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Mechanical Responses of Intestinal Smooth Muscle in a Calcium-Free Medium.* (32136)

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Investigations carried out to determine the role of calcium in smooth muscle function have led to the postulate that calcium is bound to more than one site in the smooth muscle fiber. Part of the calcium in the muscle appears to be complexed with superficial loci. The calcium at this site equilibrates rapidly with extracellular calcium ions and is involved in the regulation of membrane permeability. In addition, calcium bound to superficial loci or present in the extracellular fluid may, under appropriate conditions, migrate into the cytoplasm and activate contractile proteins. Another part of the fiber calcium appears to be combined with loci that are less accessible to the external milieu. The calcium at this site equilibrates more slowly with extracellular calcium ions, does not seem to affect membrane permeability, and can also be mobilized to activate contractile elements (1,2,3,4).

The verification and extension of these concepts are important but still incomplete. In this study we used an experimental procedure which conditions an intestinal smooth muscle preparation to exhibit a high degree of mechanical activity in a calcium-free environment. The tests subsequently performed gave some indication of the ways in which extracellular calcium ions, a high potassium medium, and acetylcholine affect the accumulation and/or release of calcium at a poorly accessible site in the muscle.

Methods. The tissue preparation used was the isolated longitudinal muscle from guinea pig ileum. A segment approximately 3 cm long was suspended in a muscle bath which contained 10 ml of a physiological salt solution. The temperature of the bath was maintained at 31-32°C. Isotonic contractions of the muscle were recorded on a standard kymograph. Tension on the muscle was approximately 0.35 g.

The physiological salt solution had the following composition: NaCl, 125 mM; KCl, 2.7 mM; $CaCl_2$, 1.8 mM; glucose, 11 mM; and tris [tris (hydroxymethyl) aminomethane, Sigma-trizma base] buffer, 23.8 mM. The solution was adjusted to pH 7.5 with 6 N HCl and was saturated with 100% oxygen. The composition of the high potassium medium was identical with that of the physiological salt solution except that all the NaCl was replaced by an equimolar quantity of KCl.

Calcium content of the muscle was measured by the flame photometric method described by Geyer and Bowie(5).

Modifications in the efflux of calcium ions from the longitudinal muscle fibers were estimated from measurements of the rate of outflow of Ca^{45} from the tissue. Ca^{45} was counted in a liquid scintillation counter.

Results. The isolated longitudinal muscle undergoes changes in muscle tone if the physiological salt solution in which it is immersed is exchanged for a bathing medium lacking in calcium ions. The magnitudes of the changes induced are dependent upon the level of calcium to which the tissue is exposed prior to the introduction of the calcium-free medium. These effects were demonstrated in the following manner. A muscle was incubated in the physiological salt solution for

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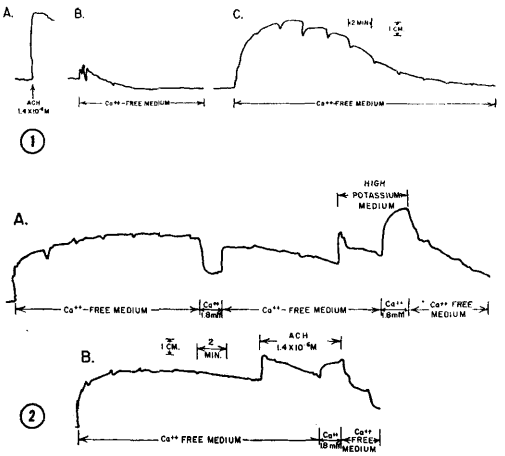

FIG. 1. Isotonic changes in muscle tone of Longitudinal Smooth Muscle. In section A, isotonic contraction produced by 1.4 $\times$ 10^{-6}M acetylcholine is shown. In section B, changes in muscle tone produced by a calcium-free medium are shown. Prior to introduction of calcium-free medium the muscle had been incubated for 2 hrs in a physiological salt solution. In section C, changes in muscle tone produced by a calcium-free medium are shown. Prior to introduction of calcium-free medium the muscle had been incubated for 2 hours in a high calcium bathing medium (36 mM $CaCl_2$). The small periodic changes in muscle tone seen in sections B and C are artifacts caused by changing the bathing medium.

FIG. 2. Isotonic changes in muscle tone of Longitudinal Smooth Muscle. Records in sections A and B show changes in muscle tone produced by a calcium-free medium. Prior to introduction of calcium-free medium the muscles had been incubated in a high calcium medium (36 mM $CaCl_2$) for a 2 hr period. In section A, effects on muscle tone of a normal calcium concentration (1.8 mM), of a high potassium (calcium-free) medium, and of a normal calcium concentration in presence of a high K medium are shown. In section B, effects on muscle tone of 1.4 $\times$ 10^{-6}M acetylcholine and a normal concentration of calcium ions in presence of acetylcholine are shown.

2 hours. Several times during the 2-hour interval the fluid was removed from the muscle bath and replaced by fresh solution. Following this period of incubation the tissue was quickly washed twice with a calcium-free solution containing 5 $\times$ 10^{-6} M EDTA and then suspended in 10 ml of this solution. Thereafter the fluid was drained from the muscle bath every 2 minutes and replaced by an equal quantity of fresh calcium-free solution. Almost immediately after being transferred to the calcium-free environment the fibers developed a transient increase in muscle tone. The size of the response was small to moderate compared with the contraction previously elicited by 1.4 $\times$ 10^{-6} M acetylcholine. An example of the responses observed is shown in the records given in Fig. 1-A and B.

The same muscle preparation was then subjected to a second 2-hour period of incubation. This time the bathing fluid contained 20 times the normal concentration of calcium ions (36 mM). At the end of the incubation period the calcium-free solution was introduced in the same manner as described above. It is evident in Fig. 1-C that the muscle, in this instance, reacted to the removal of extracellular calcium by undergoing a larger and more protracted increase in muscle tone. The magnitude of the contractile response varied from 40-100% of the response produced by 1.4 $\times$ 10^{-6} M acetylcholine; and the time required for the tone to return to baseline level varied from 10-50 minutes in 35 experiments. Similar results could be obtained when the calcium-free medium employed contained 1 $\times$ 10^{-4} M EDTA, or when the medium contained no added calcium or EDTA.

The duration of the incubation period in the high calcium medium was also found to be important. This was demonstrated by washing pairs of tissues in a calcium-free medium for one hour and then incubating them in a high calcium medium (36 mM) for different lengths of time. One member of each pair was incubated for 15 minutes; the other, for 60 minutes. When the calcium-free solution was reintroduced, the member that had been incubated in the high calcium medium for the longer period of time developed a significantly larger increase in muscle tone. In thirty-one experiments the average response of the muscles incubated for 15 minutes was 59.2% $\pm$ 3.1 (S.E.) of a reference contraction produced by acetylcholine. The average response of the muscles incubated for 60 minutes was 72.6% $\pm$ 2.9 (S.E.) of the reference contraction.

One may infer from these observations that the concentration of calcium ions in the extracellular fluid influences the quantity of calcium that accumulates in a cellular depot. The quantity of calcium in the depot, in turn, regulates the magnitude and duration

of the subsequent mechanical response. To some extent this inference is supported by the finding of Paton and Rothschild that the calcium content of the longitudinal muscle decreases if the calcium concentration of the bathing fluid is reduced(6). The inference is also supported by our observation that muscles immersed in a high calcium medium for 60 minutes accumulate significantly more intracellular calcium than those immersed in a high calcium medium for 15 minutes. This result was obtained by measuring the calcium content of 10 pairs of tissues that were washed for one hour in a calcium-free solution and then incubated in a high calcium medium (36 mM) as described above. Following the 15- or 60-minute incubation period in the high calcium medium each tissue was washed for 7 minutes in a calcium-free solution to remove extracellular calcium ions. The tissues were then blotted, weighed, and assayed for calcium content. The calcium contents of muscles incubated in the high calcium medium for 60 minutes averaged 8.75 $\pm$ 1.06 (S.E.) mEq per kg wet weight of tissue; whereas the calcium contents of muscles incubated for 15 minutes averaged 3.64 $\pm$ 0.45 (S.E.) mEq per kg wet weight of tissue.

The extent to which calcium becomes available to activate the contractile machinery, in these circumstances, seems to be governed not only by the quantity of calcium that has been accumulated in the depot but also by the ease with which the calcium dissociates or moves away from the depot site. The latter process appears to be stimulated when calcium ions are removed from the extracellular fluid.

The proposal that the dissociation or translocation of cellular calcium is facilitated by removing extracellular calcium ions was tested by another procedure. Tissues were incubated for 2 hours in a high calcium medium containing the radioactive isotope Ca^{45} (3 μc/ml.) The muscles were then subjected to a 70-minute washout period in a nonradioactive solution containing the high calcium concentration. During the next 48 minutes, washout solutions were collected every 6 minutes and analyzed for their radioactivity. For the first half of this latter period the washout solution contained 36 mM calcium ions. During the second half of this period the washout solution was calcium-free. The data in Table I show the cpm Ca^{45}/ml fluid/minute obtained at each successive 6-minute interval. The abrupt increase in the efflux of calcium ions when the extracellular calcium concentration was reduced from 36 mM to a zero level is quite apparent.

TABLE I. Change in Efflux of Ca^{45} from the Longitudinal Smooth Muscle Resulting from a Reduction in Extracellular Calcium Ion Concentration from 36 to 0 mM. Values given are averages obtained from 7 experiments.

Sampling interval (after 70 min wash)	Ca^{40} conc. of bathing medium	Cpm Ca^{45} per ml of bathing fluid per minute
0–6 min.	36 mM	10,392
6–12 "	"	7,711
12–18 "	"	4,798
18–24 "	"	4,432
24–30 "	0 mM	9,544
30–36 "	"	11,146
36–42 "	"	8,460
42–48 "	"	4,766

Thus, the immediate increase in smooth muscle tone and the immediate increase in Ca^{45} efflux observed when a calcium-free medium was introduced into the muscle bath favor the contention that a reduction in the extracellular calcium ion concentration results in an increased migration of calcium ions away from a cellular store.

Experiments were carried out to determine the effects of acetylcholine and a high potassium medium on a muscle that had been made highly responsive to a calcium-free medium. When the contractile response of the muscle in the calcium-free medium had reached its peak and started to decline toward baseline level, either 1.4×10^{-6} M acetylcholine or a high potassium medium (calcium-free) was introduced into the muscle bath. Both excitatory agents evoked an increase in muscle tone. The subsequent addition of a normal concentration of calcium ions (1.8 mM) produced another increase in muscle tone. When the same concentration of calcium ions was added in the absence of any excitatory agent, it caused a partial depression of muscle tone. These effects are illustrated in representative records in Fig. 2.

Discussion. Daniel and Irwin, after investigating the effects of chelating agents on uterine muscle contractions, concluded that the removal of calcium from a surface site in the smooth muscle membrane would initiate the release of calcium from a less accessible site in the fiber. This calcium would then enter the cytoplasm and activate contractile elements(2). The same inference was drawn from the results obtained in this study on intestinal smooth muscle. We found, in addition, that the mechanical response initiated by the sudden removal of extracellular calcium was appreciably enhanced by preincubating the muscle in a high concentration of calcium ions. The length of time that the muscle was incubated in the high calcium ion concentration influenced both the amount of calcium taken up by the fibers and the subsequent increase in muscle tone. This implies that the total quantity of calcium in the cellular depot as well as the ease with which the divalent ion leaves the depot determines the magnitude of the muscle contraction induced.

Removal of extracellular calcium not only evoked an increase in smooth muscle tone, but also increased the unidirectional efflux of calcium ions from the longitudinal fibers. Extracellular calcium is known to reduce membrane permeability to inorganic ions (7,8). It seems reasonable therefore to suggest that the cellular depot containing calcium is located in the plasma membrane and that the migration of calcium ions from this depot into the cytoplasm and into the external medium is accelerated by an increase in membrane permeability to calcium ions. In support of this suggestion both acetylcholine and a high potassium medium were found to enhance the contractile response of a muscle exposed to a calcium-free environment.

Experiments in which the interaction of calcium and excitatory agents were investigated showed that acetylcholine and a high potassium medium can convert an inhibitory effect of extracellular calcium ions on muscle tone into a stimulatory effect. A prompt augmentation of muscle tone was observed when 1.8 mM $CaCl_2$ was added to the calcium-free medium in the presence of either one of the excitatory agents. Presumably, the increase in muscle tone occurred because calcium ions were rapidly taken up from the extracellular fluid and transferred into the cytoplasm. A possible explanation for its occurrence may be that an excitatory agent, by increasing membrane permeability, nullifies the opposing effect of extracellular calcium on the membrane. This may then permit a rapid flow of calcium ions between the extracellular fluid and the cytoplasm as well as between the membrane depot and the cytoplasm. It is not possible to deduce from these experiments whether extracellular calcium ions gain entrance into the cytoplasm exclusively *via* the membrane depot or whether another pathway also exists.

Thus, the evidence obtained in this study indicates that acetylcholine, a high potassium medium, and a calcium-free medium have at least one effect in common on the longitudinal fibers of guinea pig ileum. They can induce an increase in smooth muscle tone by mobilizing calcium from an intracellular compartment which may reside in the fiber membrane. The results suggest that mobilization of this calcium is linked to an increase in membrane permeability to the divalent ions. The magnitude of the mechanical response produced is determined, in part, by the quantity of calcium present in the intracellular depot. There is also an indication that the excitatory agents enhance the movement of calcium between the extracellular fluid and the cytoplasmic fluid of the smooth muscle fibers.

Summary. Longitudinal fibers from the guinea pig ileum undergo a transient increase in muscle tone when they are transferred from a physiological salt solution to a calcium-free solution. The magnitude and duration of the contractile response is enhanced if the muscle is preincubated in a high calcium medium (36 mM). The length of time that the muscle is preincubated in the high calcium medium influences the magnitude of the response as well as the amount of intracellular calcium accumulated by the fibers. The mechanical changes initiated by removing extracellular calcium ions are accompanied by

a pronounced increase in the unidirectional efflux of calcium ions. The addition of acetylcholine or a high potassium medium enhances the tone of a muscle that is partially contracted in a calcium-free medium. The addition of 1.8 mM $CaCl_2$ depresses it. However, when the same concentration of $CaCl_2$ is added in the presence of acetylcholine or a high potassium medium it enhances muscle tone even more. We inferred from these data that acetylcholine, a high potassium medium, and a calcium-free medium, by increasing membrane permeability, accelerate the migration of calcium ions from an intracellular depot to the cytoplasm. The results suggest that they also accelerate the movement of calcium ions between the external solution and the cytoplasm of the muscle fiber.

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Production of Right Ventricular Hypertrophy With and Without Congestive Heart Failure in the Cat. (32137)

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Ventricular hyperthrophy and congestive heart failure have been induced in dogs(1), guinea pigs(2), and rabbits(3) and such experimental models have provided considerable insight into these pathologic states. However, a quantitative description of the contractile state of cardiac muscle isolated from the hypertrophied or failing heart is not available. The cat offers unique advantages for analyzing the contractile state of the myocardium in these conditions because the papillary muscle from the right ventricle is small enough to permit *in vitro* oxygenation and its fibers are oriented in parallel and thus allow definition of heart muscle function per unit of muscle cross-sectional area and length. Further, the functional characteristics of the cat papillary muscle preparation have been described in detail(4) and are sufficiently uniform to allow meaningful comparison of the myocardial contractile state of one group of cats to that of another group(5). To provide the animal model for such studies, a method for production of right ventricular hypertrophy with and without congestive heart failure in the cat has been developed and is the subject of this report. This was accomplished by imposing a chronic pressure load on the right ventricle by reduction of the lumen of the supravalvular portion of the main pulmonary artery.

Methods. The pulmonary artery of the cats was constricted by a circular clip(6) (Fig. 1) which was applied in the following manner. Animals were anesthetized with intravenous sodium methohexital (15 mg/kg). To permit endotracheal intubation and control of respiration intravenous succinylcholine (1 mg/kg) was then given and intermittent positive pressure ventilation was applied by a Harvard respirator* using air. Under sterile conditions, the chest was opened through the anterior one-third of that left intercostal space lying 2 cm cephalad from the apical impulse, the pericardium was widely excised, and a 5 mm segment of the main pulmonary artery just distal to the pulmonic valve was dissected free of the ascending aorta. This

* Harvard Apparatus Co. model 672.