The Stimulative Effect of Sodium on the Depletion Process of Calcium in the Intracellular Store of Smooth Muscle Cells of Guinea-Pig Taenia Caecum

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Abstract—The effect of readmitted Na on the Ca-depletion process in the carbachol-sensitive Ca-store was investigated using thin bundles of the K-depolarized, Na-depleted guinea-pig taenia caecum. To estimate the quantity of stored Ca, the "Ca-load Ca-release" method was employed: To load the store with Ca, 2 mM Ca was added and left (phase A), and then it was removed by washing with 2 mM EGTA-containing solution (phase B); 10^{-3} M carbachol was then applied (phase C). Instead of glucose, pyruvate was used as a nutrient. Millimolar concentrations of Na inhibited the carbachol-induced contractions when Na was present during phase B. Na present during phase A also reduced the carbachol-induced contraction, but not when Na was treated during phase C. As the period of phase B was prolonged, the carbachol-induced contraction was reduced, which suggests the depletion process of stored Ca resulting from extrusion of Ca from the cell. Na present during phase B accelerated the rate of the Ca-depletion process. Li mimicked Na with regard to the promoting effect on the depletion process, and the effect of Na was not affected by 10^{-4} M ouabain, suggesting that Na-Ca exchange would not be involved in the effect of Na. These results suggest that Na may be involved in the maintenance of cellular Ca-homeostasis through the stimulation of efflux of Ca from the store to the outside of the cell.

An intracellular Ca-store has been proposed to participate in contraction-relaxation processes (1–3). In the guinea-pig taenia caecum, both carbachol and caffeine release Ca from the store or probably the sarcoplasmic reticulum (SR) (1). Hisayama and Takayanagi (4, 5) have shown that 45 Ca taken up by microsomes from the guinea-pig taenia caecum was released by addition of a physiological concentration of Ca or thymol. On the other hand, relaxation has been also supposed to be partly brought about by Ca-uptake by the store. For example, isoproterenol-induced relaxation has been suggested to be due to stimulation of Ca-uptake by the store (3, 6, 7). Azide-insensitive, oxalate-dependent 45 Ca-uptake by microsomes prepared from the guinea-pig taenia caecum (8) and other types of smooth muscles (9) was shown to be stimulated by treatment with cyclic AMP plus protein kinase, via phosphorylation of a specific protein(s). However, because Ca is continuously entering muscle cells at either a resting or activated state, Ca taken up by the store must be somehow transported to an extracellular space to maintain cellular Ca-homeostasis.

It is well known that the longer the guinea-pig taenia caecum, as well as other smooth muscles, is incubated in Ca-free medium, the smaller is agonist-induced contraction. This phenomenon suggests the possibility that Ca in the store gradually leaks to the outside of the cells and is finally depleted (Ca-depletion) (1, 3, 10). This
suggestion is also supported by the findings of Casteels and Raeymaekers (3) that carbachol increased the rate of 45Ca-efflux from 45Ca-preloaded taenia coli when the drug was added after 6 min of efflux, but not when added after 30 min of efflux. Hence it could be assumed that the Ca-depletion process would be seemingly a model to study the Ca-mobilization from the Ca-store to the outside of the cell in the guinea-pig taenia caecum.

It was shown by Katase and Tomita (11) and Ma and Bose (12) that maintained contracture of the guinea-pig taenia coli in Na-free medium was terminated by addition of millimolar concentrations of Na, and it was suggested that external Na was involved in reducing the intracellular Ca concentration by Na-Ca exchange, energy for which was supplied by Na-influx. 45Ca-efflux data with intact muscles (13, 14) and enzyme-dispersed single cells (15) have also supported the presence of [Na]o-activated 45Ca-efflux in the guinea-pig taenia coli. Therefore, we have in the present study investigated the effect of readmitted Na on the depletion process of stored Ca in the Na-depleted guinea-pig taenia caecum, in order to obtain clues to understand the way in which Ca in the store was extruded to the outside of the smooth muscle cell.

Materials and Methods

Guinea-pigs of either sex weighing 300 to 500 g were stunned and bled, and pieces of the taeniae caeci were rapidly dissected.

A thin bundle, 100–200 μm in diameter and about 4 mm long, was prepared with the aid of forceps under a dissecting microscope and tied at both ends with fine silk threads. One end was connected to a fixed glass support in a 20 ml organ bath, the other was connected to the force-transducer. The bundle was mounted horizontally and incubated in physiological saline solution (PSS) with fine air bubbles at 32°C. Developed tension was monitored isometrically, and the size of the Ca-store was estimated by the area of tension evoked by 10–3 M carbachol (16). The basic PSS contained: (mM) Na+, 160.0; K+, 5.6; Mg2+, 2.1; Ca2+, 2.2; Cl−, 168.2; HCO3−, 6.0; and glucose, 2.8. Na-free, K-solution: K+, 165.6; Mg2+, 2.1; Cl−, 163.8; HCO3−, 6.0; pyruvate2−, 5.5; Tris (tris-(hydroxymethyl)aminomethane), 6.5; and concentrations of CaCl2 specified in the text. Na, Ca-free, EGTA (glycoletherdiamine-tetraacetic acid)-containing K-solution: K+, 165.6; Mg2+, 2.1; Cl−, 163.8; HCO3−, 6.0; pyruvate2−, 5.5; EGTA, 2.0; and Tris, 10.0. Tris base was added to neutralize EGTA and pyruvic acid (pH 7.4).

Materials used in this study were as follows: pyruvic acid, EGTA (Wako Junyaku), Tris and carbachol chloride (Sigma). Other chemicals used were of analytical grade.

Results

The quantity of stored Ca was estimated by the procedures described by many investigators (e.g., 1, 3, 6, 10) as follows: after incubation in Ca, Na-free EGTA-containing K-solution for 30–60 min (phase 0), the tissue was loaded with 2 mM Ca in Na-free, K-solution for 5 min (phase A), followed by a 10 min-washing with the above Ca, Na-free solution (phase B); then 10–3 M carbachol (a supramaximal concentration) was applied (phase C). After thorough washings, the bundle was rested (phase D). Combined period of both phase C and D was 5 min. Thereafter, the same cycles of trials (phase A to D) were repeated. As shown in a typical tracing of 4 separate experiments in Fig. 1 A and B, in glucose-containing medium, carbachol-induced transient contractions at first became larger (up to the third contraction in this figure), but then progressively declined. Introduction of 5.5 mM pyruvate instead of glucose, as suggested by Suzuki et al. (17), resulted in development of a larger contraction which is highly reproducible for at least up to 10 hr, which contrasts with the previous negative reports (3, 18).

The relationships between Ca concentrations during phase A and carbachol-induced responses during phase C are shown in Fig. 2. Each filled symbol represents tension development which was obtained by a progressive decrease in the concentration of Ca during phase A of each of the trials, whilst an open symbol shows the steady-state response which was finally obtained at a given Ca concentration until the response
became constant after the preceding concentration of Ca was decreased to the present lower one. The data in Fig. 2 indicated that the degree of the filling of the store with Ca was dependent on extracellular Ca concentration, but the difference between these dose-response relationships was clear. On the other hand, the Ca-dependent contraction became inhibited when the period of phase D was prolonged, although this was partially reversible. Carbachol-induced contraction was hardly depressed by such a prolonged Ca-deprivation (data not shown). These results indicate that constant concentration of Ca employed in phase A and constant duration of each phase in a series of experiments were necessary for obtaining reproducible data.

To study the effects of readmitted Na on Ca-depletion, we treated the preparation with 10 mM NaCl during phase B for 10 min, and then contraction was evoked by carbachol. Compared with the control response (untreated with Na, Fig. 3A), the contraction of the Na-treated tissue was greatly suppressed (Fig. 3B). Na itself did not produce any tension development (see the tension before applying carbachol in Fig. 3B). The inhibitory effect remained for 40–60 min even after Na-removal (Fig. 3C). Since NaCl was added to the bath hypertotonically, the effect of NaCl might be merely due to an osmotic effect. In order to exclude the possibility, 40 mM sucrose was added and present during phase B. The contraction evoked by carbachol was not influenced by the sucrose-treatment (data not shown). Addition of 10 mM NaCl 10 sec before applying carbachol (Fig. 3D) or just when carbachol-induced tension development peaked (Fig. 3E) did not influence the shape of the contraction.

The relationship between the concentration of Na present during phase B and carbachol-induced contraction is shown in Fig. 4. As low as 0.3 mM Na could significantly suppress the carbachol-induced contraction, and 10 mM Na almost abolished the contraction. The dose-response curve was rather concaved, but not sigmoidal. Since stored Ca was estimated by tension area, once stored Ca was fully depleted, the further effect of Na of higher concentration than 10 mM on the Ca-depletion process could not be determined using this protocol. Two mM Li during phase B for 10 min also inhibited
the carbachol-induced contraction to 57.5±1.6% (mean±S.E., N=3) as compared to the nontreated control, whereas 2 mM Na suppressed the contraction to 52.4±2.6% (mean±S.E., N=3). The inhibited contraction by Li was quickly recovered after washing as compared with Na.

Figures 5 and 6 show the promoting effect of 10 mM Na on depletion process of stored Ca from two different aspects. Closed circles in Fig. 5 shows the time-course of the rate of filling of the store with Ca which was introduced from the bathing medium. In these experiments, to estimate the carbachol-induced response precisely, Ca-induced contractions were reduced as much as possible by prolonging the period of phase 0 up to 1 hr. The maximum filling was completed within 2 min. In a parallel experiment, the store was loaded with 2 mM Ca for 2 min; then 10 mM NaCl was added; and 0.5, 1.5 or 3 min thereafter, carbachol was applied to elicit the contraction. Stored Ca was rapidly decreased by Na-readmission, and after 3 min (5 min at abscissa), reached a lower steady-state level, probably resulting from a balance between an increased Ca-efflux and a decreased Ca entry. Ca-influx was reported to be inhibited by Na (13, 14). When Ca was loaded for 5 min in the presence of Na, the carbachol-induced contraction (a filled triangle) coincided with the steady-state contraction which was obtained by Na-

Fig. 2. The relationships between the concentrations of Ca during phase A for 5 min and carbachol-induced contraction after Ca-deprivation for 10 min. Filled symbols: fractional response which was obtained by progressively decreasing Ca concentration during phase A for each of the trials. Open symbols: the steady-state response to carbachol which was finally obtained through repeating the same set of trials until the response became constant after some preceding concentration of Ca was made to decrease to the next lower one. Different symbols represent data obtained from different preparations. Inset: schematic procedure used in this experiment. xCaK: Na-free, x mM CaCl2-containing K-solution, 2GK: Na, Ca-free, 2 mM EGTA-containing K-solution. An arrow indicates an application of 10−3 M carbachol.

Fig. 3. Effects of Na-treatments on carbachol-induced contractions in Ca-free media. A. Control tension change from phase A to D. B. The carbachol-induced contraction of the preparation pretreated with 10 mM Na during phase B for 10 min. C. The next carbachol-induced contraction in the absence of Na. Tension recovery was incomplete. D. (left): The control response to carbachol. (right): The response to carbachol when 10 mM Na was added 10 sec before applying carbachol. E. (left): The control response to carbachol. (right): Na (10 mM) was added just when carbachol-induced tension development peaked.
readmission, shown as the open circle at 5 min. Furthermore, in Fig. 6, the effect of Na on the depletion process of stored Ca is shown more directly. By addition of Na, stored Ca was rapidly decreased.

To attempt to manipulate the Na-gradient across the plasma membrane, the effect of 10^{-4} M ouabain on the Na-dependent promotion of the Ca-depletion process was studied. Responses to carbachol in the presence of 5 mM Na and 5 mM Na plus 10^{-4} M ouabain for 10 min during phase B were 48.5±4.73% and 51.3±6.33%, respectively, compared with the control response (mean±S.E., N=5). The difference between these values were nonsignificant.

**Discussion**

Na–Ca interactions have been long studied in various types of smooth muscles (19). Among them, the guinea-pig taenia caecum is one of the commonly used tissues. In this tissue, removal of medium Na caused a substantial depletion of cellular Na within 30 min in this tissue (13, 18), as opposed to other types of smooth muscles (e.g., ref 20). To investigate the effect of readmitted Na on Na-depleted tissues, therefore, guinea-pig taenia caecum is suitable since a possible contribution of tightly bound endogenous Na can be expected to be negligible. Further, we used in this study a thin bundle to change chemical environments around muscle cells as rapidly as possible (1, 3).

Na seems to have influences on diverse cellular functions other than that on cellular Ca-handling, e.g., membrane electrical events (21), energy-supplying metabolism.
(17), and perhaps other unknown processes, which may in turn affect Ca-mobilization and/or muscle contractility indirectly. In the present study, we conveniently used K as a Na-substitute to avoid possible changes in the membrane potential due to changes in concentration of extracellular Na, by depolarizing the membrane almost completely, and to maintain an ionic strength in bathing milieu. Membrane depolarization was shown not to release Ca from the Ca-store (1, 3). Moreover pyruvate but not glucose was employed as a nutrient. Furchgott and Wales (22) reported that pyruvate and oxalacetate are utilized for contraction energy by the rabbit intestinal muscle. Suzuki et al. (17) more recently reported that tonic contracture of the guinea-pig taenia coli induced in Na-free K-solution was inhibited mainly through an inhibition of glucose utilization probably due to inefficiency of Na-facilitated glucose transport. Furthermore, they showed that the inhibited contracture in Na-free K-solution was recovered by introduction of pyruvate or oxalacetate (17). In accordance with their results (17), by virtue of pyruvate utilization, we can perform “Ca-load Ca-release” in the Na-deprived condition for at least up to 10 hr. Other investigators who used glucose failed to carry out repetitive carbachol-induced contractions (for example, as shown in Fig. 1A), which lead them to suggest that the ability of the store to refill with Ca would decline in stimulated preparations due to the progressive reduction of Na in the store (18). Their results are in complete contrast with our findings (Fig. 1B).

As shown in Fig. 2, carbachol-induced contractions were gradually decreased to a steady-state level by lowering concentration of Ca during phase A. Prolonged deprivation of Ca from the medium also reduced the contraction during phase A. A partial release of Ca from the store by carbachol would not account for these phenomena, since the maximum concentration of carbachol or caffeine could not evoke a contraction after the concentration of carbachol was once applied unless Ca was reloaded (1). Hence, although underlying mechanisms for the seeming deleterious effect of low or zero Ca on muscular function are now unexplained, from methodological considerations, constant concentration of Ca during phase A and constant duration of each phase in a series of experiments were adopted to obtain the reproducible data.

It was reported by Ohashi et al. (23) that D-600 or La during phase A inhibited the response to carbachol in a similar condition. From the result, it is suggested that Ca which once entered the cells would be then taken up by the store. On the other hand, it is well known that a prolonged Ca-deprivation from the bathing medium reduced the contractions evoked by stimulants in many types of smooth muscles (as in Fig. 6). This phenomenon suggests the possibility that Ca in the store would gradually leak to the outside of the cells and finally depleted in Ca-free conditions.

These results enable us to envisage a model of the Ca-cycling process, that is, Ca entering cells is actively taken up by the store, and then it extruded to an extracellular space to maintain cellular Ca-homeostasis. We have shown in the present study that Na during phase B (a Ca-depletion process)
inhibited the following contraction elicited by application of carbachol in a concentration-dependent manner. The data shown in Figs. 5 and 6 strongly suggest that the inhibitory effect of Na might be due to its promoting effect on Ca-depletion or efflux of stored Ca to the outside of the cell. Na has little effect, if any, on the muscarinic receptor or Ca-releasing mechanism itself, considering the results shown in Fig. 4D and E or our unpublished data that Na reduced the contraction evoked by caffeine as well as carbachol when Na was treated during phase B. Endo et al. (1) have suggested that caffeine acts on the store on which carbachol also acts to release stored Ca in guinea-pig taenia caecum. Taken together, Na may be involved in maintenance of cellular Ca-homeostasis through its promotive effect on the transport of stored Ca which was taken up from cytoplasm to the outside of the cell. Much the same conclusion was obtained in rabbit mesenteric artery by the finding that in Na, Ca-free solution, the contractions evoked by norepinephrine or caffeine applied repetitively persisted much longer than those evoked in the presence of Na (24). Since Na can be mimicked at least by Li, the effect is not inherent in Na. However, Na would probably operate as a natural regulatory cation in a physiological condition.

Meanwhile, cyclic AMP-mediated relaxation such as that by isoproterenol (12, 25) or papaverine (26) was reportedly weakened seriously in the absence of Na, and Sunagane et al. (26) recently suggested that the reduction of relaxant activity of papaverine results from the inactivation of a cyclic AMP-dependent Ca-effluxing mechanism due to the removal of external Na. These results will prompt us to study whether the promotive effect of Na on the depletion process of stored Ca is also applicable for accounting for the cyclic AMP-mediated relaxation.

Katase and Tomita (11) and Ma and Bose (12) have postulated the presence of Na–Ca exchange in the guinea-pig taenia caecum as described in the Introduction. Therefore, the possibility has been anticipated that Ca which was leaked from the store would be then extruded by a Na–Ca exchanger located in the plasma membrane (19, 27) or that a Na–Ca exchange mechanism could exchange Na and Ca directly between the extracellular fluid and the store (18, 19). Either hypothesis seems to be comprised of two important distinguishable arguments: what is the mechanism of Na-dependent Ca-extrusion? and where is Ca extruded to the extracellular space? At present, we do not have data for a discussion of the second point. However, it would be conceivable that Na–Ca exchange is insufficient to explain the Na-dependent promotion of the Ca-depletion process in the guinea-pig taenia caecum, as previously suggested by Aaronson and van Breemen (13, 14). When the guinea-pig taenia caecum was treated with ouabain, it is expected that on Na-readmission, Na entering cells was accumulated inside the cells and thereby the Na gradient across the membrane was dissipated, which could lead to inefficiency of Na–Ca exchanger. Our study using 10^-4 M ouabain showed that the inhibition of the Na–K pump did not have any appreciable effect on the Na-dependent Ca-depletion process. Furthermore, Li could mimic Na to the same degree with regard to the promoting effect of the depletion process of stored Ca. Consequently, these results suggest that a possible involvement of Na–Ca exchange in the promotive effect of Na on extrusion of stored Ca can be excluded.

Therefore, we should seek an alternative theory to account for the effect of Na on the Ca-store. Aaronson and van Breemen (14) discussed the effect in terms a possible non-specific competition of Ca and monovalent cations including Na for anionic binding sites in the store, as well as with regard to a possible direct stimulation of a plasmalemmal Ca-ATPase by such cations. However, in our study, we should remember that the Na effect on the Ca-depletion process can be observed sensitively even in the presence of 165.6 mM K, which seems to disagree with the first hypothesis of Aaronson and van Breemen (14). The difference between our data and those of Aaronson and van Breemen (14) is now unclear. Since their hypothesis was deduced from the results of the overall cellular 45Ca-flux experiments where ionic strength was very low due to utilization of sucrose as a Na-substitute, nonspecifically
bound \(^{45}\)Ca, which is functionally less important, may be exaggerated. In our study, however, the size of the intracellular Ca-store(s) may be underestimated, because although the Ca-store referred to in this study was the carbachol-sensitive Ca-store only, there may be an additional store which takes up Ca to initiate muscular relaxation but does not release Ca for contraction. The functional diversity of the SR is well known in skeletal muscles (e.g., ref. 28), and the same case may hold in some types of smooth muscles (e.g., ref. 29). To properly understand the role of the Ca-store(s) as a part of the Ca-extruding system, further study may be needed concerning such a Ca-sink.

In summary, we have obtained evidence suggesting that millimolar concentrations of readmitted Na would promote extrusion of Ca in the carbachol-sensitive Ca-store to the outside of the cell of the K-depolarized, Na-depleted guinea-pig taenia caecum. From these results, it may be postulated that at least two entities are involved in extrusion of cytoplasmic Ca to the extracellular space: one is the Ca-store as a Ca-concentrating system, the function of which is reportedly stimulated by a cyclic AMP-dependent mechanism, and the other transports Ca from the inside of the store to the outside of the cell through a Na-dependent mechanism, but possibly not by Na–Ca exchange. Ca may be extruded through a mechanism in which Ca is not utilized for activation of contractile proteins, but precise mechanisms remain to be clarified.

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