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# Mitosis in sand dollar embryos is inhibited by antibodies directed against the calcium transport enzyme of muscle

(cell division/microinjection/microtubules/mitotic apparatus/calcium pump)

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**ABSTRACT** **Monospecific antibodies to the calcium transport enzyme ($\alpha$-Ca pump) inhibit mitosis when microinjected into sand dollar embryos. Immunoglobulins were raised against the calcium transport enzyme (Ca pump) of sarcoplasmic reticulum (SR) from rat skeletal muscle and guinea pig ileum smooth muscle. Specific antibodies were further isolated from IgG fractions by using electrophoretically purified SR Ca-pump protein as the immobilized ligand for immunoaffinity chromatography. ELISA demonstrated that common antigenic determinants are shared by SR, SR Ca pump (of rat skeletal and guinea pig ileum smooth muscle), and isolated membrane containing "native" mitotic apparatus (MA). Preimmune sera gave negative results in identical control assays. Triton X-100 extraction of MA removes the Ca-pump antigen. SR Ca pump and the MA Ca pump have nearly identical molecular masses as determined by NaDodSO$_4$/PAGE. These $\alpha$-SR Ca-pump IgGs inhibit ATP-dependent $Ca^{2+}$ sequestration by purified SR and MA membranes. Indirect immunofluorescence of isolated native MA demonstrated coincident localization of the MA Ca pump, sequestered calcium, and membrane vesicles. Fluorescent foci were regionally concentrated within the volumes of the asters and spindle. Microinjection of the anti-Ca-pump IgGs into one of two sister blastomeres at second metaphase resulted in mitotic arrest of the injected cell accompanied by a rapid loss of spindle birefringence. Karyomeres formed and fused to form nuclei either at the site of the metaphase plate or at the position the chromosomes occupied during anaphase A. The cleavage furrow did not develop in the injected cell, while the sister and neighbor cells continued normal mitotic cycling. Injection later in mitosis yielded cells with two nuclei whose cleavage furrow relaxed completely. Routine control injections of boiled immune IgG, preimmune IgG, Wesson oil, buffer, or goat anti-rabbit IgG did not affect mitosis, birefringence of the MA, or cleavage furrow activity.**

Regulation of the assembly and functioning of the mitotic apparatus (MA) are central aspects in the cell cycle. Calcium has been shown to be of importance in the regulation of this cycle. For example, protein synthesis (1), nucleic acid synthesis (2–4), cyclic nucleotide activity (5–7), cell growth (8, 9), the assembly state of microtubules (10–12), and birefringence (BR) of the spindle and aster fibers *in vivo* (11, 12) are all affected by changes in the intracellular concentrations of calcium. The most extensively studied calcium regulatory system to date is the sarcoplasmic reticulum (SR) (e.g., refs. 13–29). In the case of muscle and the SR, a direct functional relationship between the activity of a single polypeptide, the SR calcium transport enzyme (SR Ca pump), and a specific physiological event, muscle contraction, has been established. Balanced regulation of the calcium sequestration and calcium efflux from intracellular stores provide the means of regulating the state of contraction and relaxation of the sarcomere.

Assembly of the MA and anaphase chromosome movement are hallmarks of mitosis. Both involve orderly changes in the assembly state of microtubules (30) and have been postulated to be regulated by calmodulin (31) and/or calcium-sequestering membrane vesicles (32–37). Examination of MA *in vivo* and *in vitro* with the transmission electron microscope and the light microscope has shown the MA to be rich in membrane vesicles and reticula (e.g., refs. 32–40). An indication that the membranes might be involved in the regulation of assembly and function of the MA came from the demonstration that membranes present in isolated sea urchin MA actively sequester $^{45}Ca$ in an ATP-dependent fashion (37). Still the question remains: Is calcium sequestration required for mitosis? To answer this question, monospecific, affinity-purified, anti-SR Ca-pump IgGs were prepared. These anti-Ca-pump IgGs labeled calcium containing membrane vesicles of sea urchin MA, inhibited ATP-dependent calcium sequestration by isolated SR and vesicles purified from isolated native MA, and, upon microinjection into mitotic cells, resulted in mitotic arrest, reduction of MA BR, and relaxation of the cleavage furrow.

## MATERIALS AND METHODS

Native MA were prepared from sea urchin embryos (*Strongylocentrotus purpuratus*) and characterized as described (37, 40). Sand dollar embryos (*Echinarachninus parma*) were cultured according to Just (41), except they were grown in calcium-free Jamarin-U after mechanical removal of the fertilization envelope (unpublished). This was done to ensure that the sister blastomeres would remain spherical and therefore optically identical (42). Calcium-sequestering reticula (MAR) were prepared from isolated native MA by differential sucrose-gradient ultracentrifugation and calcium oxalate loading (37, 42). Vesicles were assayed for $Ca^{2+}$ uptake in 100 mM KCl/30 mM imidazole/5 mM $MgCl_2$/1 mM MgATP/0.01 mM $CaCl_2$/30 $\mu$M antipyrylazo III, pH 7.2, at 20°C with a Hewlett Packard HP-8451 photodiode spectrophotometer (e.g., ref. 43).

Polyclonal antibodies were raised in rabbits against SR prepared from rat skeletal muscle (28) and guinea pig ileum smooth muscle (27). Preimmune and immune IgGs were prepared by two $(NH_4)_2SO_4$ precipitations (44) and DEAE-Affi-Gel blue (Bio-Rad) chromatography (42). The SR Ca-pump proteins were purified by standard procedures (25, 26) and then electrophoretically purified (42). The 105-kDa band was excised, electroeluted, and then coupled to Affi-Gel 10 (42). After washing, IgG preparations were loaded onto the columns, and the specific bound IgG fractions were eluted

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Abbreviations: MA, mitotic apparatus; SR, sarcoplasmic reticulum; MAR, calcium-sequestering reticula of the mitotic apparatus; BR, birefringence; TRITC-G$\alpha$R, tetramethylrhodamine isothiocyanate-labeled goat anti-rabbit IgG.

with 200 mM glycine hydrochloride (pH 2.5) (42). Affinity-purified IgGs were then dialyzed against calcium-free phosphate-buffered saline and stored until needed. Specificity was tested by using a portion of these IgGs as immobilized ligands that selectively removed the SR Ca-pump protein (105 kDa) from Triton X-100-solubilized SR (42). ELISA was performed with β-galactosidase-conjugated second antibody and modified to enhance sensitivity (42). NaDodSO$_4$/PAGE was performed as described (37, 42). Immunoblot analysis was conducted as described (45).

Calcium sequestered *in vivo* into native MA vesicles was localized as calcium-7-chlorotetracycline chelates, with fluorescence emissions at 530 nm (39, 41), in the presence of 10 mM EGTA (37). Indirect immunofluorescence was performed by using tetramethylrhodamine isothiocyanate-labeled goat anti-rabbit IgG (TRITC-GαR) as the second antibody. Direct pressure microinjections were made according to the methods of Hiramoto (46), with modifications described elsewhere (42, 47). Microscopic observations were made with a Nikon condenser rectified Nomarski differential interference-contrast (r-dic) and polarization light microscope (r-pol) equipped with an epi-fluorescence system. Images were recorded with Kodak 2415 and Tri-X films and on Sony-KCA60BR videotapes.

## RESULTS

**Production of Antibodies and Immunological Cross-Reactivities Between MA and SR Calcium Transport Enzymes.** Antisera were raised against SR isolated from guinea pig smooth muscle. A second antiserum was raised against SR isolated from rat skeletal muscle. This choice of antigens was made because (*i*) these are the sources of the best characterized Ca pumps (19–26); (*ii*) sufficient amounts of antigen could be obtained from each of these sources; (*iii*) they would serve as particulate, rather than soluble, antigens; and (*iv*) the cytoplasmic side of the SR proteins, especially those sites essential for calcium sequestration, would be exposed to the host's immune system. It was felt that the intramembranous and presumably highly antigenic portions of these proteins would be masked by the lipids of the intact bilayers and would, therefore, be less accessible as antigens. This approach has subsequently proven quite useful in identifying the cytoplasmic, extracellular, and luminal portions of several membrane proteins, including the 26-kDa gap junction protein (48), the 140-kDa actin binding protein of microvilli membranes (49, 50), and the SR Ca pump.

Immunoblot analysis demonstrated the monospecificity of the antibody preparation and the commonality of epitopes among the SR Ca pump and the MA Ca pump. A single immunoreactive band was found at 105 kDa in SR preparations, the molecular mass of the SR Ca pump. Blots of native MA showed a single immunoreactive species that comigrated with the SR Ca pump at 105 kDa. These points are illustrated in Fig. 1 *Upper*.

Antibodies, whole IgG fractions and affinity-purified α-Ca-pump IgGs, purified against rat skeletal and guinea pig ileum smooth muscle cross-react with the complementary muscle antigens as assayed by ELISA. Fig. 1 presents results of one such assay using antibodies directed against guinea pig ileum smooth muscle SR to probe SR isolated from guinea pig smooth muscle, on the one hand, and rat skeletal muscle on the other. These data show strong epitopic similarity between these two SR and thus the sharing of antigenic determinants. Native MA, which contained membranes, also cross-reacted with antibodies directed against the guinea pig SR (Fig. 1 *Lower*) and rat skeletal muscle (42). The level of cross-reaction is comparable to the cross-reactivity to rat skeletal muscle SR CA pump. Extraction of native MA with Triton X-100 resulted in a loss of the majority of the SR antigens.

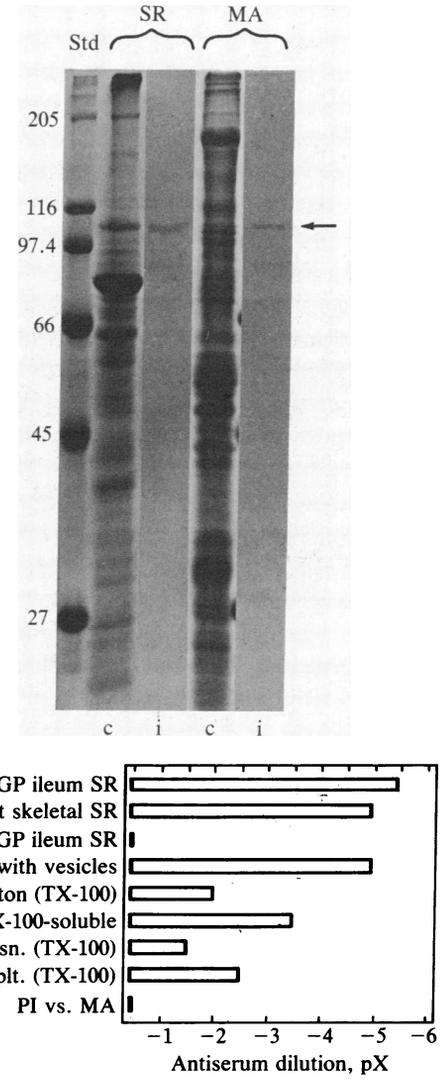


FIG. 1. Immunological cross-reactivity of the anti-Ca-pump IgG preparation assayed by immunoblotting (*Upper*) and ELISA (*Lower*). (*Upper*) NaDodSO$_4$/PAGE display of SR and MA. The Coomassie brilliant blue staining patterns (lanes c) and the immunoreactive bands that labeled with the anti-Ca-pump IgGs (lanes i) are seen in sister NaDodSO$_4$/PAGE tracks. The location of these bands is noted with an arrow. Migration of molecular mass markers (Std) is indicated in kDa. (*Lower*) ELISA for immunological reactivity of anti-Ca-pump IgG against several different antigen sets from muscle and the MA. The 50% reactive dilutions are indicated. Cross-reaction between the anti-guinea pig SR Ca-pump IgGs with the guinea pig SR (GP ileum SR) and the MA with vesicles is within the same order of magnitude as cross-reaction with skeletal muscle SR from rat (Rat skeletal SR). Washing the MA with 0.5% Triton X-100 [MA skeleton (TX-100)] dramatically reduced the reactivity of the apparatus. The activity lost upon Triton washing was found in the low-speed Triton X-100 supernatant (MA TX-100-soluble). Further centrifugation in an Airfuge demonstrated that the antigen that shares epitopes with the calcium transport enzyme can be sedimented after an hour at 97,000 rpm (Airfuge plt.), the remaining activity being found in a soluble supernatant (Airfuge sn.). Preimmune sera (PI) were negative. pX, log of IgG dilution factor.

These were present in the first Triton X-100 supernatant. The majority of the Triton-X-100-soluble antigenicity was sedimentable in an Airfuge under conditions comparable to those known to sediment skeletal muscle SR Ca pump (25, 26). Preimmune sera showed no cross-reactivity with either guinea pig ileum smooth muscle SR or native MA (Fig. 1 *Lower*).

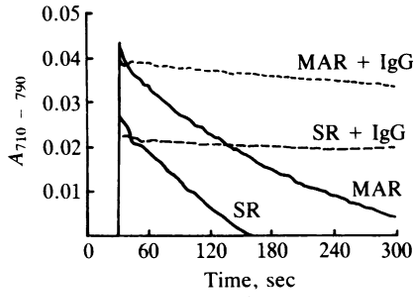


FIG. 2. Inhibition of calcium uptake by anti-Ca-pump IgG. ATP-dependent calcium sequestration is seen in control preparations of rabbit skeletal muscle SR and the calcium-sequestering vesicles of the MAR. Calcium sequestration was inhibited by preincubation with 2 ng of anti-Ca-pump IgGs of MAR (MAR + IgG) and SR (SR + IgG) for 30 min at 20°C. Calcium sequestration is seen as a reduction in $A_{710} - A_{790}$. Reaction conditions: 100 mM KCl/30 mM imidazole/5 mM $MgCl_2$/37 mM antipyrylazo III/1 mM $Na_2ATP$, pH 7.20, 20°C. SR preparations were challenged with 10 ng of $CaCl_2$, whereas MAR preparations were challenged with 20 ng of $CaCl_2$ (this was done to attenuate the MAR signals for graphical purposes). SR preparations used 10 $\mu$g of protein; MAR preparations were from $10^6$ MA. Preimmune sera had no effect on calcium uptake (data not shown).

**Inhibition of Calcium Sequestration by Monospecific $\alpha$-SR Ca-Pump IgGs.** The $\alpha$-Sr Ca-pump IgGs inhibit ATP-dependent calcium sequestration by vesicles purified from isolated SR and MAR (Fig. 2). Isolated SR preincubated with 2 ng of $\alpha$-SR Ca-pump IgG were completely inhibited in their ability to sequester calcium in an ATP-dependent fashion. Similarly, MA vesicles treated in an identical fashion were also inhibited in their calcium uptake. Control preparations not exposed to the IgG showed normal calcium sequestration activity. These inhibition data strongly suggest that the polyclonal IgGs recognize epitopes on the SR Ca pump essential for sequestration activity.

**Colocalization of the MA Ca Pump and Calcium Sequestered *in Vivo*.** The combined use of rectified Nomarski differential interference-contrast optics, fluorescence microscopy, and indirect immunofluorescence with anti-SR Ca-pump IgG demonstrated the coincidence of membrane vesicles, calcium sequestered *in vivo*, and the MA Ca pump. The conclusion that the chlorotetracycline fluorescence presented here (Fig. 3*b*) is due to calcium sequestered *in vivo* is based upon the following evidence. (*i*) Chlorotetracycline fluorescence at 530 nm is diagnostic for calcium present within the luminal spaces of, and in association with, the membranes of vesicles and reticula (39). (*ii*) The native MA were isolated and examined in a buffer containing 10 mM EGTA. Thus, the only possible source of calcium available to be complexed with chlorotetracycline would be that present within the lumen of the vesicles at the time of isolation.

In optical sections of the MA, fluorescence due to $Ca^{2+}$ (Fig. 3*b*) and the MA Ca pump (Fig. 3*c*) appears as punctate foci, nonuniformly distributed throughout the bodies of the asters and spindle of each MA. Due to the thickness of the optical sections (the micrographs presented in Fig. 3), these fluorescent foci appear as a mottled pattern. Fluorescence in Fig. 3 *b* and *c* appears to be located predominantly within the asters. Upon closer examination, astral fluorescence could be spatially divided into three zones. Zone 1 was the area occupied by the centrosome with a radius of 3 $\mu$m and centered on the mitotic pole. Zone 2 was composed of a hollow spherical shell around zone 1, extending from 3 to 8 $\mu$m from the pole. Zone 3 was the remainder of the astral volume, extended distally from 8 through 15 $\mu$m from the mitotic pole. Zone 1 contained less fluorescence than zones 2 and 3 as well as any other region of the MA. This was true for the localization of calcium and the MA Ca pump. Zone 2 was the region of predominaht fluorescence, whereas zone 3 showed intermediate fluorescence intensity for $Ca^{2+}$ and the MA Ca pump. In addition to these astral zones, $Ca^{2+}$ and the MA Ca pump were also colocalized in the central spindle and appeared along linear tracks within the spindle. Enhanced fluorescence was seen for $Ca^{2+}$ and the MA Ca pump in the region of the metaphase plate, an observation that corresponds well with the presence of membrane vesicles around the chromosomes seen with near extinction differential interference-contrast microscopy (Fig. 3*a*) as well as transmission electron microscope (42).

**Inhibition of Mitosis by the $\alpha$-Ca-Pump IgG.** Antibodies to the Ca pump inhibit mitosis when microinjected into sand dollar blastomeres undergoing mitosis. Direct pressure microinjection of the anti-Ca-pump IgG into one of the two second-cleavage, mid-anaphase blastomeres resulted in mitotic arrest of that blastomere (Fig. 4). MA BR decreased more rapidly in the injected blastomere than in the noninjected sister blastomere or blastomeres of neighboring embryos. Moreover, in the injected cell, karyomeres formed and fused to form two nuclei, each halfway between the position of the metaphase plate and the metaphase position of the mitotic poles.

While mitosis continued unabated in the noninjected control cells, the sister blastomeres that had been injected with antibodies to the MA Ca pump slowly formed a cleavage furrow (Fig. 4*B*). The contractile rings of the injected cells later halted their constriction and finally relaxed within a time span of 9 min after injection of IgG (Fig. 4 *C–D*). This observation is consistent with the fact that the contractile ring exhibits an actomyosin-type Ca-regulated contraction (51). The noninjected sister blastomere continued normal mitosis in concert with neighboring sibling embryos. The "clear" zone of the injected cell's MA remnant remained (Fig. 4*C*), with very little detectable BR, and the injected cell remained in mitotic arrest (with little change in cellular refractive index) for the next 18 hr. During that period, all noninjected control cells continued normal mitotic activity (Fig. 5*D*). Routine control injections of boiled immune IgG (Fig. 5), preimmune IgG, Wesson oil, $Ca^{2+}$-free phosphate-buffered

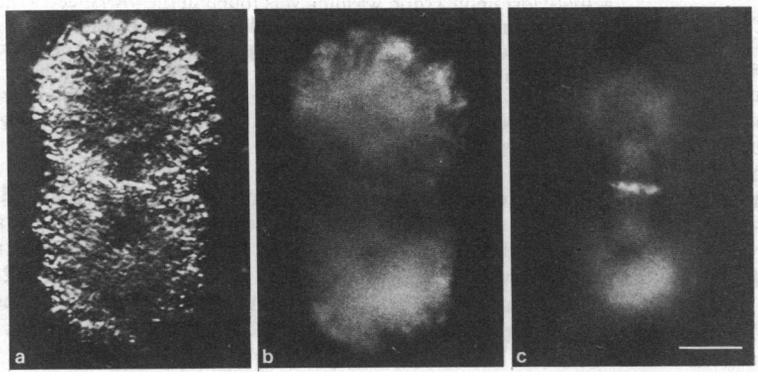


FIG. 3. Isolated bipolar metaphase mitotic apparatus seen in r-dic (*a*), fluorescence (calcium: 7-chlorotetracycline) (*b*), and indirect immunofluorescence (TRITC-G$\alpha$R: $\alpha$-SR CA-pump IgG) (*c*) light optics. Demonstrated are the colocalization of membrane vesicles resolved with differential interference-contrast optics (*a*), calcium sequestered *in vivo* (*b*), and the calcium transport enzyme, all of the MA (*c*). Note the zonal nature of the fluorescence image and the absence of fluorescence at the mitotic poles. (Bar = 10.0 $\mu$m.)

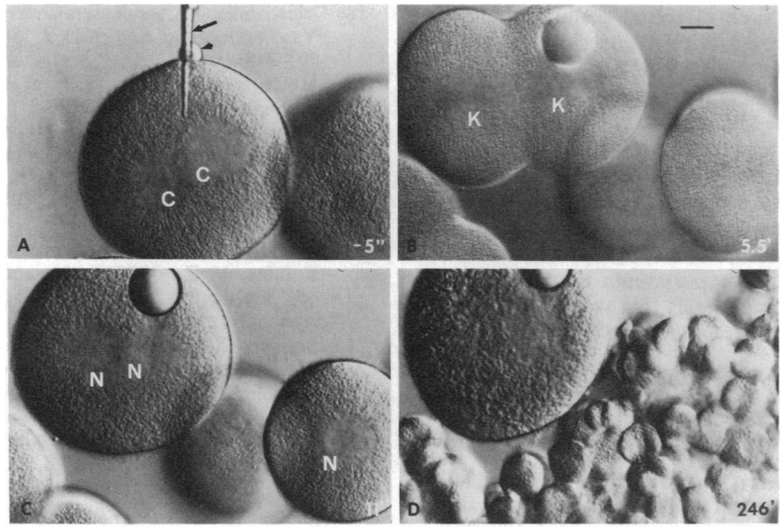


FIG. 4. Time-lapse, light microscope study with Nomarski differential interference-contrast optics of mitotic sand dollar embryos (*E. parma*) microinjected with immune anti-calcium transport enzyme IgG at mid-anaphase of the second mitotic cleavage. (*A*) Taken after penetration of the cell with the injection micropipet and 5 sec before injection. (*B–D*) After injection. Numbers in the lower right-hand corners indicate the number of seconds (″) or minutes (′) before (−) or after injection. The injected blastomere contains a refractile spherical droplet of Wesson oil. Note that the contractile ring first stops its contraction and then relaxes. Spindle BR decreases upon injection of the antibody at a rate faster than the control cell. These cells are in calcium-free sea water to preserve their rounded appearance. (Bar = 20 μm.) The injection micropipet is indicated with an arrow. An oil droplet adhering to the micropipet and the cell's exterior is indicated with an arrowhead. Injected oil is indicated with an open arrow. Chromosomes (C), karyomeres (K), and nuclei (N) are indicated.

saline, or TRITC-GαR IgG did not affect either MA BR or the injected blastomere's progress through mitosis.

## DISCUSSION

The data presented here demonstrate a functional link between calcium sequestration by an ATP-dependent Ca pump and mitosis. The MA Ca pump has been identified as a single polypeptide with a molecular mass of 105 kDa, based on immunoblotting (Fig. 1 *Upper*) with affinity-purified calcium sequestration inhibiting antibodies directed against the SR Ca pump of muscle that also inhibits *in vitro* calcium sequestration by SR and vesicles purified from MA. Furthermore, microinjection of this antibody into mitotic cells resulted in a complete and irreversible arrest of mitosis (Fig. 4). The more rapid loss of spindle BR in the injected cells suggests that the calcium-sequestering membranes leak calcium to the cytosol at a rate that ultimately results in disassembly of spindle microtubules. Thus, the continuous action of the Ca pump appears to be necessary for the maintenance of the free calcium concentration within the MA to maintain the spindle microtubules in an assembled state. In the absence of the Ca-pump activity (e.g., following α-Ca-pump IgG injection) $Ca^{2+}$ would leak out down its concentration gradient, resulting in the disassembly of spindle and astral microtubules, seen here as a rapid loss of MA BR.

It is interesting to note the differences in the localization of membrane sequestered $Ca^{2+}$ and the MA Ca pump (Fig. 3). From geometric analysis of several dozen MA labeled as in Fig. 3, it was found that the majority of the MA Ca pump is localized to a volume (zone 2) of only 14.5% of the astral volume. The differences in relative intensities of Ca-chlorotetracycline fluorescence (and therefore intramembrane calcium) in zones 2 and 3, and of the MA Ca pump in those same zones, suggest that the variations in the MA Ca pump (being located predominantly in zone 2) are due to real differences in localization and not overall MA geometry. Thus, factors regulating the MA Ca pump would be expected to regulate the state of the MA itself. Positive effectors of the MA Ca pump would stabilize MA BR and lead to the

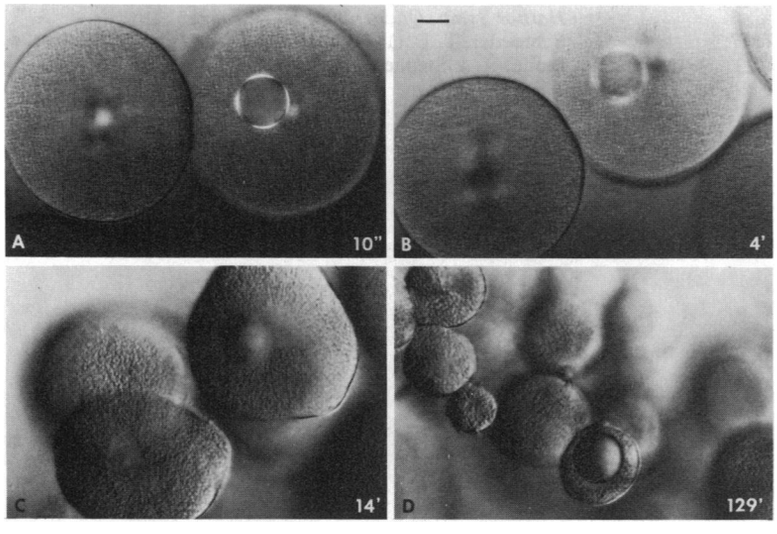


FIG. 5. Time-lapse, light microscope (Nomarski differential interference-contrast and polarization) study of mitotic sand dollar embryos (*E. parma*) injected with boiled immune anti-calcium transport enzyme polyclonal IgG. (*A*) Ten seconds after injection. (*B*, *C*, and *D*) Four, 14, and 129 min after injection, respectively. Numbers in the lower right-hand corners indicate the number of seconds (″) or minutes (′) after injection. The injected blastomere contains the refractile spherical droplet of Wesson oil. Note the injected blastomere continues to divide at a normal rate. (Bar = 20 μm.)

metaphase configuration. Such effectors could include increase in active enzyme concentration (transcriptional, translational, or posttranslational control) or a decrease in suppressor activity. Negative effectors of the MA Ca pump would tend to destabilize the MA, reduce BR, and, if spatially and temporarily regulated, lead to the orderly disassembly of the MA in anaphase. Such negative effectors could include decrease in enzyme activity due to a decrease in available ATP, increased proteolytic turnover, and/or increase in the activity of a specific suppressor. The data represented here, especially the microinjection experiments documented in Figs. 4 and 5, support the hypothesis that the MA Ca pump and the regulation of its activity *in vivo* are central facets of mitosis.

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