^{2+}-ATPase, or of calcium transport, or both in several other membranes (3–7). Jorgensen *et al.* (33) localized skeletal muscle Ca^{2+},Mg^{2+}-ATPase at the I-band (presumably on the sarcoplasmic reticulum), as well as in a roughly longitudinal pattern in A-bands. It is possible that the intermyofibrillar staining for calmodulin is due to a calmodulin-regulated Ca^{2+},Mg^{2+}-ATPase sarcoplasmic reticulum. Calmodulin is known to exist in skeletal muscle as a largely extramyofibrillar protein (34) but has not been shown to regulate its ATPase. However, cardiac sarcoplasmic reticulum Ca^{2+},Mg^{2+}-ATPase has been found activable upon the addition of rather high concentrations of calmodulin (6). Skeletal muscle may have an analogous calmodulin-regulated ATPase. Other enzymes known to be regulated by calmodulin include several protein kinase activities (11–15) and cyclic nucleotide phosphodiesterase (1, 2).

The fraction of all cellular calmodulin actually localized by the immunofluorescent technique is unknown. Soluble calmodulin may be lost from the tissue slice during washing steps, so that only a small percentage of total calmodulin is visualized. If this is true, it might be expected that active calmodulin is preferentially retained in the tissue section, because the formation of calmodulin–receptor complexes are known to occur only in the presence of Ca^{2+}. Thus, calcium-replete calmodulin would be more likely to be bound to a component of the cellular structure than would calcium-free calmodulin and would be less likely to be removed during subsequent aqueous phase procedures. The visualized calmodulin may therefore represent an "active" Ca^{2+} pool. The anticalmodulin antiserum recognizes calmodulin whether calcium is bound or free, although it does so with slightly greater affinity in the presence of ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) (17).

The immunofluorescent localization of calmodulin reveals that it is associated with glycogen and membranes, findings predictable from previous biochemical studies. The localization of calmodulin at the nucleus is quite pronounced, especially after ACTH stimulation of adrenal cortex cells. Preliminary results indicate that similarly increased nuclear calmodulin localization occurs in liver after hormone stimulation. Hormones may act not only through regulation of the concentration of intracellular Ca^{2+} but through regulation of calmodulin as well. Especially intriguing is the possibility that hormone regulation of nuclear function could be accomplished through alterations of calmodulin location, which in turn may be indicative of an altered location of "active" Ca^{2+}.

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