Ca^{++} REGULATION IN CAFFEINE-DERIVED MICROPLASMODIA OF *PHYSARUM POLYCEPHALUM*

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The shuttle streaming of the granular cytoplasm in the slime mold *Physarum polycephalum* may be produced by the interaction of cytoplasmic actin and myosin (8-12) both of which can be isolated in pure form (1, 2, 5, 6). This shuttle streaming reverses direction every 1.5-3 min which implies the existence of a control mechanism alternately allowing and inhibiting the interaction of actin and myosin in localized regions. A troponin-tropomyosin-like system has been demonstrated in *Physarum* (16) which suggests that the actin-myosin interaction could be controlled by fluctuations in the intracellular Ca^{++} concentration. Similar concentrations of free Ca^{++} are needed to initiate motility in plasmodial fragments (7) and in striated muscle. Histochemical evidence (3) suggests that high concentrations of localized Ca^{++} (4) may be contained in membrane-bounded vesicles in the plasmodium. Recently, cytoplasmic movement has been shown to be correlated with changes in the concentration of free Ca^{++} (13).

The plasmodium itself is too large an organism to monitor or to control easily. A brief incubation of the plasmodium in dilute 5-10 mM caffeine produces numerous spherical microplasmodia 50-250 μm in diameter (7). These caffeine-derived microplasmodia (CDM) possess a granular cytoplasm which displays a rhythmic, well-defined cycle of motility and they are capable of growing into large plasmodia.

CDM in an ethyleneglycolbis[β-aminoethyl ether]*N,N'*-tetraacetic acid medium free of caffeine become "inactive" i.e., their granular cytoplasm becomes nonmotile and diffuse. Caffeine, $CaCl_2$, ionophore A23187, and other divalent or monovalent cations can then be applied externally to a single CDM by micropipette. Localized and transient concentrations of Ca^{++} or intracellular Ca^{++}-effecting agents are formed at the CDM's outer surface, permitting repeated or alternating applications of different reagents to the same CDM. The possibility that Ca^{++} plays a part in the control of motility and the mechanism by which the intracellular Ca^{++} concentration is regulated are easily investigated with this technique.

MATERIALS AND METHODS

Plasmodia were cultured from sclerotia of *P. polycephalum* (Carolina Biological Supply Co., Burlington, N. C.) on agar plates. Mature plasmodia were placed in a 3-cm diameter cylinder screened with 200-μm mesh nylon at one end (Krassilk Products Inc., Elmsford, N. Y.). The screened cylinder containing the plasmodia was incubated in 10 mM caffeine, 10 mM Tris maleate buffer pH 7.1, for 30 min at room temperature. CDM were formed by removing the cylinder from the caffeine solution and gently agitating it in 10 ml of 10 mM EGTA, 10 mM Tris maleate buffer, pH 7.1

Experiments were performed in a rectangular micromanipulation chamber, 18 × 16 × 2 mm, cut into the edge of a 5-mm thick slab of aluminum. The top and bottom of the chamber were covered with 22 × 22-mm cover slips, leaving one horizontal side open to receive microtools. Micropipettes were made from 100-mm long Pyrex tubing with an inner diameter of 0.6 mm on a Leitz horizontal needle puller. The volume applied at any one time was within the range 0.4-0.72 μl over a 1-min period. Micropipette position was controlled by a Leitz micromanipulator.

All solutions were made with quartz-distilled water to minimize the concentration of exogenous Ca^{++}. All experiments were performed in a medium of 10 mM Tris maleate buffer, pH 7.1.

RESULTS

Local application of 50 mM caffeine by micropipette produces compaction of the granular cytoplasm followed by a short period of streaming in "inactivated" CDM. Repeated applications of equal volumes of caffeine do not produce identical responses but require progressively longer periods of time to initiate compaction. In addition, the duration of the response decreases. Eventually, the CDM fails to respond at all. However, the time to respond to the initial application of caffeine is independent of the time spent in the nonmotile "inactive" state in the EGTA medium.

The application of 10 μg/ml ionophore A23187 by micropipette to the exterior of an "inactive" CDM in the EGTA medium produces a compaction of the granular cytoplasm. If the exposure to ionophore is $\leq$0.36 μl, streaming persists 5-10 times longer than caffeine-induced motility. The

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granular cytoplasm does eventually become "inactive" and retains the ability to initiate motility in response to succeeding applications of caffeine, $CaCl_2$, or additional ionophore. If the exposure to ionophore is ≥0.6 μl, 90% of the treated CDM undergo violent streaming resulting in the rupture of the granular cytoplasm through the plasmalemma. Those few which do not rupture but whose cytoplasm again becomes "inactive" no longer initiate motility in response to succeeding applications of caffeine or ionophore. Succeeding applications of 10 mM $CaCl_2$ can still initiate motility.

The CDM's ability to respond to caffeine can be restored by an intervening exposure to $CaCl_2$. The CDM is repeatedly exposed to 50 mM caffeine until it ceases to respond. 10 mM $CaCl_2$ is then applied externally by micropipette producing compaction of the granular cytoplasm, streaming, and eventually rediffusion of the granular cytoplasm throughout the entire spherical volume. The same CDM is now exposed to the identical volume and concentration of caffeine as used previously. The CDM once again initiates motility in response to caffeine. Intervening exposure of the CDM to $MgCl_2$, KCl, NaCl, or Tris maleate buffer fails to restore the CDM's ability to respond to caffeine.

The same experiment can be performed with the substitution of 10 μg/ml ionophore A23187 for $CaCl_2$. As in the above experiment, the CDM is repeatedly exposed to 50 mM caffeine until it ceases to respond. Application by micropipette of ≤0.36 μl of ionophore will initiate motility and a short period of streaming. A subsequent application of caffeine once again initiates motility even though no $CaCl_2$ has been directly applied (Fig. 1).

DISCUSSION

Caffeine, which causes a release of Ca^{++} from the sarcoplasmic reticulum of vertebrate striated muscle (18), causes an initiation of motility in "inactivated" CDM in the Ca^{++}- and caffeine-free EGTA medium. Since the initiation of motility is Ca^{++}-dependent and the medium is Ca^{++}-free, the only source of Ca^{++} must be from within the CDM. Repeated applications of caffeine exhaust this store of intracellular Ca^{++} by causing repeated release of the stored Ca^{++} which is lost through chelation with EGTA. Thus, repeated applications of an equal volume of caffeine require longer periods of time to initiate motility. Because the overall supply of Ca^{++} is diminished, the motility persists for shorter and shorter periods of time. Since the initial response time to caffeine remains constant regardless of the time spent in the "inactivated" state, the intracellular store of Ca^{++} must be unavailable to the EGTA i.e., possibly membrane bound.

Experiments demonstrating the restoration of the CDM's caffeine response further substantiate the ideas that motility is Ca^{++}-initiated and that a Ca^{++}-sequestering system controls the intracellular Ca^{++} concentration. Repeated applications of caffeine cause a loss of stored Ca^{++} until insufficient Ca^{++} is released to initiate motility. The supply of intracellular Ca^{++} can then be replenished by an external application of Ca^{++}. Some of this exogenous Ca^{++} is apparently stored in the sequestering system since succeeding applications

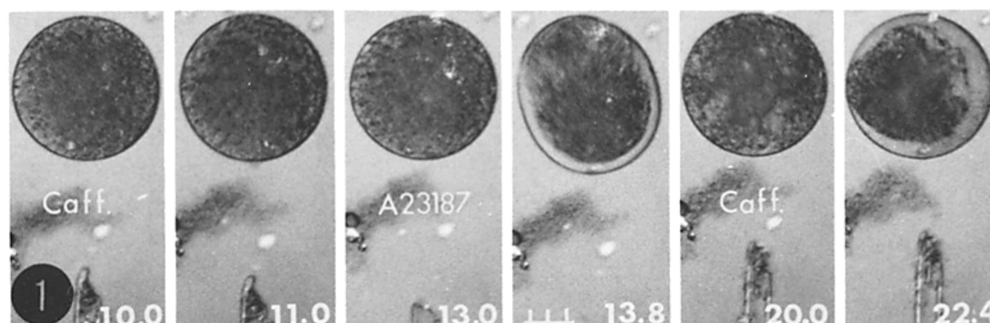

FIGURE 1 "Inactivated" CDM no longer responds to application of 50 mM caffeine (10.0) in 10 mM EGTA medium (11.0). An application of 10 μg/ml ionophore A23187 (13.0) initiates motility (13.8). The cytoplasm is nonmotile and diffuse when an equal volume of 50 mM caffeine (20.0) initiates compaction and streaming (22.4). Time in minutes. Scale division = 10 μm. × 250.

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of caffeine once again initiate motility. Other divalent and monovalent cations, even at significantly higher concentrations, do not restore the CDM's caffeine response, demonstrating that the initiation of motility depends on the presence of intracellular Ca^{++} stored in a caffeine-sensitive system.

The ionophore experiments demonstrate the existence of a biphasic Ca^{++} storage system in these CDM. After repeated applications of caffeine have exhausted the caffeine-sensitive store of Ca^{++}, an application of ionophore initiates motility in the Ca^{++}-free EGTA medium by causing a release of Ca^{++} from a caffeine-insensitive intracellular store. Some of the ionophore-released Ca^{++} appears to be sequestered in the caffeine-sensitive system since subsequent applications of caffeine can initiate motility. The ionophore also effects the caffeine-sensitive system. After a sufficiently large initial application of ionophore, neither caffeine nor ionophore can initiate motility because the ionophore has depleted the Ca^{++} stored in both drug-sensitive systems. An application of Ca^{++} can still initiate motility, which demonstrates that the motile mechanism is intact. Subsequent applications of ionophore or caffeine can then initiate motility, demonstrating that both Ca^{++}-storage systems are operational. While ionophore A23187 can cause a release of Ca^{++} from both mitochondria (11, 14) and sarcoplasmic reticulum (15) in skeletal muscle, caffeine seems to affect only the sarcoplasmic reticulum (17). These experiments therefore suggest that the CDM possess a membrane-associated Ca^{++}-sequestering system other than the mitochondria.

SUMMARY

Caffeine-derived microplasmodia possess a Ca^{++}-sequestering system which can initiate motility. The experiments presented here suggest that this system is membranous and nonmitochondrial in nature. Therefore, it is proposed that the shuttle streaming in the plasmodium is controlled by the localized release and uptake of free Ca^{++} from an intracellular storage system analogous to the sarcoplasmic reticulum.

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