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Sodium-Calcium Interactions in Mammalian Smooth Muscle*

C. VAN BREEMEN, P. AARONSON, AND R. LOUTZENHISER

Department of Pharmacology, University of Miami, Miami, Florida

I. Introduction

Force development by smooth muscle cells is directly regulated by the concentration of free calcium ions in the myoplasm (135). Evidence abounds in the literature that variations in intra- and extracellular Na^+ concentrations affect smooth muscle function by changing the levels of myoplasmic free Ca^{++}. The physiological significance of the modulation of cytoplasmic Ca^{++} concentration by sodium ions is of prime interest. There exists, furthermore, substantial evidence that the primary lesion of clinical hypertension resides in a disturbance of Na^+ metabolism (48, 62, 126, 161).

The above considerations amply justify the large research effort directed toward the study of Na^+,Ca^{++} interactions in smooth muscle. It is relatively easy to obtain viable isolated preparations from many sources that lend themselves well to measurements of force development and, although somewhat more difficult, to the recording of electrical activity. Measurements and control of intracellular ion contents and activities are, however, much harder to achieve. This situation has led to the accumulation of a large body of indirect experimental evidence with relatively few critical data to test hypothetical molecular mechanisms for ion interactions.

The purpose of this review is to: 1) organize a theoretical framework for the evaluation of experimental results; 2) present the available experimental evidence in as cohesive a fashion as possible; 3) analyze the validity of present theoretical concepts; and 4) suggest experimental lines of research open to future investigation.

II. Theoretical Considerations

A. Molecular Mechanisms

The physical chemical basis for biological interactions between Na^+ and Ca^{++} probably resides in the similarity of their bare radii (0.95–0.98 Å for Na^+ and 0.94–0.99 Å

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for Ca^{++}). This permits competition between these cations for binding to anionic sites located on or in cellular membranes. The simplest model to explain the influence of extracellular Na^+ ($[Na^+]_e$) on contractile force consists of anionic sites on the outer surface of the plasmalemma that can be occupied and neutralized by either Na^+ or Ca^{++}. The occupation by Ca^{++} initiates its inward movement (178). The fraction of the anionic transport sites occupied by Ca^{++} would then be proportional to $[Ca^{++}]_e/[Na^+]_e^2$, the proportionality constant being the ratio of the Ca^{++} affinity over the Na^+ affinity. Experimental support for this model comes from frog ventricle, where twitch tension is indeed proportional to this ratio (178). This hypothesis relates $[Na^+]_e$ to Ca^{++} influx during activation and relies on other mechanisms to remove Ca^{++} from the myoplasm during relaxation. This hypothesis was elaborated into a new model to account also for Ca^{++} extrusion from the cells in the following manner. The transport site is moveable in the membrane only when occupied by 1 Ca^{++} or 2 Na^+ (133). The transported ions on the carrier can then exchange with either 2 Na^+ or 1 Ca^{++} on either side of the membrane. If this is the only pathway available to these ions for crossing the membrane, then thermodynamic considerations predict that at equilibrium, when the Ca^{++} gradient equals two times the Na^+ gradient, the Na,Ca exchange carrier will couple the Ca^{++} and Na^+ distributions according to:

$$[Ca^{++}]_e/[Ca^{++}]_i = [Na^+]_e^2[Na^+]_i^2 \quad (I)$$

If the number of Na^+ ions able to combine with the carrier is set equal to n and the number of Ca^{++} ions able to complex to each carrier molecule is maintained at 1 the above relationship becomes:

$$\frac{[Ca^{++}]_e}{[Ca^{++}]_i} = \left(\frac{[Na^+]_e}{[Na^+]_i}\right)^n \exp(2 - n)\frac{EF}{RT} \quad (II)$$

where $[\ ]_e$ and $[\ ]_i$ are extra- and intracellular concentrations respectively, E is the membrane potential, F is the Faraday constant, R is the gas constant, and T is the absolute temperature (5). The distinguishing feature of the Na,Ca exchange carrier is that once the membrane potential and extracellular Ca^{++} concentration are known the Na^+ concentration gradient fixes the concentration of free myoplasmic Ca^{++}. Figure 1 gives the $[Ca^{++}]_i$ as a function of the ratio $[Na^+]_e/[Na^+]_i$ for integral values of n varying between 2 and 4. The value for ionic $[Ca^{++}]_e$ is assumed to be 1.5 mM (169) and the E is taken at −50 mV (77). Inspection of the curves immediately excludes the possibility for an obligatory coupling of 1 Ca^{++} leaving the cell for 2 Na^+ entering, because at the physiological $[Na^+]_e/[Na^+]_i$ ratio, which lies between 10 and 15, $[Ca^{++}]_i$ exceeds 10^{-5} M. Such high levels of Ca^{++} would keep the muscle in a continuously contracted state incompatible with normal smooth muscle function. If n = 3 the Na,Ca exchange carrier presents a theoretically possible mechanism for Ca^{++} extrusion, which can be experimentally tested. At the

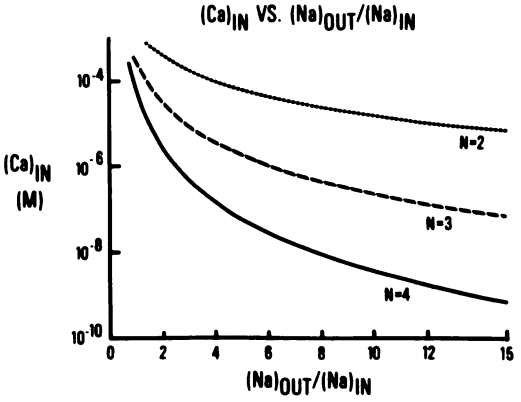

FIG. 1. The relationship between the transmembrane Na^+ gradient, given as the ratio of external and internal Na^+ concentrations (abscissae), and the concentration of ionized Ca^{++} in the cytoplasm, calculated from equation II (see text). This relationship between the Na^+ and Ca^{++} gradients is a consequence of a model in which the Ca^{++} gradient is determined solely by the Na^+ gradient. Ca^{++} efflux is coupled to Na^+ influx via an exchange carrier which may bind one Ca^{++}, or n Na^+ ions. The curves shown have been generated for n = 2, 3, and 4 (n, the number of Na^+ ions bound by a hypothetical Na,Ca exchange carrier). See text for further details.

highest $[Na^+]_e/[Na^+]_i$ ratio of 15, the resting muscle is relaxed while baseline tension develops as the ratio declines (9). The case where n = 4 has been considered for the squid axon (119). When n exceeds 2 the carrier becomes electrogenic, transferring net positive charge inward for each cycle, such that depolarization would inhibit Ca^{++} extrusion. If the hypothetical Na,Ca exchange carrier were the only mechanism for Ca^{++} extrusion from the cells (9), the curves of Figure 1 would present the lower limit of $[Ca^{++}]_i$, since the equation does not take into account a passive Ca^{++} leak or Ca^{++} channels involved in action potential generation, which do not reside within the exchange carrier.

Both the first model of competition between Ca^{++} and Na^+ for influx channels and the Na,Ca exchange model predict a rise in tension upon reduction of $[Na^+]_e$. However, only the latter model gives the quantitative relationship between the Na^+ gradient and intracellular Ca^{++} activity and consequently predicts the smooth muscle resting tension. The exchange carrier also predicts stimulation of Ca^{++} efflux by raising $[Na^+]_e$, and stimulation of Ca^{++} influx by increased $[Na^+]_i$.

Stimulation of Ca^{++} flux in one direction by a flow of Na^+ in the opposite direction has also been demonstrated in a cation exchange membrane consisting of narrow pores lined by fixed negative sites (174). This can be explained by assuming that diffusion of Ca^{++} through such a pore is proportional to the fraction of free Ca^{++}/(free + bound) Ca^{++}. In this case the binding process retards the net ion flux. Occupation of the sites by Na^+ will then increase the proportion of free Ca^{++} and thus increase the Ca^{++} flux. This mechanism couples the fluxes much less tightly and would not obey equation II. If, on the other hand, binding to a fixed negative site in a pore is an essential step in the transport, and if Na^+ and Ca^{++} compete for such sites, then coupling between oppositely directed Na^+ and Ca^{++} fluxes can be shown to become tighter such that functionally it more closely resembles the Na,Ca exchange carrier.

When considering ion transport through narrow pores, it is in theory also possible to observe inhibition of a flux of ions in one direction by ions originating from the opposite side of the membrane. This mechanism, which is based on a restriction on ions inside the pore to pass each other, has been identified for K^+ channels in the squid axolemma and was termed "single file diffusion" (78).

In general, Na^+ and Ca^{++} fluxes exhibit inhibitory effects on each other when originating on the same side of the membrane. Theoretically, they may inhibit or stimulate each other when originating from opposite sides of the membrane, depending on the model chosen.

If Ca^{++} binding sites other than transport sites are considered, then the competitive effect of Na^+ is to displace Ca^{++}. Ca^{++} binding to the outer surface of the smooth muscle cell membrane is known to have a stabilizing effect. Displacement of this Ca^{++} by extracellular Na^+ could thus lead to increased membrane permeability, depolarization, and release of intracellularly bound Ca^{++} (50). Displacement of Ca^{++} bound to intracellular membranes by Na^+ would raise the cytoplasmic free Ca^{++} in a more direct manner, which could also lead to contraction.

Finally, there exists the possiblity for allosteric effects, such that Na^+ binding to one site may change the affinity of another site specific for Ca^{++} and thereby influence Ca^{++} transport or membrane function (119). Thus there may exist some membrane sites that require Na^+ to ensure proper membrane function (8).

Changing the Na^+ gradient may affect contractility indirectly through its effect on the membrane potential, especially because Na^+ permeability of the smooth muscle plasmalemma is high relative to other tissues (35). Variations in $[Na^+]$ may also affect other ion permeabilities, thereby exerting complex effects on the membrane potential.

B. Specific Cellular Sites for Na^+,Ca^{++} Interactions

The smooth muscle myoplasmic calcium concentration is regulated by the cell surface membrane (84, 146), the sarcoplasmic reticulum (SR) (55), and possibly to a lesser extent by mitochondria (6). The relative contributions of these organelles to this regulatory function is an area of active research interest. The extent to which the Ca^{++} affinity of the regulatory molecules associated with the contractile proteins plays a part in smooth muscle function is as yet unknown. Accordingly, there exists a number of possible specific sites at which Na^+ could theoretically influence force development by smooth muscle:

1. The cell membrane active Ca^{++} extrusion pump
2. Passive "channels" in the cell membrane permeable to Ca^{++}
   a. Receptor regulated
   b. Membrane potential regulated
   c. Resting membrane leak
3. Ca^{++} binding sites at the inner surface of the plasmalemma
   a. Receptor regulated
   b. Membrane potential regulated
   c. Dependent on stability of the phospholipid bilayer
4. Ca^{++} binding sites on the outer surface of the SR
5. The SR Ca^{++}-ATPase
6. The SR Ca^{++} release gates
7. Ca^{++} binding sites on mitochondria
8. The mitochondrial Ca^{++} pump
9. The mitochondrial Ca^{++} leak
10. Ca^{++} binding sites on soluble Ca^{++} buffer molecules
11. Ca^{++} sensitive sites on the regulatory proteins of the contractile apparatus
12. Autonomic nerve terminals

The elucidation of the physiological roles of the above sites in the regulation of smooth muscle function will require the study of Ca^{++} movements with respect to these sites.

Tension measurements do not define such specific Ca^{++} movements due to the large number of the above mentioned variables. Force developed by smooth muscle should thus be regarded only as an indicator of cytoplasmic Ca^{++} activity.

III. Experimental Evidence

This section is subdivided according to general classes of smooth muscle and methods for altering Na^+ concentrations.

The division into the categories of vascular, alimentary tract, uterine, and other smooth muscle is intended not only as a convenience for readers with specific interests, but emphasizes different patterns of evidence for these tissues.

The second subdivision is a natural one since there have been only two basic manipulations of Na^+ concentrations: 1) Variation of $[Na^+]_e$ using other solutes to serve as osmotic substitutes. Due to the membrane Na^+ permeability and the operation of the Na,K pump these substitutions will also alter $[Na^+]_i$ in a time-dependent manner. Furthermore, all substitutes appear to exert specific effects related to chemical properties of the substitute in addition to the effects resulting from the altered $[Na^+]$. 2) Inhibition of the Na,K pump by ouabain or omission of external K^+. This will raise the $[Na^+]_i$ without altering $[Na^+]_e$. Again it is necessary to exercise caution in interpretation of results as some effects appear due to inhibition of the electrogenic pump and other direct actions of both cardiac glycosides and zero K^+ media, which are unrelated to the rise of $[Na^+]_i$. Although an account of all the pertinent experiments is voluminous and some discrepancies in interpretation exist, the data usually fell into clear patterns, which are outlined at the end of each section.

A. Vascular Smooth Muscle

1. Na^+ Substitution. In 1958 Bohr et al. (11) initiated these studies by varying $[Na^+]_e$ between 85 and 153 mM, using sucrose as osmotic replacement. Norepinephrine-induced contractions of the rabbit aorta were potentiated by low $[Na^+]_e$ and depressed by elevated $[Na^+]_e$. A year later

Friedman et al. (62) proposed the theory that the Na^+ gradient is a basic determinant of smooth muscle tone, its increase causing relaxation and decrease inducing contracture. In support of this they showed that acute elevation of blood [Na^+] lowered the blood pressure of an anesthetized rat and inhibited the pressor responses due to administration of norepinephrine and pitressin. Elevated $[Na^+]_e$ also decreased peripheral resistance in the dog forelimb (74). Furthermore, low blood pressure was associated with an increased Na^+ gradient after adrenalectomy (62) and high blood pressure with a decreased Na^+ gradient after aldosterone administration. No attempt was made, however, to relate the changes in Na^+ gradient to Ca^{++} transport processes. Dodd and Daniel (56) disagreed with the above hypothesis since their experiments in vitro showed no potentiation of agonist-induced contractions upon lowering $[Na^+]_e$. Instead, total Na^+ replacement with sucrose or choline caused the disappearance of histamine-, epinephrine-, and acetylcholine-induced contractions within 1½ hours. Briggs and Melvin (27) were the first to measure the effect of varying $[Na^+]_e$ on Ca^{++} fluxes. Reduction of $[Na^+]_e$ to 31 mM, using either sucrose or choline chloride as substitutes for Na^+, induced a slow contraction and increased the ^{45}Ca influx 3.2-fold. Influx was measured after 1 hour of exposure to ^{45}Ca-labelled solution followed by 10 minutes wash out in nonradioactive solution. In contrast to the above, they reported that the reduction in $[Na^+]_e$ diminished a maximal norepinephrine contraction. When all NaCl was replaced by K_2SO_4, tension and ^{45}Ca influx increased but no significant change in net tissue Ca^{++} could be demonstrated (25). Nash et al. (120) subsequently failed to show a rise in tension with a replacement of half of the NaCl by sucrose but showed a potentiating effect on norepinephrine-induced contractions. Bohr et al. (12) replaced all Na^+ in a medium perfusing rabbit aorta and mesenteric arteries with Li^+ to find a delayed rise in baseline tension and immediate potentiation of norepinephrine-induced contractions. Relaxation of the norepinephrine-induced contraction was much slower and often incomplete in the absence of Na^+. Complete Na^+ substitution with sucrose in a medium perfusing isolated portal veins caused an initial contraction associated with high spike frequency followed by complete relaxation (8). The abolition of rhythmic activity by sucrose was attributed to membrane hyperpolarization. If tris was used as a Na^+ substitute, phasic contractions were maintained for 3 to 4 hours although there was some decrease in amplitude and rise in basal tension. Biamino and Johansson (8) also made the interesting observation that Na^+, although it does not appear to carry the inward current of the action potential, is nevertheless required for Ca^{++}-mediated activation. They found that the rate of contraction, which followed addition of Ca^{++} to a Ca^{++}-free depolarizing solution, was much slower in the absence of Na^+ than in its presence. Readmission of Ca^{++} failed to induce any contraction if the rat portal vein had been exposed to Na^+-free sucrose Krebs solution for 200 minutes. The absence of Na^+ had an even greater retarding effect on relaxation after contraction in a 128 mM K^+ solution. Biamino and Johansson (8) postulated that Na^+ associated with the cell membrane facilitates Ca^{++} movements in both directions.

Sitrin and Bohr (145) demonstrated that external Na^+ may have opposing effects on the two phases of a norepinephrine-induced contraction. In 1963 Bohr (10) discovered that a norepinephrine-induced contraction of the rabbit aorta consists of two components: an initial fast (F) phase, which increases with lowered $[Ca^{++}]_e$ and falls with increased $[Ca^{++}]_e$, and a maintained slow (S) component, which falls and rises in parallel with fluctuations of $[Ca^{++}]_e$. In 1969, van Breemen (165) was able to abolish the S component but not the first F component with La^{+++}, which was shown to inhibit Ca^{++} influx. This confirmed the belief that the F component is activated by intracellular Ca^{++} release while the S component

depends on Ca^{++} influx. The subsequent finding of Sitrin and Bohr (145) was that elevation of $[Na^+]_e$ in a solution superfusing dog mesenteric artery strips increased the F and decreased the S component of the norepinephrine-induced contraction. Lowering $[Na^+]_e$ to 80 mM had the opposite effects of decreasing the F and increasing the S component. These effects developed with a rapid time course and appeared to be related to changes in $[Na^+]_e$ only. Thus these results raise the interesting possibility that competition of Na^+ for Ca^{++} binding sites on the outer membrane surface may facilitate release of Ca^{++} inside the cells while inhibiting the inward Ca^{++} movement.

In the earlier period during which the above cited research was carried out very few attempts were made to measure effects of Na^+ substitution directly on transplasmalemmal Ca^{++} fluxes. One probable reason for the lack of this type of experimentation might have been the fact that Ca^{++} flux data obtained from smooth muscle had been notoriously irreproducible. The main source of this problem appeared to be a large smooth muscle extracellular space containing numerous Ca^{++} binding sites, such that extracellular Ca^{++} exchange accounted for most of the data. van Breemen et al. (171, 172) attempted to remove the extracellular Ca^{++} while inhibiting loss of cellular Ca^{++} by washing the tissues for 60 minutes in a modified Krebs solution (tris or hepes buffers replacing carbonates and phosphates) containing 2 to 10 mM $LaCl_3$ after completion of Ca^{++} uptake and before tissue Ca^{++} analysis. Using this "La method" they showed that Li^+ substitution for Na^+ stimulated an extra Ca^{++} uptake, which leveled off at 50 μmol of Ca^{++}/kg wet wt. above the Na^+ control value. The Li^+-induced contractions were submaximal and could be reproducibly obtained only if all $[Na^+]_e$ was replaced by Li^+. If K^+ was used as a Na^+ substitute twice as much contractile force was developed and labelled Ca^{++} influx saturated at a level of 100 μmol of Ca^{++}/kg wet wt. above control. When the Ca^{++} transport sites in the membrane were protonated by lowering the pH of the bathing medium, ^{45}Ca influx was inhibited and the Na^+ substitution contraction was abolished (171). Reuter et al. (132) studied the effects of Na^+ substitution on ^{45}Ca efflux from the rabbit aorta. The washout medium contained either 0.4 mM $LaCl_3$ or was Ca^{++}-free with 0.5 mM ethylene glycol tetraacetic acid (EGTA) added in order to decrease extracellular ^{45}Ca loss during late efflux. Under these conditions choline substitution decreased the rate of ^{45}Ca efflux by a third and readmission of Na^+ stimulated the efflux. These results were interpreted as indicative of a Na,Ca exchange carrier, although Li^+ substitution without La^{+++} or EGTA in the medium had a stimulating effect on ^{45}Ca efflux. ^{45}Ca efflux from the carotid artery of the dog was inhibited by sucrose but not by Li^+ substitution. Villamil et al. (176) also found that sucrose substitutions caused large Ca^{++} gains in both extracellular and cellular Ca^{++} fractions while Li^+ failed to do so. They concluded that the results could be explained by a nonspecific Na,Ca competition for cellular and extracellular binding sites. van Breemen (166) stimulated cellular ^{45}Ca efflux with 10 mM caffeine or with metabolic inhibition. This stimulated ^{45}Ca efflux was not affected by Li^+ but was 30% reduced by choline substitution for Na^+; 10 to 20 mM Na^+ in the choline solution did overcome this inhibitory effect. When cellular ATP was rapidly depleted by a combination of 2,4-dinitrophenol and iodoacetic acid, the rabbit aorta gained 240 μmol of Ca^{++}/kg wet wt. in a linear fashion over a 3-hour period. This linear gain is expected of cells with a finite Ca^{++} permeability and an internal Ca^{++} concentration far below equilibrium but lacking energy to fuel the Ca^{++} extrusion pump. When the Na^+ gradient was abolished by Li^+ substitution the cells gained only 30 μmol of Ca^{++}/kg wet wt. in an asymptotic manner over the same time interval. After 3 hours in Li^+ the tension had returned to base line. The saturable Ca^{++} gain in choline was 70 μmol of Ca^{++}/

kg wet wt. after 3 hours (166). These results clearly indicate that ATP is necessary for maintenance of the Ca^{++} gradient but that the Na^+ gradient is not. The only argument against this conclusion is that during the La^{+++} wash an important Ca^{++} fraction was lost when Na^+ was substituted but not when metabolism was inhibited. Recent experiments have shown, however, that these results do not change qualitatively when a different quenching method (45 minutes in ice-cold solution containing 2 mM EGTA) is used which allows for much less cell Ca^{++} loss (167). Even more convincing evidence against the Na^+ gradient as an energy source for Ca^{++} extrusion is the observation that after a high K^+ contraction the aorta will relax in a Li^+ solution containing as little as 15 mM Na^+. If either caffeine or D600 were present in addition, a net Ca^{++} extrusion was seen into the above Li^+ solution (166). Even if Li^+ were accepted by the hypothetical Na,Ca exchange carrier it could not provide energy for active Ca^{++} extrusion for any appreciable length of time, since it is not extruded from smooth muscle cells (8, 167).

In 1977 Karaki and Urakawa (97) observed that the Na^+ substitution-induced contractions in rabbit aorta could be abolished by alpha-adrenergic blockade, reserpinization, and mechanical denervation. In the latter case potentiating effects of Na^+-free solution on norepinephrine-induced contractions also disappeared. They concluded that contractions following Na^+ substitution are due to the release of endogenous catecholamines and not to arrest or reversal of a hypothetical, Na,Ca exchange carrier.

Summary. Na^+ substitution in the medium bathing isolated vascular preparations produces a range of contractile effects. In the portal vein, substitution with sucrose abolishes all contractile activity while in the aorta Li^+ substitution induces a small contraction that usually relaxes over a long time period. Choline substitution for Na^+ induces a larger although still submaximal contraction.

Removal of external Na^+ induces an extra uptake of Ca^{++}, which, however, levels off at a value far below that expected from the $[Ca^{++}]_i$ going to equilibrium. The data on ^{45}Ca efflux are somewhat conflicting due to the difficulty of separating extracellular from cellular Ca^{++} exchange. Li^+ substitution appears to have a slight stimulating effect while choline inhibits ^{45}Ca efflux by 33%. The relatively low Na^+ concentration of 10 mM abolishes the retarding effect of choline substitution.

Norepinephrine-induced contractions have been reported to be both stimulated and inhibited by decreasing $[Na^+]_e$. At least some of the stimulating effects are due to liberation of endogenous catecholamines.

2. Na,K ATPase Inhibition. The initial objective for studying the effects of cardiac glycosides and K^+ removal on vascular smooth muscle was to observe indirectly the influence of varying internal $[Na^+]$ on contractile function. As will become apparent in this section, it is sometimes difficult to separate the effects due to altered $[Na^+]_i$ from those due to alterations in the membrane potential, stimulation of neurotransmitter release, and direct actions on the cell membrane.

In 1957, Leonard (109) first studied inhibition of the Na,K pump in isolated rabbit carotid artery. He found that both a K^+-free solution and strophanthidin potentiated electrically-stimulated contractions and, after some delay, induced tension without stimulation. The latter effect was more pronounced in the case of strophanthidin application than it was for zero K^+. Cardiac glycosides were later found to potentiate contractions induced by: norepinephrine in mesenteric arteries (12) and veins (117), and rabbit aorta (170); adrenergic nerve stimulation in dog cutaneous veins (24); norepinephrine, 5-hydroxytryptamine, acetylcholine, $BaCl_2$, high K^+ and low Na^+ in the dog saphenous vein (23), and by low Na^+ in the rabbit aorta (9). All of these preparations also respond with a slow delayed rise in developed force upon inhibition of the Na,K pump. The ouabain-

induced contraction of the rabbit aorta is accompanied by an increase in Ca^{++} uptake (28, 166, 170) and both the contraction and stimulated Ca^{++} influx were abolished by addition of La^{+++} or removal of Ca^{++}. Most of these studies were explained on the basis of an increased concentration of intracellular Na^+, which would then exchange with extracellular Ca^{++} to increase Ca^{++} influx and decrease Ca^{++} efflux and thus raise $[Ca^{++}]_i$. In order to test this hypothesis van Breemen (166) measured the ouabain- and zero K^+-stimulation of Ca^{++} uptake over a prolonged period and compared it with the effect of metabolic inhibition. Both zero K^+ and 10^{-5} M ouabain caused a submaximal contraction, which was maintained for at least 5 hours, and induced a slow net Ca^{++} uptake, which leveled off at 100 μmol of Ca^{++}/kg of aorta. In contradistinction, the net Ca^{++} gain induced by 10^{-3} M iodoacetic acid plus 10^{-4} M 2,4-dinitrophenol was much faster (54 μmol of Ca^{++}/kg/hr) and did not tend to level off during the same time interval of 5 hours. It was concluded from the submaximal force development and the limited Ca^{++} gain that abolition of the Na^+ gradient did not cause the Ca^+ distribution to approach equilibrium.

In the ear artery of the rabbit, ouabain produced a rapid depolarization of 20 mV, which is followed by a progressive slower depolarization (77). Removal of ouabain brought about a slow recovery. K^+ removal exhibited the same pattern of depolarization, but when K^+ was returned to the perfusate an immediate and marked hyperpolarization to −75 mV was seen. This hyperpolarization could be abolished by applying ouabain immediately after readmission of K^+. The inhibition of the Na,K pump in the ear artery was accompanied by a transient contraction that relaxed completely in spite of continued depolarization and the running down of the Na^+ gradient. Hendrickx and Casteels (77) concluded from their results that the transient contraction was caused by depolarization resulting from inhibition of the electrogenic Na,K pump. The subsequent relaxation remained unexplained but did appear incompatible with the Na,Ca exchange carrier hypothesis. Recent experiments from the same laboratory showed that the marked hyperpolarization, seen on return of K^+ to K^+-depleted cells, has no effect on ^{45}Ca efflux (56a), which rules out the possibility of an electrogenic Na,Ca exchange carrier. In the rat tail artery the zero K^+-induced contracture was more prolonged but relaxed immediately upon return of K^+ to the perfusate (63). The relaxation of this tissue was not correlated with restoration of the Na^+ and K^+ gradients after readdition of K^+ since this process was much slower, progressing with a rate constant of 0.0147 min^{-1}.

Starting with the work of Broekaert and Godfraind (29), recent observations from a number of laboratories indicate that most of the contractile effects elicited by inhibition of Na,K ATPase are neurogenic in origin. Cardiac glycosides elicit various contractile patterns in vascular smooth muscle of different origins. Veins that have spontaneous rhythmic activity, such as the portal and mesenteric veins, respond to cardiac glycosides or zero K^+ by an immediate transient contracture equal in amplitude to the spontaneous contractions. This is followed by a larger transient contraction, after which there is total loss of activity (23, 117, 160). Tsuru and Shigei (160) found that phentolamine or prior treatment of the dogs with reserpine abolished the second larger contraction but left the initial transient one intact. In the other vascular preparations, most of which do not exhibit spontaneous activity, inhibition of the Na,K ATPase induces a delayed transient contraction. Both the length of the delay and the rate of relaxation vary from tissue to tissue. For example, in the rabbit ear artery the delay is only a few minutes (77, 95) although it may be 20 to 30 minutes in the rabbit aorta or dog saphenous vein (95, 170). These contractions are also abolished by alpha-adrenergic blockade and mechanical or chemical denervation but not by tetrodotoxin (95, 160). Karaki and Urakawa

(97) observed the disappearance of ouabain potentiation of norepinephrine-induced contractions when the rabbit aorta was denervated. Bonaccorsi et al. (13) showed that the contractions induced by K^+-free solutions in the rat tail artery and femoral artery and the dog mesenteric arteries were also abolished by alpha-adrenergic blockade or chemical denervation with 6-hydroxydopamine. They demonstrated an excellent correlation between the time course of the delayed contraction and the zero K^+-induced release of ^3H-labelled norepinephrine from the nerve endings of the rat tail artery. Their conclusion was that in these arteries the neurogenic mechanism is the only one responsible for the contractile effects of Na,K pump inhibition. The only ouabain-induced contractions that were not abolished by phentolamine were seen in the guinea-pig and rat aorta and the very late contraction (after 3 hours) of rabbit aorta (29, 95). The contractions of the guinea-pig aorta induced by ouabain or K-free solution were proportional to the ratio $[Ca^{++}]_e/[Na^+]_e^2$ (126a), which suggests either the presence of Na,Ca exchange or Na,Ca competition for influx.

Summary: Inhibition of the Na,K pump induces contractions in vascular smooth muscle and potentiates contractions elicited by a large number of activating agents. The effect on membrane potential is a rapid depolarization due to inhibition of the electrogenic Na,K pump, followed by a slower progressive depolarization due to loss of the K^+ gradient. In rabbit aorta a limited net Ca^{++} uptake is induced but the Ca^{++} distribution does not go to equilibrium. Alpha-adrenergic blockade or denervation abolishes all these contractile effects except the initial transient contraction in spontaneously active veins, the contractions in rat and guinea-pig aorta, and the very late contraction of the rabbit aorta.

B. Alimentary tract smooth muscle

1. Na^+ Substitution Experiments. Partial or total replacement of Na^+ with Li^+, hydrazine, or choline, or a large increase in extracellular Na^+, or treatment with high K^+, may cause increased action potential frequency in guinea-pig taenia coli, but may simultaneously decrease muscle tension (3, 79, 80). Na^+ substitution thus appears to interfere with excitation-contraction coupling. Recognizing this problem, Axelsson (3) concluded in 1961 that the examination of ionic movements might be more appropriate than analysis of electrical activity or contraction in the investigation of smooth muscle function. With an increasing recognition of the importance of intracellular sites in controlling tension and the concomitant development of methods for more accurately measuring intracellular Ca^{++}, the focus of sodium substitution and Na^+ gradient alteration experiments has in fact moved in the direction suggested by Axelsson (3). Therefore, this section will in general follow the progression of research in this area, first, by describing experiments relating the Na^+ gradient and tension and then, by discussing the analyses correlating Na^+ and unidirectional, or net, fluxes of Ca^{++} across intestinal smooth muscle plasma membranes.

A. Na^+ SUBSTITUTION AND ELECTRICAL ACTIVITY. The literature on the effects of manipulating the Na^+ gradient on the electrical activity of intestinal smooth muscle is extensive, and will be covered only briefly here. For a complete discussion of smooth muscle electrical activity and its ionic determinants, the interested reader should consult excellent reviews by Tomita (155) and Kuriyama (105). The effect of Na^+ substitutes on plasmalemma electrical parameters is complex and depends to a large extent on the substitute used and the amount of Na^+ left in the physiological saline solution (3, 20, 31, 79, 80, 122). The effects of any particular Na^+ substitute on membrane potential, spike frequency and configuration, and slow wave activity are generally a function of the length of time that the muscle has been exposed to the substitute, probably due to the secondary effects of Na^+ substitution on ionic gra-

dients. In spite of these difficulties, several interesting ideas have emerged.

A decrease of $[Ca^{++}]_e$ from 2.5 mM to 0.2 mM depolarized taenia coli cells and inhibited spike activity (20). If Na^+ was then decreased to 10 mM (with sucrose as Na^+ substitute), the depolarization and spike inhibition were reversed. It was suggested that Na^+ and Ca^{++} compete for control of membrane potential and for sites related to spike generation.

A similar finding is that in cat intestine it is possible to vary $[Ca^{++}]_e$ between 0.8 and 4.8 mM without changing action potential amplitude, if $[Na^+]_e$ is simultaneously altered such that the ratio $[Na^+]_e/\sqrt{[Ca^{++}]_e}$ remains constant (47, 130). Curtis and Prosser (47) have also found that in a Ca^{++}-free medium containing EDTA or EGTA cat intestine displays regular plateau potentials that are blocked by Ca^{++} influx inhibitors (Mn^{++}, Co^{++}, La^{+++}, verapamil), and also by the substitution of Na^+ by Li^+, choline, and tris. These potentials appear to be due to some alteration in Ca^{++} channels due to the loss of Ca^{++} from the plasmalemma that enables them to carry a Na^+ current. It appears, therefore, that although the spike in intestinal smooth muscle is due to Ca^{++} influx (20, 106, 155) this influx may be modulated by Na^+, perhaps via competition for sites in or near the influx site.

B. Na^+ REPLACEMENT AND TENSION. In 1959, Friedman et al. (62) found that partial substitution of Na^+ with sucrose caused a transient contraction of rat colon strip, which was directly proportional to the amount of Na^+ removed. Equilibration of colon in a low Na^+ medium increased the sensitivity of the tissue to a threshold dose of carbachol, while equilibration in a high $[Na^+]_e$ medium had the opposite effect. Also, an acute increase in $[Na^+]_e$ rapidly abolished a carbachol contraction initiated in a normal Na^+ medium. They concluded that the Na^+ gradient across the plasmalemma of smooth muscle exerted a fundamental control over tone, so that a decrease in the gradient $[Na^+]_e/[Na^+]_i$ increased force development, while an increase caused relaxation. Accordingly, drugs causing contraction of smooth muscle were assumed to do so by moving a given quantity of Na^+ into the cells.

Judah and Willoughby (88) demonstrated that when a piece of guinea-pig ileum was exposed to a Tyrode's solution in which all Na^+ had been replaced with sucrose the tissue went into a sustained contracture, which immediately relaxed when Na^+ was replaced. The maximum rate of relaxation was proportional to the amount of Na^+ replaced into the solution, up to 40 mM Na^+; above this concentration the relaxation rate did not increase. The contracture was also dependent on the presence of extracellular Ca^{++} and K^+. Li^+ could not mimic Na^+ in relaxing the tissue. Treatment of ileum with 10^{-5} g/ml G-strophanthin for 10 minutes prior to the replacement of 36.5 mM Na^+ into the sucrose-Tyrode's medium markedly reduced Na^+ relaxation. Na^+ relaxation was also attenuated to some extent if $[Ca^{++}]_e$ was increased from 1.0 to 1.8 mM.

Tomita and associates (98, 157) exposed taenia coli segments to a Krebs solution in which Na^+ had been replaced by K^+. This led to a typical high K^+ depolarization and contracture. The K^+ was then reduced to normal levels (5.9 mM), and sucrose was substituted. This zero Na^+ solution repolarized the cells to near normal levels but did not relax the taenia. If as little as 5mM Na^+ was added to the sucrose-Krebs, relaxation occurred. The rate of relaxation was $[Na^+]_e$ dependent. If normal Na^+-Krebs was introduced relaxation was complete and normal electrical activity started after a 10- to 20-minute lag time. Li^+-Krebs was also able to relax the contracted taenia but Tris-Krebs could not. In this case ouabain did not block the Na^+-mediated relaxation and the Q_{10} for time to half relaxation was 1.4, implying that relaxation was not mediated by the Na^+-K^+ ATPase or any other active process.

The contracture could also be abolished by replacement of sucrose with isotonic

Mn^{++}, Mg^{++}, La^{+++}, and Ca^{++} salts. Lower concentrations of Ca^{++} (< 30 mM) caused a marked increase in tension.

Treatment of the taenia coli with sucrose-Krebs without prior high K^+ treatment did not cause a contracture even if $[Ca^{++}]_o$ was raised to 10 mM. The authors concluded that high K^+ treatment was necessary to increase the permeability of the plasma membrane to Ca^{++} and that subsequent replacement of K^+ with sucrose did not reduce the Ca^{++} permeability. Na^+ might then reduce Ca^{++} influx by competing with Ca^{++} for influx sites in the membranes, as did the multivalent cations except for Ca^{++}, which must have inhibited Ca^{++} influx through a stabilizing action (50). Na^+ might also increase Ca^{++} efflux directly through a Na^+,Ca^{++} exchange process. However, since the high K^+-sucrose contracture was not maximal another Ca^{++} extruding process must have been operating in this tissue and the authors conclude that Ca^{++} extrusion was, in addition, mediated by an active Ca^{++} pump using another mechanism.

In rat ileum, Na^+ removal alone is sufficient to cause a transient contracture followed by complete relaxation. Taniyama (151) demonstrated that these Na^+-free contractures were dependent both on an increased Ca^{++} influx and release of intracellular Ca^{++} from a storage site. The transient contractions caused by replacement of Na^+ with sucrose or choline were gradually diminished over a period of 4 hours when $[Ca^{++}]_o$ was removed, probably due to the abolition of the influx component. If Ca^{++} (2 mM) was then returned to the medium during the period in which the muscle was no longer able to respond to Na^+ removal, repeated reapplication and removal of Na^+ did not cause contractures unless Na^+ was present during a crucial period after Ca^{++} was added back. It was also found that zero Na^+ contractions could be blocked by 10^{-7} M isoproterenol. The author concluded that zero Na^+ stimulated release of Ca^{++} from intracellular storage sites, probably via a drop in intracellular Na^+. If these sites were depleted by repeated removal of Na^+ from a zero Ca^{++} medium they could not be refilled unless both Na^+ and Ca^{++} were added back to the medium.

High K^+ pretreatment decreased but did not abolish intracellular Ca^{++} release by zero Na^+, implying that release occurs both from sites sensitive to $[K^+]_o$ and from sites resistant to $[K^+]_o$. Similarly, mouse rectal strips briefly contracted in a LiCl medium and this transient Na^+-free contraction only gradually diminished in zero Ca^{++} solution (44). Acetylcholine, Ba^{++}, K^+, and Ca^{++}, when added to a zero Ca^{++} medium, caused a biphasic contraction of rectal strips. If Na^+ was removed during a 3-minute period just prior to Ca^{++} removal, acetylcholine, Ba^{++}, and K^+ contractions were decreased, as was the phasic Ca^{++} contraction. The tonic Ca^{++} contraction was, however, increased. Cheng (44) suggests that Na^+ removal causes the release of Ca^{++} from intracellular storage sites that are also involved in supplying the Ca^{++}-mediated K^+, acetylcholine, and Ba^{++} contractions. Furthermore, the depletion of Ca^{++} from these pools causes a membrane destabilization responsible for the increased Ca^{++} influx associated with Na^+ removal.

The action of isoproterenol (Iso) to relax contracture in rat rectum was shown to be cAMP dependent (152). Iso diminished ACH and high K^+ contractures in a zero Ca^{++} medium, as did dibutyryl cAMP, and aminophylline potentiated these effects. It was suggested that an increase in cAMP inhibited the release of Ca^{++} from intracellular storage sites involved in ACH and K^+ contractions. Replacement of medium Na^+ with Li^+ or choline antagonized the relaxation caused by these agents, and it was proposed that this effect might be due to membrane depolarization, an inhibition of Na^+,Ca^{++} exchange, or as noted above, a decrease in Ca^{++} binding by intracellular storage sites. Takayanagi et al. (150) found that Iso could not relax taenia coli in a depolarizing high K^+ medium ($[K^+]_o$ > 20 mM) if Ca^{++} was present in the solution,

although it is not clear whether this inhibition of relaxation was due to depolarization or to a deficiency in $[Na^+]_e$.

Tomita and Sakamoto (156) studied zero Na^+ contracture and Na^+-dependent relaxation in the circular muscle of guinea-pig stomach. They found that replacement of Na^+ with choline caused a sustained increase in tension that could be abolished by treatments thought to prevent Ca^{++} influx, such as high Ca^{++}, zero Ca^{++}, and 0.5 mM Mn^{++}. These results suggest that Na^+-free contracture is due to an increased Ca^{++} influx. If the Na^+-free contracture was terminated by the addition of only 5mM Na^+, subsequent removal of Na^+ caused a contraction of much smaller amplitude. The authors suggest that the initiation of contracture may require both a certain level of intracellular Na^+ and an inwardly directed Na^+ gradient of critical magnitude. These results may alternatively be explained in light of the model for intracellular Ca^{++} release described above. Low concentrations of external Na^+ might block Ca^{++} influx competitively at the plasma membrane, but may not raise intracellular Na^+ to the level required for refilling of the intracellular Ca^{++} pools, which respond to zero external Na^+. Thus, small amounts of Na^+ could relax a zero Na^+ contracture but not allow muscle cells to recover their intracellular stores of releasable Ca^{++} to the extent necessary for another zero Na^+ contracture.

The ideas of Taniyama (151) and Cheng (44) are intriguing, but more work is needed before the concept of intracellular release of Ca^{++} by zero Na^+ stimulus can be regarded as proved. One major problem with these studies is that they do not rule out the possibility that enough extracellularly bound Ca^{++} may have persisted in their zero Ca^{++} solution to mediate residual zero Na^+, Ba^{++}, K^+, and cholinergic contractions, if these stimuli increase the influx of this bound cation (69, 70). Neither author used a Ca^{++}-chelating agent to ensure that the anionic sites in the glycoprotein matrix in the extracellular space and the outer face of the membrane were Ca^{++}-free.

The existence of a large concentration of cation binding sites on the outer surface of the membrane has also been a serious problem confronting those who have attempted to distinguish different Ca^{++} compartments in smooth muscle. Goodford (69), using Na^+ and K^+ to displace Ca^{++} from anionic sites on the cell surface, found 4 mEq/kg of such sites in taenia coli. Sparrow (148) found 12 mEq/kg of anionic sites, using Ca^{++} and Mg^{++} as competitive agents. Goodford and Wolowyk (70), analyzing the displacement of uranyl cations by Mg^{++}, Ca^{++}, Na^+, and K^+, subsequently found evidence for two arrays of anionic sites on the outer plasmalemmal surface of taenia coli fixed in glutaraldehyde. Their data suggested that one array had a much higher cation affinity than the other, and that both types of sites demonstrated the relative cation affinity order $Ca^{++} \simeq Mg^{++} >> K^+ > Na^+$. van Breemen et al. (170) used short labelling periods in isotonic sucrose media containing $^{45}Ca^{++}$ and found 8.1 ± 0.3 mmol/kg Ca^{++} binding sites on the surface of rabbit aorta cells; they also demonstrated that a significant fraction of this pool remains bound even after 90 minutes incubation in a zero Ca^{++} solution. Brading and Widdicombe (22) examined the effect of La^{+++}, which is known to displace cations from extracellular anionic sites (170, 172), on ionic content, and on the uptake and washout of ions in taenia coli. They found a total of 15 to 20 mEq La^{+++} sensitive sites per kg wet wt. Normally, these sites are occupied by (in mmol/kg) 1.37 Ca^{++}, 0.48 Mg^{++}, 1 K^+, and 12 to 15 Na^+. Considering the relative affinities of these negatively charged binding sites for Ca^{++} and Na^+ and the relative concentrations of these cations in physiological media, it is very probable that competition occurs between Na^+ and Ca^{++} for these sites.

C. Na^+ SUBSTITUTION AND Ca^{++} FLUXES. An approach that has been widely used in the study of Na,Ca interactions involves

the use of labelled isotopes, especially $^{45}Ca^{++}$ and $^{22}Na^{+}$, to measure Na^{+} and Ca^{++} fluxes across intestinal smooth muscle cell membranes. In this way, manipulations of the Na^{+} gradient, for example, can be related to measurable changes in tissue Ca^{++} content and directional fluxes. Ma and Bose (111) equilibrated a large number of taenia coli segments in a series of physiological media, all of which contained identical specific activities of $^{45}Ca^{++}$. The taenia were first labelled in a normal Na^{+} medium and then in a medium containing sucrose as a Na^{+} substitute, plus 70 mM K^{+}. The K^{+} was then washed out. Finally, the sucrose was replaced by Na^{+}. The taenia exhibited a contracture in the high K^{+} medium, which persisted in the sucrose, but was abolished when Na^{+} was replaced. During each step some taenia were removed and the intracellular label quantitated using the La^{+++} method (170) to quench extracellular Ca^{++} Na^{+}-free contracture was found to be associated with a large increase in cellular Ca^{++}, while Na^{+}-mediated relaxation was accompanied by a very rapid net extrusion of Ca^{++}, so that cellular Ca^{++} returned to precontracture levels within 2 minutes of Na^{+} replacement.

In order to establish whether relaxation subsequent to Na^{+} replacement was due to an increased Ca^{++} efflux or whether a decrease in Ca^{++} influx alone was enough to relax the tissue, Ca^{++} influx blockers were used in an attempt to relax the zero Na^{+} contracture. Both 10 mM Mg^{++} and Mn^{++} were able to cause only a limited relaxation, as was $4 \times 10-^{7}$ M D600. The authors, therefore, concluded that zero Na^{+} contraction was due to an increased Ca^{++} permeability and a decreased extrusion of Ca^{++}, suggesting that a Na,Ca exchange carrier might be responsible for some Ca^{++} extrusion. However, the results of these experiments may be criticized, in that the concentrations of Ca^{++} influx blockers were very much lower than those that have been shown to be maximally effective (98, 118). Relaxation of the taenia coli contracted under identical conditions has been obtained with 10^{-5} M D600 or 2 mM La^{+++} (unpublished results from authors' laboratory).

The experiments of Ma and Bose (111), as well as those to be described below, have been designed to establish whether the inward Na^{+} gradient is necessary for the extrusion of Ca^{++} from intestinal smooth muscle, as was suggested for vascular smooth muscle by Reuter et al. (132). If Ca^{++} extrusion is singly dependent on the Na^{+} gradient, removal of this gradient should abolish Ca^{++} efflux and cause a progressive increase in $[Ca^{++}]_i$, approaching equilibrium distribution. Ma and Bose (111), have found a Ca^{++} gain upon Na^{+} removal and their data support the conclusion that this gain is indeed partially due to a decreased Ca^{++} extrusion.

Goodford and associates (7, 67, 68) found that replacement of all medium Na^{+} with Li^{+} caused a transient increase in the total tissue Ca^{++} content of taenia coli (from 3.1 $\pm$ .02 to 3.7 $\pm$ 0.2 mmol/kg wet wt.) during the initial phase of contracture initiated by this substitution. However, within 2 hours after Na^{+} substitution, all of the additional Ca^{++} was extruded, leaving 3.0 $\pm$ 0.2 mmol/kg wet wt. During this period the efflux of $^{45}Ca^{++}$ was not significantly altered by Na^{+} substitution. It was found that $^{45}Ca^{++}$ uptake into taenia coli could be described in terms of three components. Na^{+} substitution increased the two rapid components of uptake during the period in which tissue Ca^{++} was increased, but this increase was not sufficient to explain the large accumulation of Ca^{++} by the tissue. It was, therefore, suggested that some of the uptake of label must have occurred into the slowly exchanging compartment. The relative size and kinetics of this compartment suggested that it was intracellular and that the Ca^{++} uptake was not due to an effect on extracellularly bound cation. However, it is possible that this extracellular pool may have obscured any changes in transmembrane Ca^{++} efflux caused by Na^{+} substitution.

Casteels and van Breemen (43) have also used $^{45}Ca^{++}$ to study the effects of reducing external Na^+ on the intracellular Ca^{++} content of taenia coli. They found that replacement of all but 7 mM Na^+ with choline caused a small plateau type increase in $^{45}Ca^{++}$ content over that seen with normal Na^+. The authors also investigated the effect of Na^+ replacement on Ca^{++} efflux.

Taenia that had been fully labelled with $^{45}Ca^{++}$ were treated with dinitrophenol and iodoacetate. These procedures increase cytoplasmic $^{45}Ca^{++}$ activity by releasing bound $^{45}Ca^{++}$ from some intracellular sequestration site(s) and cause a corresponding increase in the rate of efflux of label from cells (173). The replacement of all medium Na^+ with Li^+ enhanced the metabolic inhibition-mediated rate increase, implying that the Na^+ gradient was not involved in Ca^{++} extrusion.

Cooling taenia coli to 0°C causes the cells to take up a large amount of Ca^{++}. If the temperature of the medium is then raised to 37°C, the cells will extrude the Ca^{++} they have gained. Replacement of Na^+ with tris, or of all but 7 mM Na^+ with Li^+, did not prevent this net extrusion found upon raising the temperature. The authors concluded from their data that an inwardly directed Na^+ gradient did not appear to be necessary for Ca^{++} extrusion.

The apparent discrepancy between the results of Reuter et al. (132) and those of Casteels and van Breemen (43) may be explained by the work of Raeymaekers et al. (131), which indicated that the effects of Na^+ substitutes on $^{45}Ca^{++}$ efflux from taenia coli are predominantly caused by extracellular phenomena. Since a large component of $^{45}Ca^{++}$ efflux from labelled taenia coli is due to the washout of $^{45}Ca^{++}$ from the extracellularly bound pool described above, it is possible that Na^+ substitutes with differing affinities for Ca^{++} binding sites will exchange with extracellular Ca^{++} at varying rates, thus producing apparent differences in Ca^{++} efflux. The results of Raeymaekers et al. (131) suggest strongly that the apparent effects of Na^+ substitutes on Ca^{++} efflux are due to extracellular exchange. Substitution of Li^+ for Na^+ stimulated Ca^{++} washout, while sucrose, K^+, tris, and choline variably decreased the rate of Ca^{++} washout. If the cell membranes were made leaky by prolonged treatment with metabolic inhibitors or by boiling the tissues for 2 minutes, the effect of Na^+ substitution on Ca^{++} efflux was similar to that found in healthy tissue. Lowering the pH from 7.4 to 5 caused a transient increase in Ca^{++} efflux, which would be expected if protons were competing with Ca^{++} for extracellular binding sites. In agreement with the above, Curtis and Prosser (47) reported that replacement of NaCl by LiCl caused an increase in $^{45}Ca^{++}$ efflux in labelled cat jejunum and that this increase was prevented by procedures that remove extracellularly bound Ca^{++} (e.g., EGTA treatment). These authors did not see a similar exchange of Li^+ with extracellular Ca^{++} in taenia coli. Cesium was also able to release extracellular Ca^{++} in jejunum but tetraethylammonium (TEA) and sucrose could not. The authors used lithium treatment to remove extracellular Ca^{++} and then replaced the tissue into media containing Na^+ or sodium substitutes (tris or arginine). Na^+ substitution was not found to change the efflux of $^{45}Ca^{++}$ in this situation, in which efflux was presumably transmembrane. It was concluded that the Ca^{++} efflux is not Na^+-dependent in cat jejunum.

Brading and Widdicombe (22) have attempted to establish the presence of Na, Ca exchange in taenia coli by studying the dependency of the $^{24}Na^+$ efflux from taenia coli on extracellular Ca^{++}. The Na, Ca exchange mechanism suggested by Reuter et al. (132) should be able to mediate Na, Ca exchange in either direction if the ionic concentrations in the cellular and extracellular compartments are manipulated correctly.

Taenia were loaded with $^{24}Na^+$ by prolonged incubation in a medium containing zero $[K^+]_e$. This procedure caused a complete replacement of cellular K^+ with Na^+ (4, 36). The tissues were then allowed to

efflux into a Krebs solution containing no K^+ or Na^+, so that both Na,K and Na,Na exchanges (37) were prevented. The rate of $^{24}Na^+$ efflux first decreased (an extracellularly bound label was washed out) and then showed a secondary increase (16, 17, 21). This delayed increase in $^{24}Na^+$ was dependent on the Na^+ substitute employed; it was present in sucrose, dimethyldiethanol ammonium choloride (DDA), and tris chloride-substituted media, but not in Mg^{++} or Li^+ containing solutions. Furthermore, this secondary stimulation of efflux was $[Ca^{++}]_e$-dependent, and prevented by La^{+++} (5 mM)-pretreatment.

Brading (19) suggests that the large outwardly directed Na^+ gradient causes entry of Ca^{++} into cells due to a Na,Ca exchange system. The subsequently increased $[Ca^{++}]_i$ is assumed to interact with the inner surface of the plasmalemma to cause an increased Na^+ permeability and efflux rate. However, comparison of the efflux curves before the membrane breakdown does not provide evidence for a component of $^{24}Na^+$ efflux that is coupled to Ca^{++} influx. The secondary increase in Na^+ efflux could also be explained if these extreme experimental conditions sequentially increased Ca^{++} and Na^+ permeabilities of the cell membrane. In order for the delayed potentiation of Na^+ efflux to occur, tissues must be both Na^+-loaded and exposed to a medium with less than 15 mM Na^+ (19). At this time, the ratio of $[Na^+]_e/[Na^+]_i$ is 0.1, as opposed to a normal ratio of 10 to 15.

A nonspecific increase in plasmalemma permeability was indeed detected in taenia coli with a reversed Na^+ gradient, as shown by the penetration of ^{14}C-sucrose and ^{60}Co EDTA into cells and by an increased flux of $^{42}K^+$ across the membrane. Brading (19) proposed that the increase in $[Ca^{++}]_i$ due to Na,Ca exchange precedes and causes the general membrane alteration, which permits the secondary increase in Na^+ efflux, and a simultaneous uptake of Ca^{++}. She has, however, not been able to show such a sequential mechanism. If Na^+ gradient-reversed tissues were exposed to zero Na^+ media containing either $^{45}Ca^{++}$ or ^{14}C-sucrose, the uptake of $^{45}Ca^{++}$ did not precede that of ^{14}C-sucrose, implying that no measurable Na,Ca exchange was occurring prior to membrane alteration. Assuming muscle tension to be an indicator of Ca^{++} uptake, Brading (19) has shown that Ca^{++} uptake precedes the nonspecific increase in membrane permeability by about 5 minutes. These experiments are not conclusive, since in this case muscle tension may not have been a function of Ca^{++} influx but of Ca^{++} released from internal stores.

The increase in membrane permeability was prevented by 5 mM La^{+++}, as shown by its ability to block the increased Na^+ efflux found in Na^+ gradient-reversed taenia coli after the addition of $[Ca^{++}]_e$. La^{+++} also delayed, but did not prevent, the onset of tension that immediately followed Ca^{++} addition. Brading suggests that the tension in the presence of La^{+++} was due to a La^{+++}-resistant Na,Ca exchange. It is alternatively possible that the tension development in this situation was caused by intracellular Ca^{++} release or to a penetration of La^{+++} into the cells. This latter effect has been noted with similar La^{+++} concentrations in conditions favoring increased membrane permeability in smooth muscle (118, 159).

Brading and Widdicombe (21) obtained evidence for $^{45}Ca^{++}$ efflux coupled to Na^+ influx in the following manner. They loaded the cells with $^{45}Ca^{++}$ by exposing Na^+-rich tissues to labelled zero Na^+ medium. The cells became leaky and took up a large amount of $^{45}Ca^{++}$ and were subsequently "resealed" with 5 mM La^{+++}. The tissues so prepared showed a $[Na^+]_e$-dependent $^{45}Ca^{++}$ efflux component. There remains, however, some uncertainty concerning the extent to which cells challenged by these procedures reflect physiological mechanisms.

It is interesting that Casteels et al. (39) have also noted that an increase in $[Ca^{++}]_e$ from 2.5 to 10 mM caused an increase in the rate of $^{24}Na^+$ efflux from Na^+-rich taenia coli, but not from Na^+-normal

tissues. These authors suggest that $[Ca^{++}]_o$ might affect the Na^+ permeability of the plasmalemma, but their experiments do not address the possibility that a general membrane breakdown may have occurred in the Na^+-rich preparation.

Recently, van Breemen et al. (167) have used Na^+ substitutes to investigate the relationship between the Na^+ gradient and cellular Ca^{++} content in taenia coli. Intracellular Ca^{++} was measured by washing the tissues in cold EGTA-containing medium for 45 minutes after completion of the experiments to remove extracellularly bound Ca^{++}. It was found that total replacement of medium Na^+ with Li^+ caused a small but significant gain of Ca^{++} by the cells, which reached a plateau between 30 and 60 minutes, and a subsequent slow secondary Ca^{++} accumulation, which may have been due to cell damage. Replacement of all Na^+ with sucrose caused a much larger Ca^{++} gain by the taenia cells (175 μM/kg), which also reached a constant level within 20 to 30 minutes. A large secondary accumulation of Ca^{++} was noted after 1 hour in sucrose medium. In one series of experiments, various concentrations of Na^+ were added to the sucrose solution, and cell Ca^{++} was measured. Addition of more than 20 mM Na^+ caused a net extrusion of all the Ca^{++} gained during the sucrose incubation and a subsequent maintenance of cell Ca^{++} at control level. Intracellular Na^+ was measured during this period and it was found that the ratio $[Na^+]_o/[Na^+]_i$ was 2.5, assuming that all intracellular Na^+ was freely dissolved in the cytoplasm; 140 mM Li^+ was able to mimic the effect of Na^+, but at a slower rate.

If small concentrations (1 mM) of Na^+ were replaced into the sucrose medium during the plateau period, cell Ca^{++} increased further due to a large increase in membrane permeability to Ca^{++}. The authors concluded on the basis of these experiments and others to be described below that extracellular Na^+ may both influence Ca^{++} influx and stimulate Ca^{++} efflux by interacting with Ca^{++} in an ion exchange pore lined by anionic binding sites. However, this mechanism alone cannot explain the maintenance of Ca^{++} homeostasis in the taenia under physiological conditions.

SUMMARY. The evidence described above suggests that Na^+ and Ca^{++} interact at a variety of sites in intestinal smooth muscle and that Na^+ may influence Ca^{++} homeostasis in complex ways.

The removal of medium Na^+ seems to increase the Ca^{++} permeability of the plasma membrane, resulting in an increased Ca^{++} influx and a gain of Ca^{++} by cells. It is not clear whether the increased influx is due to alterations in the Na^+ gradient or due to the specific absence of either intracellular or extracellular Na^+.

A decreased $[Na^+]_i$ may cause Ca^{++} to be released from intracellular sequestration sites, resulting in a transient contraction. It is also possible that this Ca^{++} release may influence the Ca^{++} permeability of the plasmalemma. $[Na^+]_i$ may also be important in the reuptake of Ca^{++} into intracellular reservoirs.

Taenia coli cells that have been loaded with labelled Ca^{++} during an incubation in a zero Na^+ medium will extrude the additional Ca^{++} when as little as 20 mM Na^+ is added back to the medium, and several types of smooth muscle tissue will relax when as little as 5 mM Na^+ is resubstituted. The basis of this net extrusion is not yet understood; it may be due solely to a reduction of Ca^{++} influx or to both a reduction of influx and a stimulation of efflux. This latter effect, which has not yet been unequivocally demonstrated, would be consistent with a Na,Ca exchange hypothesis in intestinal smooth muscle.

Regarding the Na,Ca exchange model proposed for smooth muscle in 1973, several studies in this section demonstrate that the presence of a Na^+ gradient in the physiological range is not necessary for maintenance of the very low $[Ca^{++}]_i$ found in intestinal smooth muscle. Na^+ may, however, exert widespread effects on intracellular

and transmembrane Ca^{++} fluxes and thus be required for normal smooth muscle function.

Substitution of Na^+ with K^+ is of special importance since it does not require the introduction of a nonphysiological molecule. Since, however, the literature on elevated $[K^+]$ in intestinal smooth muscle is very extensive and complex the next section is devoted to this problem.

2. *Effects of $[K^+]_e$ Elevation.* A great deal of effort has been directed toward the development of a model that would explain the increased tension observed in intestinal smooth muscle upon elevation of $[K^+]_e$.

The increase in tension following exposure of intestinal smooth muscles to a medium containing excess K^+ (high K^+ medium) is generally considered to include an initial rapid (phasic) component and a sustained (tonic) component. In taenia coli, Gabella (64) has noted an initial transient drop in tension before the phasic contraction, which he suggests is caused by a K^+-mediated release of an inhibitory transmitter from nonadrenergic nerve endings. In an extensive series of experiments, he has also established that the guinea-pig taenia coli will show a measurable contractile response to as little as 0.5 mM extra $[K^+]_e$. Maximal isometric tension was produced by $[K^+]_e$ in the range of 20 to 25 mM, and maximal duration of contracture was produced by concentrations of 20 to 70 mM. In these experiments, $[K^+]_e$ of more than 36 mM were obtained by replacing some NaCl with KCl; at lower $[K^+]_e$, K^+ was added hypertonically. Taenia coli which had been exposed to more than 55 mM $[K^+]_e$ did not relax when the high K^+ media were washed out with normal K^+ media; instead they showed a larger aftercontraction that increased in amplitude and duration as a function of the K^+ concentration to which they had been previously exposed. Therefore, under certain conditions an increase in Na^+ gradient may be associated with contraction rather than ralaxation.

Gabella (64) found that maximal tension increases were produced at the $[K^+]_e$ that depolarized the taenia to about −32 mV. Higher $[K^+]_e$ appeared to decrease contracture duration if isometric recording methods were employed. Isotonically recorded contracture was well maintained between 20 and 110 mM $[K^+]_e$.

In high K^+ media, taenia coli from guinea pig depolarizes within 30 seconds to reach a stable membrane potential (134). The resting potential decreases linearly with the increase in log $[K^+]_e$ at concentrations above 30 mM; the change in potential per 10-fold increase has been measured by several investigators and found to be between 26 and 51.5 mV (32, 80, 103, 134). The slope is not linear below 30 mM $[K^+]_e$ and is less than the 59 mV per 10-fold change in $[K^+]_e$ predicted by the Nernst equation if K^+ were the sole determinant of membrane potential. Thus, Na^+ and Cl^- permeabilities contribute significantly to the maintenance of the resting membrane potential (103). Holman (80) has found, however, that the slope of the line relating log $[K^+]_e$ to the potential is similar whether K^+ is added hypertonically to the medium or whether Na^+ is decreased to maintain isosmoticity as K^+ is added.

The relationship of the depolarization of the membrane to the contracture produced by high K^+ treatment remains obscure. Kuriyama et al. (106) cite work done by Ito and associates on pulmonary artery, which showed that a high K^+ contracture can be produced even if the membrane potential is clamped at resting levels. Electrical stimulation without increased $[K^+]_e$ also caused a contracture, although both responses were less pronounced than that to high K^+ alone. It was also noted (106) that in spontaneously active smooth muscle, high K^+ produces large phasic and small tonic contractures (e.g., circular muscle of antrum), while other types of smooth muscle show small phasic and large tonic responses (e.g., longitudinal muscle of fundus). In the circular muscle preparation, the K^+ response is very much membrane potential-depend-

ent, while in the fundus it is less potential dependent. This suggests that K^+ contractures may be mediated mainly by depolarization in more electrically active types of smooth muscle and by some other pathway in more quiescent tissues. However, Matthews and Sutter (117) have found that 50 mM $[K^+]_e$ is able to contract taenia coli that has been maximally depolarized by ouabain. Thus, even excitable tissues may respond to high K^+ via a membrane potential-independent pathway.

Controversy also exists concerning the sources of the Ca^{++} that activate the contractile apparatus during the two phases of K^+ contracture. As with other stimuli, high K^+ may cause contraction either via an increased net influx of Ca^{++} or via potentiation or initiation of Ca^{++} release from some intracellular sequestration site(s). Evidence has accumulated that the phasic and tonic components of the contractile response of smooth muscle to high K^+ may be mediated by somewhat different Ca^{++}-supplying systems.

Two major models have been developed to account for high K^+ contraction of intestinal smooth muscle and the influence of various agents upon this phenomenon. The first was proposed initially by Chujyo and Holland in 1963, and has subsequently been elaborated by Holland, Karaki, Urakawa, and associates (45, 65, 93, 94, 96, 121, 140, 163). In 1967, Imai and Takeda (85) published an alternative model that has also received considerable support from a variety of studies in the literature. These hypotheses and pertinent evidence will be described below; at this time, neither can be regarded as proved. Most of this work has been done using guinea-pig taenia coli.

Chujyo and Holland (45) found that the contracture of taenia coli caused by the addition of 35 mM K^+ (raising $[K^+]_e$ to 40 mM) to the medium was attenuated by reduction of $[Ca^{++}]_e$. Dinitrophenol also reduced the K^+ response, affecting the tonic response more than the rapid initial tension development. High K^+ also increased ^{45}Ca influx but did not change total tissue Ca^{++} or ^{45}Ca efflux significantly. These inherently contradictory findings probably resulted from a lack of accuracy in the total tissue Ca^{++} measurement. Dinitrophenol blocked the increased ^{45}Ca influx and reduced total tissue Ca^{++} during K^+ treatment, although an increased ^{45}Ca efflux was not detected.

It was proposed that exposure of taenia coli to a high K^+ medium might cause the release of bound Ca^{++} from intracellular stores. This Ca^{++} would contribute to tension development and then be inactivated. The pool of stored Ca^{++} must be refilled by an influx of extracellular Ca^{++} and the process of release and refilling would continue as long as $[K^+]_e$ remained elevated. Reduction of $[Ca^{++}]_e$ would not deplete this pool unless $[K^+]_e$ was elevated. Metabolic inhibition by dinitrophenol might slow the refilling process enough to abolish the sustained component of K^+ contracture but would not inhibit initial Ca^{++} release and tension development.

Subsequent work has led to the further elaboration of this scheme. Removal of glucose from the medium was discovered to selectively abolish the tonic component of high K^+ contracture in taenia coli. With this method of separating the two phases of response, Urakawa and Holland (163) found that ^{45}Ca uptake increased during both phases, while total tissue Ca^{++} increased only during the tonic component. Pfaffman et al. (128) were also able to selectively inhibit the tonic contraction by decreasing the temperature of the medium, by replacing medium NaCl with LiCl, and by treating taenia coli with 2×10^{-6} M ouabain. Shimo and Holland (140) noted that the amplitude and rate of development of the phasic contraction were related to the $[K^+]_e$ and the spike frequency whereas the tonic response was dependent on the membrane depolarization. They suggested that the phasic response was caused by the release of Ca^{++} from a limited pool and that the rate of this process was a function of $[K^+]_e$ and of the spike frequency. The tonic contraction would be dependent on a mem-

brane potential-dependent Ca^{++} influx, which would require metabolic energy and intracellular Na^+. Metabolic energy would be required for the function of a coupled Na^+ efflux, Ca^{++} influx process, and for the continuing activation of the contractile apparatus by Ca^{++}.

Karaki et al. (93) examined the effect of 40 mM excess $[K^+]_e$ on total tissue Ca^{++}, ^{45}Ca efflux, ^{45}Ca uptake, and ^{45}Ca uptake measured after a 4 minute wash in nonradioactive medium. The label remaining in the taenia coli after the wash was referred to as the "tightly bound fraction" (TBF). Incubation in the high K^+ solution increased total tissue Ca^{++} and $^{45}Ca^{++}$ uptake only during the sustained phase of contraction; $^{45}Ca^{++}$ efflux was not affected consistently by high K^+. TBF increased after 15 minutes. These results were consistent with the hypothesis of phasic Ca^{++} release and tonic Ca^{++} influx suggested earlier (140). In later experiments, Karaki and Urakawa (96) presented evidence that the TBF increased slowly throughout high K^+ treatment. This store of "accumulated Ca^{++}" exchanged with the extracellular medium with a half time of 7 minutes. The authors suggested that this pool represents Ca^{++} that has been utilized in contraction and subsequently rebound. This Ca^{++} might then be extruded from the cell. van Breemen and Daniel (168) and Lüllmann and Siegfriedt (110) found a similar shift of Ca^{++} to a slowly exchanging fraction in rat uterus and guinea-pig small intestine, respectively, during high K^+ stimulation.

Imai and Takeda (85) discovered that reduction of $[Ca^{2+}]_e$ to 10^{-8} M, using EGTA, suppressed the phasic response to high K^+ completely while leaving a component of the tonic response unaltered. Earlier, using the same tissue (taenia coli), Chujyo and Holland (45) had been able to eliminate both components of contraction if tissues were pretreated in a zero Ca^{++} medium to which excess K^+ was subsequently added. Imai and Takeda (85) noted that Ca^{++} removal abolished the action potentials that accompanied the phasic response, whereas Shimo and Holland (140) did not see a similar inhibition of spike activity during this period of high K^+ response when they removed Ca^{++} from their solution. In these latter experiments, the action potentials may have been dependent on the presence of a large extracellularly bound pool of Ca^{++} that is lost only gradually in zero Ca^{++} media but which Imai and Takeda (85) presumably removed with EGTA.

The phasic response was again totally eliminated by pretreatment of the taenia coli with multivalent cations (cadmium, nickel, cobalt) while the tonic phase was only partially inhibited. In a Ca^{++}-free medium, the residual tonic response to high K^+ was potentiated by these cations. The role of these ions was discussed in terms of their ability to block Ca^{++} influx and also to "reactivate" some aspect of the excitation-contraction coupling system that might be inhibited in a Ca^{++}-free milieu. Their results prompted the authors to propose that the phasic tension increase subsequent to elevation of $[K^+]_e$ could be due to an influx of Ca^{++} associated with the increased spike activity noted (41, 80, 134) during the initial rapid membrane depolarization. This influx would trigger the release of Ca^{++} from some sequestration site or bound state into the myoplasm. The tonic phase would be due to both influx of Ca^{++} and to a sustained influx-independent Ca^{++} release.

The model of Imai and Takeda (85) is consistent with other experimental evidence that has been reported. Mayer et al. (118) found that both D600 and La^{+++} abolished both phasic and tonic contractions elicited by incubation of taenia coli in a solution in which KCl replaced all NaCl, suggesting that both components are influx dependent. Diltiazem, a coronary vasodilator, was shown to block both phases of the the response of taenia coli and stomach strip from guinea pig to high K^+, although the phasic response was less sensitive to this agent (112). The inhibition of the phasic response was accompanied by blockade of the action potentials ordinarily oc-

curring during this period. In addition, subsequent work has supported the suggestion of Imai and Takeda that the action potentials in taenia coli carry an inward Ca^{++} current (20).

^{45}Ca efflux experiments also have provided some support for Ca^{++} influx during the initial rapid component of high K^+ contraction. van Breemen et al. (173) found that incubation of labelled taenia coli in a medium containing 80 mM $[K^+]_e$ caused an immediate increase in ^{45}Ca efflux from the tissue. This effect was prevented if Ca^{++} was removed from the medium. The authors concluded that high K^+ increased Ca^{++} influx and that the unlabelled Ca^{++} entering the cell displaced bound ^{45}Ca into the cytoplasm, stimulating its extrusion. This implies that the primary event underlying the phasic response to K^+ is Ca^{++} influx. The inability of other authors to find a stimulation of ^{45}Ca efflux by high K^+ may be related to differing $[K^+]_e$ employed as well as to difficulties related to separating intracellular and extracellular components of tissue isotope washout. Huddart and Saad (81) noted that quinine (0.5 - 5 mM) acts similarly to La^{+++} in that it showed the paradoxical property of preventing or cutting short a high K^+ (200 mM) contracture while potentiating the increased ^{45}Ca efflux caused by the K^+ treatment of rat ileum strip. They explained this effect by proposing that La^{+++} and quinine act similarly to displace an extracellularly bound Ca^{++} pool that might provide a source of the Ca^{++} influx necessary for both phases of the K^+ response.

Since action potentials in taenia coli and other types of spontaneously active intestinal smooth muscle are generally thought to be mainly mediated by an inward Ca^{++} current, and since there is an initial increase in spike frequency upon elevation of $[K^+]_e$, it is not unreasonable to conclude that Ca^{++} influx may be increased during the phasic response. It is not clear, however, whether the tension response is dependent on this Ca^{++} or whether this influx acts to trigger a larger release of intracellular Ca^{++} that is solely responsible for initial contraction. The inability of several workers to find an increased cell Ca^{++} content during the phasic response suggests that the influx is small (93, 163, 167) while the large increase in Ca^{++} efflux could reflect a more significant intracellular Ca^{++} release.

Both models envision the tonic phase of high K^+ contracture as being dependent on both Ca^{++} influx and intracellular Ca^{++} release, although the earlier scheme (45) suggests that these two effects are operating in series while the latter implies that they are in parallel. As of yet, there is no conclusive evidence for either hypothesis. It is worth noting, however, that removal of extracellular Ca^{++} may have caused an alteration in the structure of the plasmalemma during the experiments of Imai and Takeda (85). This may have led to an intracellular Ca^{++} release not ordinarily seen during high K^+ treatment.

One source of the variability in the literature dealing with the effects of high K^+ treatment may be the degree to which Na^+ is removed from the medium when K^+ is added. Karaki et al. (93) suggested that the discrepancies between their results and those of Imai and Takeda may have been due to the differences in $[K^+]_e$ and/or $[Na^+]_e$ used in the respective studies. In some of the latter, Na^+ was present in the medium only in the form of 8 mM $NaHCO_3$, although the authors note that they found no change in their data if all the Na^+ was left in the medium when K^+ was elevated (by the addition of 126 mM K_2SO_4 in both cases).

The concentration of Na in the medium may be very important in high K^+ experiments since $[Na^+]_e$ reduction may cause effects in addition to those caused by the elevation of $[K^+]_e$. This is especially true in that Na^+ substitution may exert its effects independently of the membrane potential (98). The interactions of $[Na^+]_e$ with high K^+ contracture in taenia coli have been described by Urakawa and associates in a series of papers (65, 92, 164).

Urakawa et al. (164) compared the ten-

sion response, tissue Ca^{++} content, and ^{45}Ca uptake caused by either the addition of 40 mM K^+ to normal Na^+ Tyrode's solution (hyper 40 K^+ medium) or the replacement of all medium Na^+ by K^+ (iso 152 K^+ medium). They found that in the iso 152 K^+ medium the tonic phase of the contracture reached a maximum and then declined to a low level. In the hyper 40 K^+ solution, the tonic phase did not decline in amplitude during the incubation. In a labelled hyper 40 K^+ medium, total tissue Ca^{++} did not change and ^{45}Ca uptake was elevated over a 30-minute period, while in the iso 152 K^+ total tissue Ca^{++} decreased and ^{45}Ca uptake did not change, as compared to the control tissues left in normal Tyrode's solution. ^{45}Ca uptake was measured after a 4-minute wash designed to remove extracellular Ca^{++}. Subsequently, it was shown that the tonic response to iso 152 K^+ treatment was not abolished by pretreatment of the taenia with ouabain, although this agent was able to inhibit the tonic response to hyper 40 K^+. It was suggested that the Na^+ gradient might play a different role in the two types of contraction. Karaki et al. (92) studied the relationship between $[Na^+]_e$ and the tonic phase of high K^+ contracture using several procedures. In one, $[K^+]_e$ was elevated progressively while $[Na^+]_e$ was reduced to maintain isosmolarity. It was found that the tonic tension decreased as $[Na^+]_e$ was lowered even though $[K^+]_e$ was concomitantly being increased. Tissue Ca^{++} and ^{45}Ca uptake (TBF) were also measured using this procedure and results similar to those reported by Urakawa et al. (164) were noted. In another series of experiments, $[K^+]_e$ was set at 42.7 mM and $[Na^+]_e$ was varied between 50 and 300 mM without any osmotic compensation. There was again a quasilinear decrease of tonic tension as $[Na^+]_e$ was lowered from 160 to 50 mM. Finally, $[K^+]_e$ was set at 42.7 mM and $[Na^+]_e$ was varied between 0 and 149 mM; Na^+ substitutes (Li^+ choline, tris, or sucrose) were employed to maintain isosmolarity. Although the results varied somewhat according to the Na^+ substitute used, there was again a general decrease in tonic contraction tension as $[Na^+]_e$ was reduced.

These results are consistent with those found by Gabella (64), since in this latter study, Na^+ was removed to maintain isosmolar conditions at the higher $[K^+]_e$ that gave abbreviated isometric K^+ contractures. In all of these experiments the phasic response was not sensitive to Na^+ removal. Riemer et al. (134) have also noted that elevation of $[K^+]_e$ from 32.45 to 59 mM, with a simultaneous reduction of $[Na^+]_e$, leads to a reduction of the sustained response of taenia coli to high K^+. Brading (18) has found that Na^+ substitution in high K^+ solutions inhibited the response of taenia coli to the stimulus in that the threshold concentration of extracellular Ca^{++} needed to produce the K^+ contraction is elevated if Na^+ is replaced. On the other hand, when $[Ca^{++}]_e$ was elevated, the presence of Na^+ in the medium prevented taenia coli from reaching a maximal contracture tension; when Na^+ was removed, maximal tension was reached. She, therefore, suggests that Na^+ may be necessary for both Ca^{++} influx and efflux. However, these observations might also be related to Na^+-dependent release or sequestration of Ca^{++} within cells.

Taniyama (151) has made the anomalous observation that removal of extracellular Na^+ increases the tonic contraction while decreasing the phasic contraction during the response of rat ileum to high K^+. This is similar to the effect of Na^+ substitution on the norepinephrine-induced contraction of mesenteric arteries, which was discussed in an earlier section (145). It, therefore, appears that different smooth muscle types may show a wide range variability with respect to the dependency of high K^+ contractures upon Na^+.

van Breemen et al. (167) have recently measured changes in the Ca^{++} content of taenia coli during total replacement of medium Na^+ with K^+. They found that the phasic response was not accompanied by a net uptake of labelled Ca, although the cells did accumulate about 100 μmol/kg wet wt.

of Ca^{++} during the tonic contracture. The muscle then relaxed completely at a time when the Ca^{++} content was still increasing. After 100 minutes in the high K^+ zero Na^+ medium, tissue Ca^{++} had decreased to near control levels. It therefore seemed likely that the phasic contraction is initiated by Ca^{++} release while the tonic high K^+ response may be accompanied by complex changes in membrane Ca^+ permeability and intracellular Ca^{++} sequestration. In addition, it became obvious that the taenia can actively extrude accumulated Ca^{++} in the total absence of Na^+.

SUMMARY. Incubation of intestinal smooth muscle in a medium containing an elevated concentration of K^+ has been generally found to cause a biphasic tension response. Upon application of high K^+, there is an immediate increase in tension that reaches a peak rapidly and then declines (phasic response) to a steady, sustained contracture (tonic response). Taenia coli are very sensitive to $[K^+]_e$; as little as 0.5 mM excess $[K^+]_e$ will give a response, and a maximal response occurs between 20 and 70 mM $[K^+]_e$. The high K^+ contracture is related to the depolarization of the cell membrane caused by the decrease in the K^+ equilibrium potential, but the stimulus also seems to work via a pathway that is independent of the membrane potential. The phasic portion of the contracture probably is due to a release of Ca^{++} from intracellular storage sites; this release may be triggered by a Ca^{++} influx. The tonic response is mainly influx-dependent, although there is some evidence for a release of intracellular Ca^{++} during this time.

The tonic response has been shown to be dependent on the presence of Na^+ in the medium, although it is not clear whether the Na^+ requirement involves intracellular or extracellular Na^+. A large increase in intracellular Na^+, due to inhibition of the Na,K ATPase caused by ouabain treatment, also blocks the tonic response. The residual tonic response remaining after Na^+ removal is not sensitive to ouabain. Therefore, it seems possible that Na^+ may exert differential effects on the influx of Ca^{++} that occurs during the tonic response to high K^+. In the presence of an elevated $[K^+]_e$ extracellular Na^+ may promote Ca^{++} influx while intracellular Na^+ may reduce Ca^{++} influx. Alternatively, an increase in intracellular Na^+ may allow cells to sequester influxing Ca^{++} more rapidly or completely so that this Ca^{++} does not reach the myofilaments and thus does not cause a tension response.

3. Ouabain and Zero K^+. Inhibition of the Na,K ATPase by ouabain or zero K^+ treatment causes a gradual breakdown of the Na^+ and K^+ gradients in smooth muscle (4, 34). Blockade of the Na,K pump of intestinal smooth muscle exhibits a pattern of effects similar to that seen in blood vessels capable of spontaneous rhythmic activity in the presence of phentolamine (95, 97). van Esveld (175) first reported that digitalis had a dual effect upon the motility of intestinal smooth muscle, characterized by an initial increase in tension (phase I) lasting 15 to 20 min, followed by a relaxation (phase II). Measurements of electrical activity in taenia coli during phase I, utilizing both sucrose gap techniques (138) and direct recordings by intracellular microelectrodes (34), demonstrated a parallelism between the early tension development and an increase in spike frequency. Phase I effects are probably related to an initial depolarization of tissues by 10 to 15 mv (39, 117), consistent with the concept that the ouabain-sensitive pump is electrogenic in taenia coli (36) and that spike frequency is related to the amplitude of the depolarized phase of the "slow wave" characterizing the resting membrane potential of the taenia (105). With ouabain there is a subsequent continuing depolarization of intestinal smooth muscle to about 25 to 30 mv (34), whereas with zero K^+ incubation the cells repolarize to control levels or become slightly hyperpolarized (80, 158). The hyperpolarization is probably related both to an increased $[K^+]_i/[K^+]_e$ ratio and an increase in the K^+ permeability of the membrane and is dependent on the presence of

a permeable anion in the medium. After about an hour in the K^+-free media, the cells begin to depolarize and reach a final steady potential of about −10 mv within 3 hours (36). It is also possible that the electrogenic Na,K pump is still operating during phase II, utilizing K^+ that leaks out of the cells (38).

A number of studies have also dealt with the effects of ouabain or zero K^+ treatment on zero Na^+ contracture and associated Ca^{++} movements.

Judah and Willoughby (88) replaced $[Na^+]_o$ with sucrose to cause contracture of guinea-pig ileum and then treated the tissue with 10^{-5} M ouabain for 10 minutes before replacing $[Na^+]$ in the bathing medium. The typical Na^+-mediated relaxation of the tissue was greatly inhibited by ouabain. It is difficult to explain this inhibition on the basis of a reduced Na^+ gradient because it is unlikely that ouabain would affect the instantaneous distribution of Na^+ existing immediately after Na^+ repletion; the effect of the drug may have been exerted via some effect on membrane potential or on Ca^{++} permeability. Conversely, Katase and Tomita (98) found that 2×10^{-6} g/ml ouabain or zero K^+ pretreatment only slightly slowed Na^+-mediated relaxation of Ca^{++}-loaded taenia coli incubated first in high K^+ medium and then in normal K^+, zero Na^+ medium.

Several studies have established that after the transient increase in tension caused by ouabain and zero K^+ intestinal smooth muscle relaxes completely (4, 34, 117, 138, 158, 175). Unless these treatments decrease the Ca^{++} permeability of the membrane to a negligible level, this relaxation implies that the Na^+ gradient is not responsible for maintaining the low cytoplasmic Ca^{++} concentration found in relaxed smooth muscle. This relaxation is not due to a direct effect of the drug on the contractile apparatus, since application of high K^+ to ouabain relaxed smooth muscle will cause contracture (117). Brading (18) has found that several doses of carbachol will also contract taenia relaxed in zero K^+. In the absence of stimuli, the Na^+-loaded taenia coli will only take up extra Ca^{++} when extracellular Na^+ is lowered so that Na^+ gradient becomes $[Na^+]_i/[Na^+]_o = 10$. She has shown that this Ca^{++} uptake is probably due to an increase in nonspecific membrane permeability.

Attempts have been made to measure changes in cellular Ca^{++} concentrations during Na^+ gradient breakdown. van Breemen et al. (170) used 2×10^{-5} M ouabain, zero K^+, and metabolic inhibition to block the Na^+,K^+-ATPase and examined Ca^{++} content in taenia coli. Depletion of ATP with dinitrophenol and iodoacetate caused a significant breakdown of Na^+ and K^+ gradients within 1 hour. During this time the $^{45}Ca^{++}$ content of the tissue did not increase significantly over that found without metabolic inhibition. A subsequent linear increase in $^{45}Ca^{++}$ paralleled an increase in the penetration of ^{14}C-sorbitol into taenia cells and was thus probably due to some nonspecific increase in membrane permeability caused by ATP depletion. Metabolic inhibition of taenia by substitution of N_2 and sorbitol for O_2 and glucose also reduced the Na^+ gradient within 1 hour without significantly raising intracellular Ca^{++} as measured with the lanthanum method. Finally, reduction of the Na^+ gradient with ouabain and zero K^+ did not significantly increase tissue Ca^{++} content.

In a similar study, Casteels et al. (42) also used the lanthanum method to quantitate changes in the intracellular Ca^{++} content of taenia coli during ATP depletion in a glucose and O_2-free medium. After 30 minutes in this solution, cell Ca^{++} increased from 90 to 145 μM/kg, while $[ATP]_i$ decreased from 2.4 to 0.6 μM/g. The Na^+ and K^+ gradients were not greatly altered during this period. Treatment of the muscle with a K^+-free solution or one containing 2×10^{-5} ouabain caused a slow increase in cell Ca^{++}, which was slowly reversed when the tissue was returned to the normal medium. It was found that the Ca^{++} permeability of the plasma membrane was augmented by K^+ depletion and it was suggested that this

phenomenon might explain the enhanced net uptake of Ca^{++} found during Na^+,K^+-ATPase inhibition. On the basis of these experiments, both van Breemen et al. (170) and Casteels et al. (42) concluded that maintenance of the transmembrane Ca^{++} gradient depended on ATP and not on the inward Na^+ gradient. These results are not conclusive, however, since extracellular Ca^{++} was not washed out except in the experiments with N_2 and sorbitol. In order to strengthen these conclusions, Casteels and van Breemen (43) eliminated the Na^+ electrochemical gradient by treating taenia coli for 4 hours with a zero K^+ medium containing $^{45}Ca^{++}$. At various times during the incubation, segments of tissue were removed and cellular $^{45}Ca^{++}$ was measured by the La^{+++} method. $^{45}Ca^{++}$ content throughout this incubation was identical to that found during an incubation in a normal K^+ medium containing the same specific activity of $^{45}Ca^{++}$. van Breemen et al. (167) have used a more accurate method of quantifying total intracellular Ca^{++}, based on washout of extracellular $^{45}Ca^{++}$ in ice-cold EGTA, to examine the effect of Na^+ gradient breakdown and metabolic inhibition on cellular exchangeable Ca^{++}. Cellular Na^+ and K^+ were measured simultaneously. Treatment of taenia with 10^{-4} M ouabain or zero K^+ for 2 hours greatly reduced the Na^+ and K^+ concentration gradients without affecting the Ca^{++} content of taenia coli. After the 2-hour ouabain treatment, the intracellular Na^+ concentration was approximately 130 mM/L of cell water. At this time, the tissues were placed in a medium containing 70 mM K^+ and 70 mM Na^+. The Na^+ gradient was thus reversed and membrane permeability to Ca^{++} may have been increased (117, 118). In spite of the reversed Na^+ gradient, no significant Ca^{++} gain was observed over the period of 1 hour. Subsequent addition of 1 mM iodoacetate and 0.1 mM dinitrophenol to this solution to deplete ATP caused a sustained linear increase in cellular Ca^{++}. Raeymaekers et al. (131) loaded taenia coli cells with Na^+ using a 3- to 4-hour incubation in zero K^+ solution. The tissues were then labelled with $^{45}Ca^{++}$ during a further 2-hour incubation in a radioactive zero K^+ solution. Other tissues were incubated for 3 to 4 hours in a normal K^+ solution and then labelled for 2 hours. After labelling, both sets of tissues were washed for 3 minutes in a zero K^+, zero Ca^{++} solution to wash out some extracellular Ca^{++}. It was found that high Na^+ tissues had not accumulated more $^{45}Ca^{++}$ during the 2-hour period than had the control tissues. Furthermore, the efflux of Ca^{++} from Na^+-enriched tissues was similar to that from normal Na^+ tissues, whether the efflux solution contained Na^+ or sucrose as a Na^+ substitute. Although these results suggest that the Na^+ gradient is not a factor in Ca^{++} extrusion, it is not certain that the method of washing off extracellular Ca^{++} did not leave a large portion of this pool bound to the cell, as well as resulting in the loss of some cell Ca^{++}

Burton and Godfraind (33) found that incubation of taenia coli in a labelled Krebs medium containing 10^{-4} M ouabain increased the uptake of both Na^+ and $^{45}Ca^{++}$, while reducing total tissue Ca^{++}. Similar effects were found in a medium with $[K^+]_o$ reduced to 2.7 mmol/L. A 5-minute incubation in ouabain increased total tissue Ca^{++} significantly. They suggested that initially, pump inhibition increased $[Na^+]_i$, enhancing Ca^{++} influx via an exchange carrier. Eventually, as $[Na^+]_i$ rose, Na^+ may have displaced Ca^{++} from intracellular buffering sites and this Ca^{++} may have been extruded, again via an exchange process. If, however, the Na^+ gradient was greatly reduced during pump inhibition, it would not be able to provide sufficient energy to extrude Ca^{++} via an exchange carrier, implying that some other mechanism might have been responsible for the net extrusion of Ca^{++} noted.

James and Roufogalis (86) measured the cellular content of Ca^{++}, Na^{++}, K^+, and Mg^{++} during incubation of guinea-pig ileum in 10 mM ouabain. They used a modification of the La^{+++} method, which they showed preserved the cellular contents of

these ions, while removing extracellularly bound cation. During the initial excitatory phase there was a significant increase in the Ca^{++} and Na^+ contents of this tissue. The Ca^{++} content then fell to the pre-ouabain level and stayed there, while Na^+ fell and then began to increase as K^+ decreased. The authors concluded that ouabain contraction is caused by an increased Ca^{++} influx and that ouabain does not inhibit the Ca^{++} extrusion mechanism.

SUMMARY. Treatment of intestinal smooth muscle with ouabain or zero K^+ has been shown to cause an initial increase in tension associated with enhanced electrical activity, a membrane depolarization of 10 to 15 mV, and increased net influx of Ca^{++}. This phase is followed by a sustained inhibition of electrical and mechanical activity. During the complete abolition of Na^+ and K^+ concentration gradients and a major drop in membrane potential in ouabain, the Ca^{++} content of intestinal smooth muscle is maintained at control levels.

A. INHIBITORY EFFECT OF OUABAIN OR ZERO POTASSIUM. While the excitatory or phase I stage of the response to zero $[K]_e$ or ouabain is generally considered to be dependent upon initial alterations in membrane potential, the mechanisms responsible for the inhibition which characterizes phase II have not been resolved. Casteels (34) demonstrated that when the rate of stimulation is maintained constant by supramaximal electric field stimulation the early (phase I) potentiation disappears. Under these conditions an early inhibitory effect is unmasked and ouabain suppresses maximal force development within 5 minutes after application. The profound inhibitory effects of Na,K pump blockade are further demonstrated by the ability of ouabain to relax a high K^+-induced contracture completely. The relaxation observed is slower in onset than the inhibition seen with Ca^{++} influx blockers such as D600 or La^{3+}. Complete relaxation is seen only after 15 to 20 minutes of exposure to the glycoside. Thereafter, if ouabain is removed from the high K^+ media bathing the taenia coli contracture is reestablished with a time course similar to that of the onset of inhibition (15). In the absence of external Na^+ ouabain does not relax a high K^+ contracture. If Na^+ is now reintroduced, relaxation proceeds at a rate which exceeds that observed when ouabain is applied with external Na^+ present initially (14, 15). Furthermore, the state of inhibition achieved is dependent upon the concentration of Na^+ added to the ouabain-treated preparation. Thus, Bose found that 10 mM Na^+ decreased tension by 12%, 40 mM Na^+ reduced it by 20%, and 140 mM Na^+ produced 100% inhibition when added to a ouabain-treated high K^+ contracture in the taenia coli. Although the high K^+, normal Na^+ solution was hypertonic, the increased osmolarity alone was shown to produce no inhibition (14). When ouabain was applied during a high K^+ contracture, left for 20 minutes, and then washed from the tissue with hypertonic high K^+ media containing normal (138 mM) Na^+, a delayed transient inhibition was seen. When the ouabain was washed out with a high K^+ solution containing hypertonic lithium (138 mM) a progressive inhibition resulted demonstrating complete relaxation without spontaneous recovery (15). The radioisotope ^{22}Na was used to monitor tissue Na^+ content during initiation and recovery of inhibition in response to transient exposure to ouabain. The temporal profile of inhibition and subsequent recovery exactly paralleled the gain and removal of excess tissue ^{22}Na (15). Finally, when the taenia was placed in sucrose medium containing normal K^+ (5 mM) and no sodium, subsequent addition of ouabain resulted in only phase I excitation and sustained contracture without inhibition.

Godfraind and Godfraind-deBecker, (66) reported that a number of cardiac glycosides including digitoxin, ouabain, and K-strophanthoside profoundly antagonized the contractions produced by histamine and acetylcholine in the guinea-pig ilium. This inhibitory action was characterized by a dependence upon the concentration of the glycoside, duration of exposure, and the

number of contractions elicited during exposure. Thus the mechanism involved seems more complex than simple competitive inhibition at receptor sites. The inhibition was more effective against acetylcholine than histamine (66). The effect of Na,K pump blockade on agonist-induced contractions in the taenia was further investigated by Griffin et al. (72). These authors reported that ouabain inhibited contractions induced by acetylcholine, histamine, serotonin, and bradykinin. Furthermore, omission of potassium from the bathing media produced an antagonism to histamine contractions which was similar to that caused by ouabain. The dose-response characteristics of ouabain inhibition were again incompatible with simple competitive inhibition. Specifically, the degree of inhibition was dependent upon duration of exposure to ouabain. Furthermore, the inhibitory effect of ouabain was abolished if Na^+ was omitted from the bathing solution. Readmission of Na^+ in the presence of continued exposure to ouabain produced inhibition. The temporal profile of both onset of inhibition and recovery after removal of ouabain for histamine contractions followed the same pattern as that found for electrical and high K^+-induced contractions (14, 39, 72). Omission of potassium produced an inhibition that was similar in rate of onset; however, recovery from zero K^+ upon readmission of K^+ was more rapid than that seen with recovery after ouabain exposure. This is compatible with a time-dependent dissociation of ouabain from the membrane (72).

Hurwitz and Joiner (83) elicited contractions in the guinea-pig ileum by Ca^{++} removal after exposure to high Ca^{++} solutions. Griffin et al. (72) reported that 10^{-6} M ouabain inhibited this type of contraction only if ouabain is included in the Ca^{++} loading solution prior to Ca^{++} removal. However, if ouabain was added only with the zero Ca^{++}, no inhibition was seen (72). This finding is perhaps simply a reflection of the time requirement for maximal inhibition following ouabain treatment. However, inhibition of this type of contraction further indicates the diversity of contractile stimuli that are inhibited by Na,K pump blockade.

Phase I (excitation) is associated with depolarization whether pump blockade is effected by ouabain or omission of external K^+ (4, 34). However, the electrical properties of the smooth muscle during phase II (inhibition) are markedly different depending upon the method used to block the Na,K pump. Casteels reported that ouabain produced a progressive depolarization, stabilizing at −25 to −30 mV. When ouabain was washed from the media, the membrane hyperpolarized to −60 to −65 mV within 3 to 5 minutes. This hyperpolarization was seen before any alteration in ionic composition could be measured and was taken as evidence for the electrogenicity of the Na,K pump (34). Potassium removal initially induces depolarization, concomitant with phase I contractions. Subsequently, the tissue placed in zero K^+ repolarizes or hyperpolarizes during the phase II inhibition. Thus the inhibition is not dependent upon alterations in membrane potential. However, it has been reported that after prolonged exposure to K^+-free media (in excess of 1 hour) both electrical and somewhat reduced mechanical activity may spontaneously reappear (4).

Taenia coli in zero K^+-induced phase II inhibition will undergo depolarization when exposed to high K^+ media. However, the mechanical response to high K^+ depolarization is greatly reduced or abolished (4). Thus, there is a dissociation between mechanical and electrical events following a period of Na,K pump blockade. If the external Ca^{++} concentration is raised to 5 mM, contractility is partially restored. Further increases in external Ca^{++} are not effective in restoring contractility above 50% of control (4).

The partial recovery of tension produced by raising $[Ca^{++}]_e$ possibly indicates that the high K^+-induced increase in calcium permeability is at least partially maintained in the presence of phase II inhibition. Thus

this may suggest that pump blockade or elevated $[Na^+]_i$ does not produce inhibition by blocking high K^+-activated Ca^{++} channels. However, partial blockade would not be excluded by this observation. Additional evidence suggesting that ouabain inhibition is not mediated by a blockade of Ca^{++} influx is provided by the observations of Karaki et al. (94). These authors reported that preincubation with 2.5×10^{-6} M ouabain for 30 minutes did not reduce the high K^+-induced (40 mM isotonic) increase in ^{45}Ca uptake or increase in total Ca^{++} content. However, ouabain pretreatment reduced the "tightly bound fraction" of tissue ^{45}Ca (TBF, described above) to control levels. Pfaffman et al. (128) and others (92) have shown that ouabain pretreatment selectively abolishes the tonic component of the high K^+ contractions, leaving the phasic component intact. As stated in the preceding section, evidence suggests that the phasic component involves Ca^{++} release, while the tonic component is more dependent upon Ca^{++} influx. This apparent contradiction may be related to a dissociation of Ca^{++} flux and tension development. This interpretation would suggest that phase II inhibition involves a redistribution of tissue Ca^{++} away from the contractile elements. The response to high K^+ during ouabain inhibition is similar to the response seen after preincubation in Na^+-free, Li^+-Tyrode, or glucose-free media. All three manipulations selectively abolish only the tonic component of the high K^+ contraction (128). However, it is not known at this time whether a common mechanism is involved.

Axelsson and Holmberg (4) reported that preincubation in zero K^+ did not reduce the level of adenosine triphosphate (ATP) or creatinine phosphate (CP) as measured by enzymatic assay of freeze-dried tissue. However, when paired tissue samples were stimulated for 1 minute in high K^+ solution after 3 hours in normal media or K^+-free Krebs, the zero K^+-pretreated tissue had statistically lower ATP and CP levels (1.48 ± 0.13 vs. 1.35 ± 0.17, $P < .05$ and 0.97 ± 0.29 vs. 0.76 ± 0.29, $P < .05$ μmol/g wet wt. respectively). It should be pointed out that such findings could reflect a primary metabolic influence of zero K^+ treatment or a reduction in metabolic stores secondary to the activation of an energy dependent process, such as active Ca^{++} extrusion. Briggs (26) reported that in glycerinated uterine smooth muscle elevated $[Na^+]_e$ resulted in reduced tension development. However, in these experiments, under conditions in which KCl was completely replaced by NaCl, tension was decreased only by 50%. Axelsson and Holmberg (4) found no difference in contractile response when glycerinated taenia strips were bathed in high K^+ (Na^+-free) or high Na^+ (K^+-free) media. Thus it appears that the total inhibition seen with Na,K pump blockade and concomitant elevation of cytoplasmic Na^+ is dependent upon the presence of intact membranes.

SUMMARY. Blockade of the Na,K ATPase pump of intestinal smooth muscle by either ouabain or zero K^+ produces a profound inhibition of tonic contractions produced by electrical stimulation, high potassium, acetylcholine, bradykinin, serotonin, histamine, and membrane destabilization achieved by alteration of $[Ca^{++}]_e$. Phase II inhibition is not simply related to alterations in the electrical properties of the tissue; ouabain produces a sustained depolarization and zero K^+ treatment results in a repolarization or hyperpolarization. Also, pretreatment with zero K^+ blocks the contraction but not the depolarization produced by high K^+. The inhibition produced by ouabain and zero K^+ is reversible and the temporal profile of both onset of inhibition and recovery most closely parallels concurrent alterations in intracellular Na^+ content. Furthermore, inhibition will not occur if Na^+ is omitted from the media and application of ouabain in Na^+-free sucrose results in a sustained contracture. A reduction in the magnitude of the Na^+ gradient per se does not seem to be involved, since lowering $[Na^+]_e$ does not cause inhibition. These findings suggest that the inhibition is produced secondary to an ele-

vation in cytoplasmic Na^+ concentration. Increasing the Na^+ content of glycerinated taenia coli does not produce comparable inhibition. Thus the inhibition requires the presence of intact membranes. Such inhibition could be effected by blockade of Ca^{++} influx or stimulated removal of activating Ca^{++} from the area of the myofilaments. Zero K^+-induced blockade of subsequent high K^+ contractions is partially reversed by elevating $[Ca^{++}]_e$, suggesting that the inhibition does not abolish the high K^+-induced increase in Ca^{++} permeability. Direct measurement of ^{45}Ca uptake during inhibition also suggests that pump blockade does not reduce high K^+-mediated Ca^{++} influx. This finding, coupled with the observation that ouabain selectively inhibits the tonic component, may suggest that the inhibition involves a redistribution of tissue Ca^{++} away from the contractile elements. In total, this evidence suggests that in intestinal smooth muscle elevation of cytoplasmic Na^+ concentration may produce inhibition by stimulation of the efflux or sequestration of activating calcium. However, partial inhibition of calcium influx cannot be excluded.

C. Uterine Smooth Muscle

1. Na^+ Substitution. The resting tension of uterine smooth muscle is relatively insensitive to alterations in external sodium between 50% and 100% normal (53, 114, 125). A 50% decrease in external Na^+ (70 mM) has been reported to have either no effect upon resting tension (125) or to produce a transient contraction that accompanies a concurrent increase in rate of spontaneous stimulation (53, 114). Such transient contractions subside within 15 minutes. Thereafter, both tension and rate of spontaneous activity return to control levels and such preparations remain stable for periods in excess of 4 hours (114). Further reductions in external Na^+ concentration to values between 70 and 10 mM produce a biphasic response, which is characterized by an initial tonic contracture followed by a period of recovery or relaxation in which the normal rhythmic contractions reappear (1, 114). The initial contracture is again associated with an increase in electrical activity characterized by a continuous discharge of action potentials and may have a duration in excess of 45 minutes (114). The tonic contraction relaxes as the continuous electrical discharge subsides. Thereafter, cyclic trains of action potentials are coupled to phasic contractions that are superimposed upon an increased base line tension. This pattern of rhythmic activity will continue unchanged for periods in excess of 2½ hours (114). Reduction of external Na^+ below 10 mM or complete removal of Na^+ usually produces a monophasic response, characterized by a sustained contracture and an initial burst of continuous spike discharge followed by complete inhibition of electrical activity (114, 125). Osa (125) found that when mouse uterine tissue was exposed to zero $[Na^+]_e$ in the presence of 1.5 mM $MnCl_2$, the rapid contraction and initial increase in electrical activity were not seen. Under these conditions tension slowly increased and peak tension was reached after 2 hours. The final tension achieved was slightly less than that seen in zero Na^+ controls. Similarly, sequential short applications of 1.2 mM $MnCl_2$ during a zero Na^+ (Tris) contraction produced progressively decreasing amounts of relaxation, suggesting that the zero Na^+ contraction may be composed of two components. The effects of severe reduction in external Na^+ are, in part, dependent upon the nature of the Na^+ substitute utilized. Thus Osa (125) has reported that contracture is seen when external Na^+ is substituted with sucrose, tris chloride, or choline chloride. In contrast, total replacement of Na^+ with Li^+ does not lead to sustained contracture, but rather the initial tonic contraction decays in the presence of persistent spike activity (125). Furthermore, after 30 minutes in the Li^+ medium the tension is reduced to levels below that seen prior to Na^+ removal. A specific inhibitory action of Li^+ is also suggested by findings of Freer and Smith (59), which show that under appropriate condi-

tions Li^+ may inhibit agonist-induced contractions. These authors report that in Ca^{++}-deficient media (0.2 mM) inhibition of both angiotensin II- and acetycholine-induced contractions were observed when as little as 10% of the Na^+ was replaced by Li^+. Complete inhibition of contractions was observed with 50% Li^+ substitution. The inhibitory action of Li^+ was reduced at increased Ca^{++} concentrations and no inhibition was seen at normal Ca^{++} levels (59). Csapo and Kuriyama (46) report that reduction of $[Na^+]_e$ to 24 mM by sucrose replacement resulted in a gradual decrease in tension in the presence of sustained spike activity. However, such a dissociation of spike activity and tension was not seen when choline chloride was used as a Na^+ substitute (46). In addition to effects of Na^+ substitutes, concurrent alterations in external chloride may also influence the response to experimental manipulation of external Na^+. Kao and Gluck (90) observed a potentiation of the phasic contraction when $[Cl^-]_e$ was replaced by nitrate, bromide, iodide, and thiocyanate.

The resting membrane potential of uterine muscle is very sensitive to changes in external K^+ concentration and demonstrates a linear relationship with log $[K^+]_e$ at concentrations of 10 mM and above. Furthermore, the Cl^- distribution across the membrane influences resting potential and substitutions with less permeant anions such as benzene sulfonate or sulfate cause depolarization, whereas substitution by nitrate or iodide ions results in hyperpolarization (1, 90). The membrane potential as measured by intracellular microelectrodes most closely parallels the K^+ equilibrium potential in myometrial tissue from animals in late pregnancy and at $[K^+]_e$ above 15 mM. However, at physiological $[K^+]_e$, there is a marked deviation in the observed value from the calculated K^+ equilibrium potential. This deviation is most apparent in nonpregnant uterine tissue. At $[K^+]_e = 5.9$ mM the KCl potential is −80 mV, while the measured potential is −60 mV during gestation and −42 mV in the nonpregnant uterus (40). Such observations imply that the permeabilities of ions other than KCl are significant and that alterations in permeabilities may occur with changes in hormonal influences (40, 113). A reduction in external Na^+ concentration to only 25% of normal has no effect upon the resting potential of uterine smooth muscle (53, 114). Further reductions to as low as 10% have been reported to exert either no effect (53, 114) or to produce a slight depolarization (1,125). Lowering $[Na^+]_e$ to 5 mM or complete replacement with tris chloride or sucrose results in a marked depolarization with resting potential stabilizing at −25 to −35 mV (125). This observation is contrary to predictions based upon the Goldman equation. Such findings would be consistent with the interpretation that severe reduction or removal of external Na^+ increases the permeability of an inwardly directed cation. Osa has reported that 1.2 mM $MnCl_2$ abolishes the zero Na^+-induced depolarization (123, 124).

Uterine smooth muscle will undergo a rapid phasic and sustained tonic contraction when depolarized by media containing elevated K^+ concentrations. Maximal isotonic and isometric contractures are obtained with solutions containing 20 to 30 mM K^+. Progressive elevation in K^+ concentration results in a concomitant depression of the tonic component. Furthermore, uterine smooth muscle exhibits after-contractions when the depolarizing stimulus is removed, and such after-contractions are more pronounced in both amplitude and duration as the K^+ concentration is increased. In this regard, uterine smooth muscle resembles other smooth muscle preparations; however, the duration of the after-contraction phenomenon may appear more exaggerated in this tissue (64). If uterine smooth muscle is made to contract in high K^+ media and subsequently placed in Na^+-free (i.e., sucrose) media containing normal K^+, the tonic contracture is well maintained. At this point reintroduction of external Na^+ causes a prompt relaxation (111).

The spontaneous action potentials that are involved in the generation of rhythmic contractions are insensitive to blockade by tetrodotoxin (1,125). Reductions in $[Na^+]_e$ to 50% of normal have no effect upon either the amplitude or rate of rise (53, 114, 125).

When uterine tissue is placed in media containing excess Ca^{++} the amplitude of spontaneous action potentials is increased. Conversely, if external Ca^{++} is reduced, the result is a corresponding decrease in spike amplitude (1, 40, 46, 115). While reduction of $[Na^+]_e$ to 70 mM (50%) has no effect upon the characteristics of action potentials, total omission of external Na^+ completely inhibits spontaneous activity, concurrently producing membrane depolarization (114, 125). There are contradictions in the literature regarding the effect on the action potential of reducing $[Na^+]_e$ to less than 50% of normal. Early work measuring electrical properties of myometrial strips utilizing intracellular microelectrodes and external surface electrodes suggested that replacing external Na^+ with sucrose or choline to values as low as ⅛ normal Na^+ did not alter the general configuration of action potentials and resulted in either no change or an increase in the amplitude of the biphasic, surface electrode recording (53). In contrast to these findings, other investigators have reported that reductions in external Na^+ to values of 34 mM, 24 mM, and 15 mM result in a progressive decrease in spike amplitude and rate of rise and fall as measured by both intracellular recordings and sucrose gap techniques (3, 73, 114, 125). Finally, it is reported that while reduction of $[Na^+]_e$ decreases the amplitude and rate of rise of spontaneous action potentials, this treatment concurrently increased both parameters of evoked spikes (1). Similarly, it has been reported that excess $[Na^+]_e$ will increase action potential amplitude and rate of rise (46, 90, 104). Thus it appears that both Na^+ and Ca^{++} currents are involved in the uterine action potential. Further support for this comes from the observation that electrical stimulation increases ^{22}Na permeability in this tissue (89, 91). Voltage clamp recordings utilizing the sucrose gap technique in rat uterine strips demonstrate a decrease in the peak transient current in Na^+-free solutions, further suggesting that the Na^+ conductance is increased during the action potential (2).

Direct measurement of net Ca^{++} flux in isolated rat uterine horns demonstrated that when external Na^+ concentration was varied between 10 and 200 mM (by choline chloride replacement) no effect upon tissue Ca^{++} content was seen. Furthermore, ^{45}Ca efflux rate from preloaded uterine tissue was not affected by changes in external Na^+ concentration (169).

2. Na,K ATPase Inhibition. The response of uterine smooth muscle to Na,K pump blockade can in general be characterized by an initial contracture (phase I) followed by relaxation and a period of inhibition of spontaneous activity (phase II). However, the response differs from that seen in intestinal smooth muscle in that the duration of contraction is greatly prolonged and the onset of inhibition is delayed by hours.

Daniel (49) studied the relationship between active ion transport and contractions in isolated rabbit, cat, and rat uterine smooth muscle and observed variations in the responses that were associated with differences in species and method of pump blockade. In both estrogen-dominated rabbit and pregnant cat uteri 10^{-6} to 10^{-5} M ouabain produced initial contractions that were either immediate in onset and of up to 1 hour duration or of delayed onset and persisted for hours. Rat uteri were less sensitive to the glycoside, but similar responses were produced at 10^{-3} M ouabain (49). Complete refractoriness to spontaneous and acetylcholine-induced contractions was observed after 3 to 4 hours of exposure to 10^{-5} M ouabain in the rabbit and cat preparations and after 1 to 1½ hours with isolated rat uterine horns. Exposure to zero K^+ media produced initial contraction followed by relaxation in cat and rat tissues. However, the rabbit uterus did not show enhanced automaticity or contractility in

response to zero K^+ in this study. Daniel et al. (52) reported that 10^{-5} to 5×10^{-4} M ouabain will potentiate a variety of agonist-induced contractions in the rat uterus. This potentiation was similar to that seen with 10^{-4} M $ZnCl_2$. Both $ZnCl_2$ and ouabain produced contractions at higher concentrations, which were antagonized by adrenaline. The authors suggested that in this case the action of ouabain may not involve blockade of Na,K ATPase but may act through adenyl cyclase. Taylor et al. (153) report that as in the taenia, 10^{-3} M ouabain produces a progressive depolarization in isolated rat myometrial strips and zero K^+ treatment results in no change in resting membrane potential. Total tissue Ca^{++} content as measured by ^{45}Ca exchange demonstrated no net increase in tissue Ca^{++} after 1, 3, and 6 hours of exposure to 10^{-3} M ouabain in isolated rat uteri (169).

SUMMARY. The resting tension of uterine smooth muscle is unaltered by reduction in $[Na^+]_e$ to 50% control (i.e., 70 mM). Reductions in $[Na^+]_e$ to 10 mM produce biphasic responses, characterized by an initial transient contracture followed by a relaxation and return of spontaneous rhythmic contractions superimposed upon an increase in base line tension. Complete removal of Na^+ or reduction below 10 mM produces a monophasic contracture. The addition of Mn^{++} at various times during the zero Na^+ contracture produces a relaxation of progressively reduced amplitude. The zero Na^+ contraction is not sustained if Li^+ is used as a Na^+ substitute. This observation, in addition to the report that Li^+ will inhibit angiotensin II and acetylcholine contractions in 0.2 mM $[Ca^{++}]_e$, suggests that Li^+ may be a specific inhibitor in uterine tissue. The slow development of Li^+ inhibition in zero Na^+ may suggest that Li^+ acts intracellularly.

The resting membrane potential of the uterus is insensitive to reductions in $[Na^+]_e$ to values of 10 mM. However, lowering $[Na^+]_e$ to 5 mM or complete replacement with sucrose or tris chloride results in a marked depolarization. This observation is contrary to predictions based upon the Goldman equation and could indicate an increase in Ca^{++} permeability. However, a decrease in K^+ permeability would also produce depolarization. Magnesium ions block the zero Na^+-induced depolarization, presumably by blocking Ca^{++} influx (123).

Uterine smooth muscle contracts when exposed to high K^+ media. Maximal contractions are produced by solutions containing 20 to 30 mM K^+. When uterine smooth muscle is transferred to sucrose, the tonic contraction is maintained. Repletion of external Na^+ will relax this type of contraction.

There is evidence suggesting that both Na^+ and Ca^{++} currents generate the action potential in uterine smooth muscle. The amplitude and rate of rise of the action potential are increased when $[Na^+]_e$ is raised. However, only severe reductions in $[Na^+]_e$ to values of 34 mM or below alter these parameters. Complete removal of $[Na^+]_e$ abolishes action potentials.

Total Ca^{++} content of uterine tissue (uncorrected for external binding) is not altered when $[Na^+]_e$ is varied from 10 to 200 mM. Also ^{45}Ca efflux and rate of accumulation are not affected by alterations in $[Na^+]_e$.

Uterine smooth muscle exhibits a prolonged contraction when exposed to ouabain or K^+-free media. In this respect, this tissue is similar to arterial smooth muscle. However, after prolonged exposure to ouabain or zero K^+ (1½ to 4 hours), uterine tissue is relaxed and spontaneous and acetylcholine-induced contractions are inhibited.

D. Smooth Muscle of the Ureter

1. Na^+ Substitution. Under physiological conditions, the smooth muscle of the ureter possesses no intrinsic resting tone. Contractions in this tissue are elicited in response to propagated action potentials. The spontaneous action potentials that have been recorded in isolated smooth muscle cells of the guinea-pig ureter are of long and somewhat variable duration (i.e., 500–1000

msec). They consist of an initial rapid depolarization with superimposed small spikes that decay into a prolonged plateau or "slow phase" (101, 108, 143). The initial "spike phase" is thought to be dependent upon an inward Ca^{++} current and is abolished by application of 2 mM $MnCl_2$ (107, 143). Mn^{++} treatment may enhance the amplitude of the plateau but abolishes the contractions that normally accompany the action potential. Verapamil (10^{-5} M) and omission of external calcium will also inhibit contractions. Verapamil reduces the amplitude of the spike component and shortens the duration of the plateau (143). Omission of external Ca^{++} completely abolishes the action potential (101, 108). When external Na^+ is removed by total replacement with sucrose or tris chloride, the plateau or slow component is selectively abolished. During the first few minutes of exposure to zero Na^+ the resting potential is slightly hyperpolarized and the action potentials appear as single spikes of reduced amplitude. Under these conditions, peak tension is decreased, possibly due to the reduced duration of the action potentials. Within 5 to 10 minutes after Na^+ removal the membrane gradually depolarizes and the action potentials may increase slightly in amplitude and duration. The contractions are augmented and while tension amplitude remains less than that seen with normal Na^+ media, the duration of contractions may be prolonged. However, at no time does the plateau phase of the action potential return (101, 108, 143). Although external Na^+ is required for the plateau, this phase of the action potential is not sensitive to tetrodotoxin (101, 107). Thus omission of external Na^+ results in a depression in the peak tension of spontaneous contractions, which is probably due to a concomitant shortening of the action potential duration. Removal of external Na^+ does not cause an increase in base line tension in isolated ureter (143).

Epinephrine, norepinephrine, and histamine potentiate both amplitude and duration of spontaneous contractions in the guinea-pig ureter. These agents cause a concomitant increase in the duration of the plateau phase of spontaneous action potentials (141) but have little or no effect upon the rapid component. These agonist-induced effects are abolished when external Na^+ is replaced with tris chloride or sucrose (142, 144). $MnCl_2$, 2 mM, also abolishes the effects of norepinephrine and epinephrine and inhibits the action of histamine on the duration of the slow phase of the action potential, suggesting that Ca^{++} may be involved in the response.

When isolated ureter is placed in media containing progressively elevated K^+ concentrations, both phasic and tonic contractions are elicited. The threshold concentration for a contractile response is in excess of 20 mM K^+ and in this respect the ureter is different from the taenia coli and uterus (177). At K^+ concentrations between 25 to 40 mM, repeating transient phasic contractions are produced with no elevation in base line tension. Further elevation in external K^+ produces a complex contraction pattern consisting of two sequential phasic components and a sustained tonic component of reduced magnitude. Both phasic and tonic components are dependent upon external Ca^{++} and are abolished in zero Ca^{++} media or 10^{-5} M verapamil (87, 149, 177). Washizu (177) reported that the maximal tonic response occurred between 80 and 100 mM KCl and that increasing $[K^+]_o$ to 160 mM led to a decline in tonic tension. In these experiments, isotonicity was maintained by removing NaCl and a reduction in $[Na^+]_o$ may be involved in the attenuated response. Sunano (149) and Sekiyama (139) have reported that external Na^+ was required for the maintenance of the tonic contraction. Washizu (177) also found that when tissue was preincubated in Na^+-free Li^+ Tyrode's, high K^+-induced phasic contractions were reduced in magnitude and tonic contractions are abolished. However, the effect of Li^+ substitution may again be in part due to a specific inhibitory effect of

this ion, since tonic contractions of reduced magnitude are well maintained in media in which Na^+ is completely replaced by K^+ ions.

When the external Ca^{++} concentration is increased from 2.0 to 4.0 mM during the tonic phase of the high K^+ contraction, one sees a rapid contraction followed by repeated fluctuations in tension. Conversely, when $[Ca^{++}]_o$ is returned to 2 mM a similar oscillation preceded by a rapid relaxation is observed. When the fluctuations subside, the tension is maintained at the same level seen prior to the perturbation. Such observations have prompted the suggestion that in the ureter the rate of Ca^{++} extrusion and/or sequestration is appropriately altered in response to changes in the cytoplasmic Ca^{++} concentration and that the complex pattern of high K^+ contractions are in part related to such an adaptation (149). Similarly, such a mechanism would be reflected in the lack of resting tension of this tissue, the apparent failure of omission of external Na^+ to influence resting tone, the elevated threshold for tonic high K^+ contractions, and the greatly reduced tonic component of the high K^+ contractions. Such a highly adaptive Ca^{++} extrusion system would be of physiological importance in this tissue. Electrically propagated phasic contractions are essential for the peristaltic action of the ureter. However, at least a few cells of the ureter smooth muscle near the luminal surface may at times be exposed to high concentrations of both K^+ and Ca^{++}. If, under these conditions, the ureter were to sustain a tonic contracture occlusion and impaired renal function would result. Thus the relatively low sensitivity to elevated $[K^+]_o$ may have a protective function in this tissue.

2. Na,K Pump Blockade. When the Na^+ gradient is reduced by blockade of the Na,K-ATPase by either 10^{-7} M ouabain or omission of external K^+, the slow component or "plateau phase" of the action potential is abolished. The amplitude of spontaneous contraction is also reduced, possibly due to the marked reduction in action potential duration. Ouabain and zero K^+ treatment do not seem to increase resting tension (143).

Pretreatment of isolated ureter with either ouabain or zero K^+ reduces both the potentiation of contractions and elongation of spontaneous action potentials in response to histamine (144).

SUMMARY. The action potential of the ureter consists of two components. The initial rapid spike component is sensitive to media Ca^{++} and agents that block Ca^{++} influx and is not prevented by removal of external Na^+. The second component of the action potential is a prolonged plateau phase that is selectively reduced or abolished by removal of external Na^+ or a reduction in the Na^+ gradient by Na,K^+ pump blockade. Alterations in external Na^+ or the Na^+ gradient across the sarcolemma exert an effect upon the Ca^{++} flux associated with spontaneous action potentials, possibly by changing the duration of this plateau. The actions of agents that affect the slow component are also dependent upon the Na^+ gradient. (The Na^+ current may be blocked by the removal of external Ca^{++} or addition of verapamil and the action of agents which lengthen the Na^+ plateau is blocked by $MnCl_2$.) Thus, in the ureter, reduction in $[Na^+]_o$ or the Na^+ gradient produces an inhibition of spontaneous contractions. The resting tension is unaffected by either omission of external Na^+ or Na,K pump blockade. The presence of external Na^+ may be required for maximal tension development during the tonic phase of high K^+-induced contraction. Thus the tonic component of contraction is completely abolished in tissues that have been preincubated in Na^+-free, Li^+-substituted media. However, it is possible that Li^+ may have a specific inhibitory effect in this case.

Apparently, the smooth muscle of the ureter possesses an unusually efficient mechanism for eliminating elevations in cytoplasmic Ca^{++} activity. Evidence based upon tension development suggest that the

efficiency of this process is not dependent on the Na^+ gradient.

IV. Analysis

Na^+-Ca^{++} interactions in smooth muscle involve a variety of cellular mechanisms that cannot be adequately described by the single hypothesis of a Na^+,Ca^{++} exchange carrier. Active Ca^{++} extrusion is seen to proceed in the absence of a Na^+ gradient and is probably mediated by a Ca-ATPase. However, the possibility remains that both Na^+,Ca^{++} exchange carrier and Ca-ATPase are involved in Ca^{++} extrusion with the caveat that they be arranged in series. A parallel arrangement in the plasmalemma would lead to a reduction in the $[Ca^{++}]_i$ below that predicted from equation II (if n = 3) by the Ca-ATPase, which in turn would cause reversal of the Na^+,Ca^{++}-exchange carrier such that it would contribute to Ca influx instead of extrusion. If, on the other hand, Ca is first translocated into the lumen of the SR by a Ca-ATPase pump and if special connections exist between the SR and surface membrane, then a Na^+,Ca^{++} exchange carrier could mediate Ca extrusion against the reduced gradient between SR and extracellular space. This hypothetical series pathway for Ca extrusion involving both the SR Ca-ATPase and a Na^+,Ca^{++} exchange mechanism would then be in parallel with the plasmalemmal Ca-ATPase that is interposed between the cytoplasm and extracellular space.

Other sites where Na^+ and Ca^{++} appear to interact are passive membrane transport channels and binding sites both inside and outside the smooth muscle cells, which are important in regulating the cytoplasmic Ca^{++} concentration.

Figure 2 provides a schematic representation of how a number of these Ca^{++} transport and sequestering systems may be interrelated with respect to control of cytoplasmic ionic calcium. This scheme is intended to emphasize multiple cellular loci for ion interaction and to compare theoretical models. In keeping with the state of the art, it is not intended to be comprehensive or final.

All Ca^{++} flux studies have shown the smooth muscle cell membrane to be permeable to Ca^{++}. Consequently the large electrochemical gradient ($\Delta\bar{\mu}$ Ca^{++} = 9000 cal/mol Ca^{++}) drives a passive Ca^{++} flux inward. There is general agreement that external Na^+ ions have an inhibitory action on Ca^{++} influx as have protons, La^{+++}, and a variety of other multivalent cations. Figure 2, ① depicts the role of Na^+ at the Ca^{++} leak as competition with Ca^{++} for a selective cation exchange pore across the cell membrane. Ca^{++} also enters the cell during activity via membrane potential-sensitive (Fig. 2, ⑦) and receptor-activated channels (Fig. 2, ⑤ and ⑥). Maintenance of homeostasis requires that a mechanism exists that pumps Ca^{++} out of the cell to balance Ca^{++} influx. Figure 1 depicts three possible mechanisms for active Ca^{++} extrusion: a Na,Ca exchange carrier ②, a Ca-ATPase ③, and a hypothetical pathway between the SR and surface membrane that might involve both mechanisms (⑧ and ⑩).

Many studies have directly or indirectly tested the hypothesis that Ca^{++} extrusion is accomplished by a Na,Ca exchange carrier as originally proposed by Reuter and Seitz (133). The main feature of the exchange carrier is that it couples Na^+ influx to Ca^{++} efflux, such that energy available from $\Delta\bar{\mu}$ determines $\Delta\bar{\mu}$ Ca^{++} and thus the contractile state. Figure 3 summarizes the tests of this theory. Using the relationship between $[Ca^{++}]$ and smooth muscle contraction first obtained by Filo et al. (57) in glycerinated smooth muscle in 1965 and which has remained virtually unchanged in more recent investigations (71), the $[Ca^{++}]_i$ of Figure 1 has been converted to percentage of maximum tension (*curves*, Fig. 3). The individual points are values obtained from the literature. Although these points are estimates, Figure 3 tends to minimize the discrepancies between experimental observations and theoretical predictions rather than to overestimate

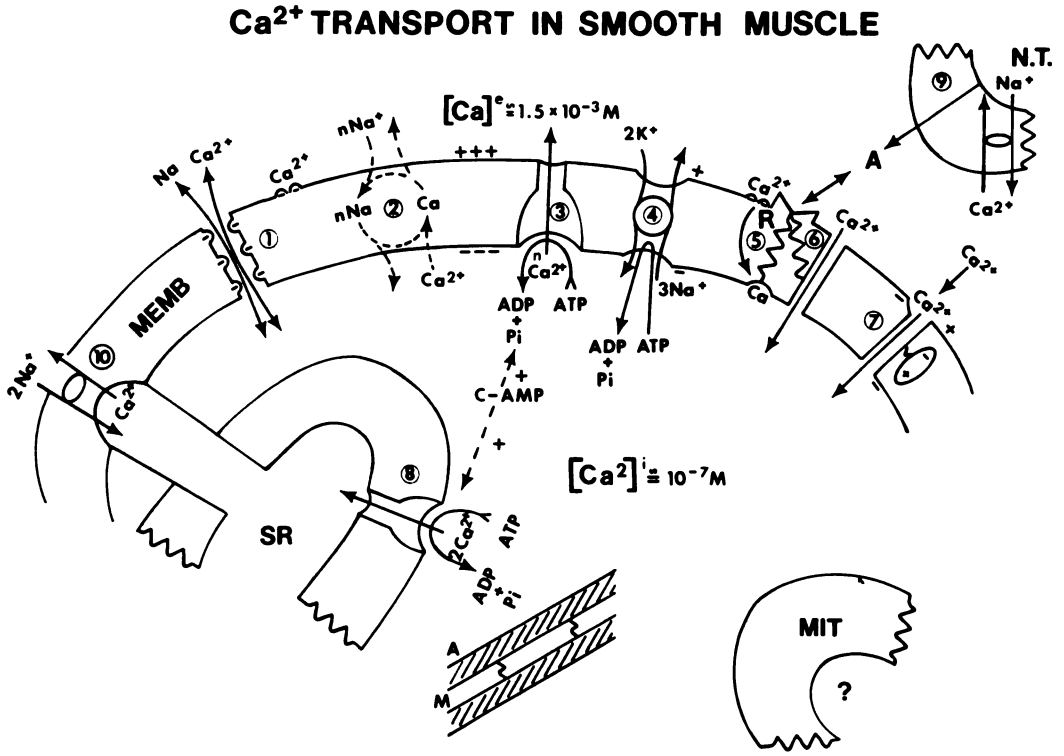

FIG. 2. Hypothetical Ca^{++} transport mechanisms involved in the regulation of cytoplasmic calcium that could be affected by sodium ions: ①, a pore lined with fixed negative sites which have affinity for both Na^+ and Ca^{++}; ②, the Na,Ca exchange carrier which couples the inward movement of n Na^+ to 1 Ca^{++} moving out of the cell (n refers to the number of Na^+ ions bound by a hypothetical Na,Ca exchange carrier). Since evidence in the literature does not support its presence in smooth muscle a *dashed line* is used; ③, an active Ca^{++} extrusion pump energized by ATP hydrolysis. No stoichiometric coefficients are as yet available for this pump; ④, Na,K-ATPase pump for active Na^+ extrusion and active K^+ accumulation; ⑤ and ⑥, a receptor complex for smooth muscle agonists. Activation of the receptor, R, leads to release of Ca^{++} bound on the inner surface of the membrane and opens a channel, ⑥, that allows Ca^{++} to enter from the extracellular space. The *squiggles* linking the receptor with the Ca^{++} binding sites and channel represent unknown energy transduction pathways. The channel may be the same as the one that refills the internal storage sites from Ca^{++} bound to the outer cell membrane surface, ⑤; ⑦, a voltage-dependent Ca^{++} channel; ⑧, the sarcoplasmic reticulum (SR) Ca-ATPase; ⑨, the neurohormonal release mechanism that involves Ca^{++} flux across the nerve terminal membrane; ⑩, a hypothetical special pathway for Ca^{++} between the SR and extracellular space. Its features are a) greater Ca^{++} permeability of the SR membrane facing the plasmalemma, b) restriction on lateral diffusion of Ca^{++} in the special cytoplasmic region between the SR and plasmalemma, and c) a Na,Ca exchange mechanism which facilitates Ca^{++} transport across the plasmalemma. See text for further discussion of these proposed mechanisms.

them. For example the *solid lines* are calculated with the assumption that Em = −50 mV. Most manipulations of the Na^+ gradient depolarize the membrane, such that when the exchange carrier is electrogenic at coupling values of n larger than 2, the *lines* of Figure 3 would underestimate the predicted force development (depolarization would shift the curves for n = 3 and n = 4 to the right). Experimental evidence thus rules out the possibility that a Na,Ca exchange carrier is the sole mechanism responsible for active Ca^{++} extrusion from smooth muscle cells. Estimation of the Na^+ gradient depends on the fraction of cellular Na^+ that is dissolved in the cytoplasm. However, even when as much as 10 mmol of Na^+/L cell water is assumed to be com-

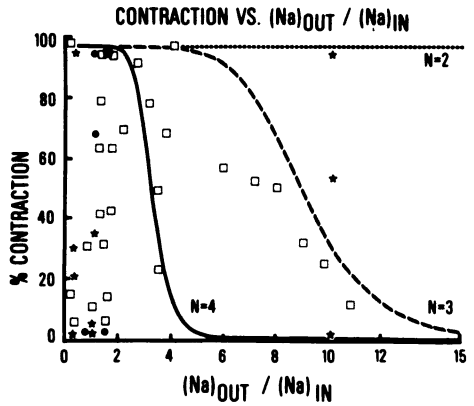

FIG. 3. The theoretical relationship between the transmembrane Na^+ gradient, expressed as the ratio $[Na^+]_o/[Na^+]_i$ (abscissae), and muscle tension, expressed as percentage of maximal contraction. This graph has been derived from Figure 1 using the relationship between $[Ca^+]_i$ and muscle tension obtained originally by Filo et al. (57). As in Figure 1, n refers to the number of Na^+ ions bound by a hypothetical Na, Ca exchange carrier. The points shown are drawn from the literature. In some studies, the value of $[Na^+]_i$ was not measured in a particular series of experiments. In these cases, values of $[Na^+]_i$ were taken from Casteels (35) or Friedman (61). Reported values of $[Na^+]_i$ in smooth muscle vary widely, even in identical tissues under similar conditions. Therefore, these points must be regarded as estimates. The points are classified according to general muscle type. The symbols are: ★, vascular smooth muscle; □, intestinal smooth muscle; and ●, uterine smooth muscle. It can be seen that there is no correspondence between the theoretical relationships expressed by the curves and the data obtained experimentally. The sources from which the points were taken are: *vascular*—Biamino and Johansson (8), Friedman (61), van Breemen (166); *intestinal*—Axelsson and Holmberg (4), Brading (19), Casteels (34), Friedman et al. (62), Judah and Willoughby (88), Riemer et al. (134), Taniyama (151), van Breemen et al. (167); and *uterine*—Bose (15), Daniel (49).

partmentalized such that it is not available for contribution to the cytoplasmic Na^+ activity, Friedman (61) calculates a physiological ratio Na_e/Na_i of 10 for the rat tail artery. Thus under physiological conditions the exchange of 3 Na^+ for 1 Ca^{++} at 100% efficiency would be incompatible with normal function.

In 1966 Schatzmann (137) discovered that Ca^{++} extrusion from the red blood cells is energized by ATP hydrolysis. The presence of a similar Ca-ATPase type pump (Fig. 3, ③) in smooth muscle is indicated by the prompt linear cell Ca^{++} gain observed upon complete metabolic inhibition (43, 166). Other lines of evidence further support ATP-driven Ca^{++} extrusion. In smooth muscle microsomal preparations enriched in elements of plasma membrane, positive correlation has been demonstrated between ATP-dependent Ca^{++} transport and surface membrane markers (51, 82). In the squid axon it is becoming evident that ATP plays a role in Na^+-dependent Ca^{++} extrusion (5a 55a). At present, it does not seem warranted to postulate a similar role for ATP in smooth muscle since active plasmalemmal Ca^{++} transport proceeds in the absence of both intra- and extracellular Na^+. Furthermore, net Ca^{++} extrusion from the uterus was shown to be stimulated under conditions of raised [cAMP] (102, 116). This fact indirectly supports involvement of Ca-ATPase in extrusion since cAMP stimulation of Ca-ATPase of cardiac SR has been well documented (99), while no evidence exists to support cAMP stimulation of Na, Ca exchange.

Suggestions of a Ca^{++} extrusion route from the SR to the extracellular space via a specialized region near the cell surface have appeared briefly in the literature at various times (18, 132). Morphological evidence for special junctional areas between SR and plasmalemma has also been presented (55). Since the Ca^{++} in the SR lumen reaches millimolar concentrations, the special transport route would have a much lower energy demand, which could be met by the Na, Ca exchange carrier. Figure 2 ⑩ schematizes this hypothesis and emphasizes the requirement for special permeability properties of the regions of junctional SR membrane. SR is capable of lowering the $[Ca^{++}]$ near its outer surface to below 10^{-7} M. Thus unmodified SR facing a leaky membrane with a Ca^{++} extrusion mechanism of low affinity would simply continue to gain Ca^{++} until saturation and thus lose its function. However, if the junctional SR membrane had greater Ca^{++} permeability (perhaps induced by the elevated $[Ca^{++}]$ of

the junctional cytoplasm) and lateral diffusion in this area were restricted, then a Na,Ca exchange carrier would be able to complete the extrusion process. This model has in principle a Ca-ATPase pump in series with a surface membrane pump, which could either be a Na,Ca exchange carrier or another Ca-ATPase. Proof for such a complex extrusion mechanism would be very arduous since it would require isolation of purified intact junctional regions. However, a specialized pathway of this kind is favored by the observation that very large net Ca^{++} fluxes can enter and leave smooth muscle cells without affecting contractile tension. For example, 175 μmol of Ca^{++}/kg wet wt. enter the guinea-pig taenia coli from a sucrose solution and all of this Ca^{++} is lost again upon replacing Na^+ or Li^+ while the muscle remained totally relaxed (167). Both inhibition of influx and stimulation of efflux appeared to be involved in the above net Ca^{++} loss. Li^+ was able to substitute for Na^+ in the induction of Ca^{++} loss from the cells which had been Ca^{++}-loaded during prior sucrose exposure. More Ca^{++} flux studies of this phenomenon could throw further light on the hypothetical Ca^{++} transport pathway linking the SR and plasma membrane. One complicating factor to be considered in the interpretation of Na^+ substitution experiments is the possibility of a Na^+, proton exchange mechanism in smooth muscle, such as has been demonstrated for the snail neuron (154). Omission of $[Na^+]_e$ could then conceivably lower intracellular pH, which might directly affect the myofilament Ca^{++} sensitivity. However, contraction was maintained in sucrose following high K^+-induced contractures (111).

A very interesting and well-documented principle emerging from this literature survey is that Na^+ is required for smooth muscle activation. Prolonged Na^+ depletion was observed to abolish activation by norepinephrine, histamine, angiotensin, bradykinin, acetycholine, and even high K^+ depolarization. In the schematic representation of Figure 2 this would mean that refilling of the agonist-sensitive Ca^{++} stores ⑤ and the patency of the receptor-activated and depolarization-activated pores ⑥ and ⑦ all require Na^+. Agonist-sensitive Ca^{++} stores appear to be refilled from the outer membrane surface (171) but their exact location is presently unknown (both superficial SR and plasmalemma have been suggested, but proof would await the isolation of the entire receptor-Ca^{++} release function).

In order to explain the Na^+ requirement for smooth muscle activation, it has been postulated that Na^+ ions exert a dual effect on Ca^{++} membrane channels (167). The assumption is that pores lined by fixed negative sites and specific for both Ca^{++} and Na^+ traverse the plasmalemma. The fixed negative charge inside the pores may be neutralized by n calcium ions or 2 n sodium ions, which behave as unbound counter ions. Thus if Na^+ ions are present they will, due to their higher concentration, exert a greater local osmotic pressure to counteract the lateral pressure of the lipid bilayer. This may be responsible for increasing pore diameter and thus permeability to both Na^+ and Ca^{++}. At much higher $[Na^+]_e$, competition between Na^+ and Ca^{++} for entry would reduce the Ca^{++} permeability. An additional consideration is that Ca^{++} may form ionic bonds with the fixed negative membrane group, which would lead to further closure of the pores. In support of the above scheme it has been shown for the guinea-pig taenia coli that Ca^{++} permeability does not vary monotonically with $[Na^+]_e$, but reaches a distinct maximum near 1 mM Na^+ (unpublished result from the authors' laboratory).

Besides the influence of Na^+ on plasmalemmal Ca^{++} transport, $[Na^+]_i$ appears to promote Ca^{++} sequestration by the SR. Studies on isolated smooth muscle SR are much needed to confirm this mechanism since there appears to be no precedent for such a role. The opposite phenomenon, i.e., Na^+-induced Ca^{++} release, has been reported for cardiac SR (127). If, however, Na,Ca exchange did mediate Ca^{++} efflux

from the SR (Fig. 2, ⑩), then Na^+ would be an appropriate charge neutralizer for the SR Ca-ATPase (Fig. 2 ⑧).

Future research on Na,Ca interactions will undoubtedly focus on specific Na^+ and Ca^{++} fluxes as closely related to the specific mechanism under investigation as possible. Two routes are open toward this goal: 1) specific blockade of the different mechanisms regulating cytoplasmic Ca^{++} concentrations; and 2) isolation of organelles and receptors. The relatively recent finding that most contractile effects of alterations in the Na^+ gradient could be blocked by phentolamine or denervation stress the importance of specific blockade. Specific inhibitors for Ca^{++} channels are being discovered (58) and it is hoped that a specific active Ca^{++} pump inhibitor can be found.

A great deficiency in the field of Na,Ca interactions has been the lack of good quantitative data on both Na^+ and Ca^{++} gradients. Here we may look forward to more refined use of the X-ray diffraction electron probe, ion selective microelectrodes, and more rigorous cellular Ca^{++} and Na^+ quenching techniques.

This review has undoubtedly suggested more questions than it has answered. However, it is the hope of the authors that it has diminished existing conflicts by showing the broad lines of experimental agreement and that it will contribute toward future research into the various mechanisms of Na,Ca interactions in smooth muscle.

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