Studies on Calcium and Sodium in Uterine Smooth Muscle Excitation under Current-Clamp and Voltage-Clamp Conditions

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ABSTRACT The objective of these studies was to define the roles of calcium and sodium in uterine smooth muscle excitation. The double sucrose-gap technique was used for current-clamp and voltage-clamp experiments. It was shown that neither sodium nor calcium alone is capable of supporting excitation in estrogen-dominated uterine smooth muscle. Calcium dependence was explained in part by increased membrane "leakage" current in calcium-free solution and calcium control of the voltage dependence of the early transient conductance. High concentrations of TTX did not affect the magnitude of the peak transient current while La++, Mn++, and Co++ greatly reduced or abolished it and decreased the steady-state current. From these and other data it was concluded that the regenerative mechanism in uterine smooth muscle has the functional characteristics of a single transient conductance channel whose activation requires the presence of both sodium and calcium. Insensitivity to TTX indicates that the molecular structure of the channel is unlike that in certain sodium-dependent systems, while the effects of La++, Mn++, Co++, and Ca++ reveal a similar dependence of conductances on extracellular polyvalent cations.

INTRODUCTION

Calcium dependence of action potential electrogenesis is a characteristic of most excitable cells. However, the qualitative and quantitative roles of calcium in this process vary with the type of tissue, and are generally not fully understood. The most generalized role of calcium appears to be its action as a membrane "stabilizer." From the point of view of excitable membranes this means control of resting membrane permeability and, as will be noted below, control of the voltage dependence of active ionic conductances (Koketsu, 1969; Frankenhuëser and Hodgkin, 1957; Hagiwara, 1966).

In addition to these regulating roles, there are numerous speculations about the role of calcium as a carrier of depolarizing membrane current in smooth
muscle (Bülbring and Tomita, 1970; Kumamoto and Horn, 1970; Job, 1969; Brading, Bülbring, and Tomita, 1969; Bülbring and Tomita, 1969; and Nonomura, Hotta, and Ohashi, 1966), cardiac muscle (Reuter, 1967; Rougier, et al., 1969; Beeler and Reuter, 1970; Mascher and Peper, 1969), and certain invertebrate neurons (Geduldig and Jung, 1968; Geduldig and Gruener, 1970). The most decisive evidence for calcium spike electrogenesis is in barnacle (Hagiwara, 1966; Hagiwara et al., 1969) and crayfish (Fatt and Ginsborg, 1958) muscle fibers.

The present voltage-clamp and current-clamp experiments were undertaken in a further effort to define the roles of sodium and calcium in the generation of uterine smooth muscle action potentials. The dependence of action potentials and the transient inward current on both external calcium and sodium has been observed. Furthermore, the ability of cobalt, lanthanum, and manganese to block this current has been established while tetrodotoxin (TTX) is without effect. It is concluded from these and other data presented below that spike electrogenesis in uterine smooth muscle is consistent with the concept of a single transient conductance channel which requires the presence of both sodium and calcium for activation. Furthermore, the nature of the calcium dependence is severalfold: (a) calcium controls "leakage" permeability, (b) there is evidence that calcium controls the voltage dependence of the transient current, and (c) depending on the validity of the experimental assumptions (see below), it may be argued that there is a depolarizing calcium current.

MATERIALS AND METHODS

Strips of myometrium from ovariectomized, estrogen-treated (100 μg estradiol benzoate/day for 5 days) rats were used in all experiments (Anderson, 1969). The sucrose-gap chamber, voltage-clamp circuit, and data acquisition and computer analysis systems were as described earlier (Anderson, 1969) unless stated otherwise. In the present experiments, hyperpolarizing and depolarizing holding currents were also used. These were provided by applying a variable fraction of + or − DC voltage from a Philbrick R-300 regulated power supply across a 100 megohm isolation resistor which was connected directly to the current pool electrode. Current-voltage plot data are presented uncorrected for leakage current (Anderson, 1969). All solutions were maintained at 32°C ± 1°C. Resting tension applied to the muscle strip in the chamber was just sufficient to remove the slack, thus facilitating control of liquid junctions defining the limits of the “node” and sucrose gaps.

Solutions

(a) Normal Krebs (mM): NaCl 113.46, KCl 4.74, KH₂PO₄ 1.18, MgSO₄ 1.18, CaCl₂ 2.54, NaHCO₃ 24.87. (b) Sodium-free solutions were prepared by substituting either dimethyl diethanol ammonium chloride (DDA, Lorente de Nó, 1949; Kleinhaus and Kao, 1969) or Tris for NaCl and NaHCO₃. (c) Low calcium and calcium-free solutions
were prepared by decreasing the amount or omitting CaCl₂ from the test solution. In some calcium-free experiments EDTA (0.2 mM) was also added. (d) Mn⁺⁺, Co⁺⁺, and La⁺⁺⁺ test solutions contained (mm): 138.33 NaCl, 4.74 KCl, 1.18 MgSO₄, and 2.54 CaCl₂. The concentrations of Mn⁺⁺, Co⁺⁺, and La⁺⁺⁺ used are indicated in the appropriate figures. (e) High calcium solution (mm): 138.33 NaCl, 4.74 KCl, 1.18 MgSO₄. The actual concentration of Ca⁺⁺ used is indicated in the appropriate figures. (f) Tetrodotoxin (TTX) was added to normal Krebs solution to give a final concentration of 1 or 2 × 10⁻⁶ M.

A preliminary report of these studies has been presented (Anderson et al., 1970).

RESULTS

Current-Clamp Experiments

1. EFFECT OF SODIUM-FREE SOLUTIONS

Test solutions in which Tris was substituted for sodium produced a marked hyperpolarization (≈10 mv) (Fig. 1 a), which was reversible in normal Krebs (Fig. 1 b). Since it can be readily demonstrated that a current which moves the resting membrane potential 10 mv more negative inside, abolishes the effectiveness of a given stimulating current (Fig. 1 c), additional experiments were carried out in which a holding current was applied such that there was no change in resting membrane potential during exposure to sodium-free Tris solutions (Fig. 1 d and e).

From these data it is apparent that failure of action potential activity in sodium-free Tris is not due to the hyperpolarizing response in this solution. Furthermore, in these experiments and in the calcium-free data presented below, neither increased stimulus strength nor change in holding current was effective in restoring action potential activity.

Because of the several objections to Tris as a sodium substitute (cf. Casteels, 1970), it seemed desirable to repeat these experiments with another substitute. In these experiments DDA was substituted for sodium. These results are in agreement with the Tris data with the exception of a less marked hyperpolarization (Fig. 1 f). Similar results are also obtained when choline is substituted for sodium (unpublished observations).

2. EFFECT OF CALCIUM-FREE SOLUTIONS

The dependence of estrogen-dominated uterine smooth muscle action potentials on external calcium is shown in Fig. 2 a and b. The time course of response to calcium-free wash was such that usually within 4–5 min the tissue was inexcitable to external depolarizing current pulses. There was very little change in resting membrane potential under these conditions with a maximum depolarization of approximately 2 mv.
Figure 1. Effects of sodium-free solutions and holding current on action potentials. (a) Tris-substituted sodium-free solution. Action potentials in this figure were taken at approximately 8 sec intervals after exposure to sodium-free solution. The change in resting membrane potential reflects the hyperpolarization associated with Tris-substituted solutions. (b) Recovery of action potential activity with return to previous resting membrane potential in normal Krebs solution. Action potentials cover a 2 min recovery period. (c) Effect of passing hyperpolarizing holding current on action potential activity. As the resting membrane potential is made more negative inside, action potential activity is abolished. (d) Tris-substituted sodium-free experiment in which the resting membrane potential was kept constant by applying an external holding current. Under these conditions, action potential activity again fails during exposure to sodium-free solution. (e) Recovery of normal Krebs. (f) Action potentials from DDA-substituted sodium-free solution. This composite picture was obtained by manually offsetting the voltage channel on the CRO for each action potential. The hyperpolarization associated with DDA-sodium-free solution was approximately 2 mv.

Voltage-Clamp Experiments

1. SODIUM-FREE EXPERIMENTS

As reported earlier (Anderson, 1969) the effect of Tris-substituted sodium-free Krebs solution on the transient current-voltage relation is to markedly
reduce the amplitude of the transient inward current and to shift the equilibrium potential along the voltage axis toward a more negative value. These experiments have been repeated with DDA as the sodium substitute. Fig. 3 again clearly shows the dependence of the transient current on external sodium.

2. CALCIUM EXPERIMENTS

As predicted from the calcium-free action potential data, the transient regenerative current was dependent on extracellular calcium (Fig. 4a–c). The decrease in net transient current in low calcium and calcium-free solutions was not reversed by changing the holding potential to either more negative or more positive values from the resting potential level. In Fig. 4c a small net transient inward current remained after 9 min in calcium-free EDTA solution. However, under current-clamp conditions depolarizing current pulses failed to elicit clearly regenerative responses beyond the electrotonic potential induced by the depolarizing current pulse. The inability of the small residual active

![Figure 2. Effect of calcium-free solution on action potentials. (a) Action potential responses to a suprathreshold constant current pulse during the 10 min exposure to calcium-free solution. (b) Action potential responses during the approximately 10 min recovery period.](image)
membrane current to initiate a regenerative action potential may be due to:
(a) simultaneous increase in membrane leakage permeability or (b) capacitance loading by inactive cells. The small increase in leakage current in calcium-free solution coupled with the marked decrease in net transient current,
Figure 4. Current-voltage relations of myometrium in low and calcium-free solutions.
(a) 1.0 mM Ca\(^{2+}\) (n = 10), (b) 0.1 mM Ca\(^{2+}\) (n = 5), (c) calcium-free plus EDTA (0.2 mM) (n = 10).
will decrease the ability of the active current to trigger regenerative responses. This interpretation is supported by computed action potential model experiments (H-H type membrane, Hodgkin and Huxley, 1952) in which it is readily demonstrated that a small increase in leak conductance can block the action potential (unpublished observation). However, this inhibition is readily overcome by increasing the stimulus strength, while in the uterus this is not an effective means of restoring action potential activity. Alternatively, it may be that the residual regenerative current in the unclamped uterus is ineffective in producing a spike because of the large distributed capacitive loading by cells which have lost excitability in calcium-free wash. It should also be pointed out that with continued exposure to Ca++-free solution all inward current is lost; however, under these conditions recovery in normal Krebs solution is often very poor.

The decrease in net transient current has at least three possible explanations: (a) increased leakage current, (b) control of the voltage dependence of the transient conductance, and (c) decrease in the availability of calcium as a carrier of depolarizing membrane current.

(a) Calcium and Membrane Leakage Current Leakage current has been shown to be a linear function of voltage in the hyperpolarizing direction and for small depolarizing steps (Anderson, 1969). On this basis it was assumed that leakage current was linear for all values of membrane potential.¹

It is clear that under these conditions an increase in membrane leakage current will decrease net inward current. Steady-state hyperpolarizing currents plotted in Fig. 4 show an increase in leakage current in calcium-free solution. Fig. 5 shows data from another series of experiments designed to determine the time course of this calcium-free effect. It is apparent from this figure that the increase in leakage current lags behind a marked decrease in net transient inward current. Furthermore, leakage correction after 5 and 10 min in calcium-free solution restored the peak transient current only 2.5 and 5.0%, respectively, of the normal Krebs control value. Thus, the calcium dependence of early inward current is only in small part explained by an increase in leakage current.

(b) Effects of Calcium on the Voltage Dependence of Membrane Conductances It has been well demonstrated in nerve (Frankenhaeuser and Hodgkin, 1957) that a fivefold increase in calcium outside shifts the peak transient conductance-voltage relation 10–15 mv to the right along the voltage axis. The voltage dependence of potassium conductance is similarly affected.² In barnacle

¹ This is the rationale frequently used for leakage current correction in axon voltage-clamp data (Frankenhaeuser and Huxley, 1964; Chandler and Meves, 1970; Narahashi et al., 1964; Moore et al., 1964). However, there are reports that leakage current is nonlinear over a certain range of membrane potentials in the depolarizing direction (Adelman and Taylor, 1961; Goldman and Binstock, 1969).

² Recent voltage-clamp experiments (Hille, 1968) in the frog node of Ranvier, however, failed to show a calcium-dependent shift of steady-state currents.
Figure 5. Effects of calcium-free solution (with 0.2 mM EDTA) on peak transient current \( I_p \), leakage current (leak) (i.e., steady-state hyperpolarizing current), and spike height (A.P.). Changes in \( I_p \) and leak were monitored with alternating 20 mV depolarizing or hyperpolarizing voltage-clamp pulses. Membrane action potentials were taken at intervals between voltage-clamp pulses in the current-clamp recording mode. The stimulating current was at least twice threshold. On the ordinate, 0% corresponds to the values of \( I_p \), leak, and A.P., in normal Krebs and 100% is the value after 10 min in calcium-free solution. (a) Ca\(^{++}\)-free effect. The two \([\square]\) symbols at 5 and 10 min refer to leakage-corrected \( I_p \) data. (b) Recovery in normal Krebs solution.
muscle (Hagiwara and Naka, 1964) a 6-7 mv shift in the transient current was observed with a twofold increase in external calcium. The effect of a threefold increase in external calcium is illustrated in Fig. 6. In this experiment there was a 6-7 mv shift to the right along the voltage axis. However, this response was quite variable.

Lowering [Ca++]o from 2.54 to 1.0 mM did not significantly change either the magnitude or the voltage dependence of transient current (Fig. 4 a). However, 0.1 mM [Ca++]o (Fig. 4 b) and Ca++-free solutions (Fig. 4 c) caused a marked reduction in peak transient current. Measurement of transient current shifts in the voltage axis under these conditions is limited by the simultaneous reduction in the absolute magnitude of peak inward current.

3. TTX EXPERIMENTS

It is well established that TTX specifically blocks the early transient conductance increase in lobster (Narahashi et al., 1964) and squid (cf. Kao, 1966) axons. Other experiments show that the basis of this specificity is the molecular structure of the transient conductance channel rather than the ions carrying current (Moore and Narahashi, 1967; Moore et al., 1967; Hagiwara et al., 1969).

In the present studies 1 X 10^{-6} M TTX, a concentration several hundred times greater than that producing complete blockage of excitation in squid axons, had no significant effect on the magnitude of the peak transient inward current (Fig. 7).
FIGURE 7. Myometrial current-voltage relations in $1 \times 10^{-5}$ M TTX ($n = 9$). The magnitude of the peak transient current did not change significantly during the 12 min exposure to TTX.

FIGURE 8. Effects of La+++ (2.4 mM) on the transient and steady-state current-voltage relations ($n = 4$). La+++ completely abolished the transient inward current and decreased the steady-state current.

4. La+++ EXPERIMENTS

La+++ (2.4 mM) reversibly abolished all regenerative action potential activity during a 4–5 min test period. This effect was associated with complete inhibition of net transient inward current and decreased steady-state current under voltage-clamp conditions (Fig. 8). This ability of La+++ to block excitation has been previously demonstrated in both sodium (Takata et al., 1966) and calcium (Hagiwara and Takahashi, 1967) regenerative systems. These
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data and the present experiments are in agreement with the prediction of Lettvin et al. (1964) that the high membrane-binding affinity for La+++ should limit or block the normal voltage-dependent conductance increases.

5. Mn++ EXPERIMENTS

Since the demonstration that Mn++ blocks the calcium spike in crayfish (Fatt and Ginsborg, 1958) and barnacle muscle (Hagiwara, 1966; Hagiwara and Nakajima, 1966), manganese inhibition (partial or complete) of spike electrogensis has been taken as evidence for the presence of a calcium component of depolarizing membrane current. Thus in taenia coli (Bülbring and Tomita, 1969; Nonomura et al., 1966) and cardiac muscle (Rougier et al., 1969; Ochi, 1970) Mn++ studies have suggested the presence of a calcium current. However, recent experiments by Tarr et al. (1969) have also shown that Mn++ (10 mM) blocks the TTX-sensitive sodium current as well as the slow inward TTX-insensitive current in cardiac muscle. In the present studies 1 mM Mn++ also blocked the action potential, usually within 4–5 min. Under voltage-clamp conditions this was associated with inhibition of the transient inward current and marked reduction of the steady-state current (Fig. 9). These effects were reversible in normal Krebs solution.

6. Co++ EXPERIMENTS

Cobalt has also been shown to block the transient current and decrease the steady-state current in uterine smooth muscle (Fig. 10). These data are similar to the effects of cobalt recently described by Hagiwara et al. (1969) in barnacle muscle fibers. Interpretation of these results, as with La+++ and Mn++ data, depends on one's ability to distinguish between the “stabilizing” action of polyvalent cations on the one hand and their apparent ability to selectively and competitively inhibit calcium currents on the other. Only in the light of other data can this qualitative distinction be made.

DISCUSSION

The implicit assumption in the interpretation of Na+ and Ca++ substitution experiments is that the specific ionic conductances are independent of one another. However, much of the present data is not compatible with a strict application of this principle. That is, the transient inward current requires the presence of both sodium and calcium.

There is, however, a widely accepted rationale for interpretation of calcium-dependent excitation systems in which calcium is not a significant carrier of depolarizing membrane current (Frankenhaeuser and Hodgkin, 1957). These studies have considered the action of calcium on the electrogenic

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* See Segal (1969) for a discussion on this point.
process in terms of controlling the voltage dependence of other ionic conductances-specifically sodium and potassium. Narahashi's (1966) studies on the calcium dependence of cockroach giant axon excitation are particularly relevant in this context. In this paper he tests the possibilities that calcium acts either indirectly through controlling or stabilizing actions on membrane
conductances, or that it is significantly involved as a carrier of depolarizing membrane current. His results indicate that the calcium dependence of this system is accounted for by the indirect mechanism described by Frankenhaeuser and Hodgkin (1957) and that calcium does not carry a significant amount of depolarizing membrane current. Furthermore, Hagiwara (1966) has shown that this controlling or stabilizing action of calcium holds for calcium electrogenic systems as well as transient sodium conductances. In the present experiments, action potential activity was not restored in either Na⁺- or Ca²⁺-free solution by externally applied depolarizing or hyperpolarizing holding currents or increased stimulus strength. Furthermore, voltage-clamp data are in support of these observations in that the transient inward current was greatly reduced or abolished in both sodium and calcium-free solutions. The observation that changing the holding potential (either more negative or positive inside from the resting potential), failed to restore the transient current, further supports the notion of calcium-sodium dependence.

These results are explained in part by the effect of calcium-free wash on leakage currents. It has been suggested previously (Anderson, 1969) that leakage current may limit the ability of the active transient conductance to depolarize the membrane. Kao et al. (1970) have also recently suggested that calcium may influence excitability in uterine smooth muscle by controlling membrane leakage permeability. However, in the present experiments the peak transient current decreased much more than could be accounted for by increased leak; in fact, as shown in Fig. 5 there was a marked decrease in peak transient current before increased leakage was observed.

In high calcium solution the negative slope region of the transient current was shifted to the right or to more positive values of membrane potential. This effect is qualitatively similar to the stabilizing response described earlier in nerve (Frankenhaeuser and Hodgkin, 1957) and in barnacle muscle (Hagiwara, 1966). This similarity of response to elevated levels of calcium supports the notion that calcium interacts with excitable membranes at a level of organization which does not distinguish between highly differentiated conductance channels.

Voltage-clamp experiments under conditions of varying external sodium and calcium concentrations have led to the following conclusions: (a) neither sodium nor calcium alone is capable of sustaining excitability in uterine smooth muscle; (b) calcium controls the voltage dependence of active membrane currents; and (c) in the absence of highly sensitive, ion-specific measurements or ion-specific blocking agents it is not possible to decisively establish the nature and relative roles of sodium and calcium in uterine smooth muscle excitation.

*See Van Breeman and McNaughton (1970) and Krejci and Daniel (1970) for a discussion of calcium flux measurement in smooth muscle.*
The following discussion of TTX and polyvalent cation experiments reviews the rationale for their use and the implications of these results in understanding the ionic events of excitation in uterine smooth muscle.

In the present experiments TTX did not block the transient inward current. This insensitivity to TTX is similar to that described in certain other sodium-dependent excitation systems (Kao and Furhman, 1967; Kao, 1966; Keatinge, 1968) and in barnacle muscle (Hagiwara et al., 1969). It is well known that TTX blocks the normal transient sodium conductance in squid and lobster axons. However, a variety of evidence suggests that the basis of transient current sensitivity to TTX is not the species of ion-carrying depolarizing membrane current but rather the molecular organization of the membrane (Hagiwara et al., 1969; Moore et al., 1967).

The apparent ability of various polyvalent cations to mimic or antagonize actions of calcium on excitable membranes has provided the rationale for their use in a number of tissues and experimental designs. Reference data for the present discussion are from tissues in which the ions carrying membrane current are known. La$^{+++}$, Co$^{++}$, or Mn$^{++}$ inhibition of spike electrogenesis in barnacle (Hagiwara and Takahashi, 1967) and crayfish (Fatt and Ginsborg, 1958) muscle fibers has been taken as evidence for "competitive inhibition" of the transient calcium conductance. This effect is tightly coupled with a stabilizing action in which the polyvalent cations have a specific order of potency (La$^{+++}$ > Co$^{++}$ > Mn$^{++}$ > Ni$^{++}$ > Ca$^{++}$), presumably a reflection of their membrane binding constants (Hagiwara and Takahashi, 1967; Blaustein and Goldman, 1968). Recent voltage-clamp studies on barnacle muscle (Hagiwara et al., 1969) have clearly shown these actions of Co$^{++}$; i.e., inhibition of the transient inward calcium current and shift in the voltage dependence of the conductances to more positive values of membrane potential.

In sodium-dependent TTX-sensitive systems La$^{+++}$ (Takata et al., 1966; Blaustein and Goldman, 1968), Co$^{++}$ and Ni$^{++}$ (Blaustein and Goldman, 1968) effects are also viewed in terms of competition between ions for membrane-binding sites. La$^{+++}$ has been shown to block action potential activity in the lobster axon (Takata et al., 1966) and, furthermore, this effect is associated with marked reduction of the transient inward sodium current and steady-state current. Thus the effect of La$^{+++}$ on action potential activity is the same in either sodium or calcium spike systems. Furthermore, Co$^{++}$ effects are qualitatively the same in both tissues; i.e., the voltage dependence of the conductances shifted to the right and the peak transient inward current was reduced (Hagiwara et al., 1969; Blaustein and Goldman, 1968).

In the present experiments La$^{+++}$, Co$^{++}$, and Mn$^{++}$ greatly reduced or completely blocked the transient inward current and decreased the steady-state current. With the view that TTX selectively blocks Na$^{+}$ conductances...
and polyvalent cations selectively block calcium conductances, these data, together with the high and low calcium experiments are compatible with the calcium spike hypothesis.

However, results not explained by this model include: (a) sodium dependence of the regenerative mechanism; (b) non-ion-specific effects of polyvalent cations on membrane ionic permeabilities; (c) non-ion-specific basis of TTX sensitivity.

Similar efforts to identify the ionic components of action potentials in cardiac muscle (Rougier et al, 1969) and Aplysia neurons (Geduldig and Gruener, 1970) have led to the conclusion that there are independent parallel sodium and calcium (or sodium/calcium) transient conductance channels. In both tissues a sodium-dependent, TTX-sensitive current was identified which had voltage-dependent characteristics different from those of the slower calcium (or sodium/calcium) channel which in turn was inhibited by Mn++ (Rougier et al., 1969) or Co++ (Geduldig and Greuner, 1970) ions.

In summary, the regenerative mechanism in uterine smooth muscle has the functional characteristics of a single transient conductance channel whose activation requires the presence of both sodium and calcium. Insensitivity to TTX indicates that its molecular structure is unlike that in certain sodium systems while the effects of Mn++, Co++, La++, and Ca++ reveal a similar dependence of conductances on extracellular polyvalent cations.

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