On the Mechanisms Whereby Temperature Affects Excitation-Contraction Coupling in Smooth Muscle

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abstract Moderate cooling of smooth muscle can modulate force production and may contribute to pathophysiological conditions, but the mechanisms underlying its effects are poorly understood. Interestingly, cooling increases force in rat ureter, but decreases it in guinea pigs. Therefore, this study used ureteric smooth muscle as a model system to elucidate the mechanisms of the effects of cooling on excitation-contraction coupling. Simultaneous recordings of force, intracellular $[Ca^{2+}]$, and electrical activity were made in intact ureter and ionic currents measured in isolated cells. The increase in force amplitude in rat ureter with cooling was found to be due to a significant increase in the duration of the Ca^{2+} transient. This in turn was due to a marked prolongation of the action potential. In guinea pigs, both these parameters were much less affected by cooling. Examination of membrane currents revealed that differences in ion channel contribution to the action potential underlie these differences. In particular, cooling potentiated Ca^{2+}-activated Cl^- currents, which are present in rat but not guinea pig ureteric smooth muscle, and prolonged the plateau of the action potential and Ca^{2+} entry. The force-Ca^{2+} relationship revealed that the increased duration of the Ca^{2+} transient was sufficient in the rat, but not in the guinea pig, to overcome kinetic lags produced in both species by cooling and potentiate force. Ca^{2+} entry and release processes were largely temperature-insensitive, but the rate of relaxation was very temperature-sensitive. Effects of cooling on myosin light chain phosphatase, confirmed in experiments using calyculin A, appear to be the predominant mechanisms affecting relaxation. Thus, smooth muscle is diverse in its response to temperature, even when experimental variables, such as the mode of stimulation, are removed. Although the biochemical and mechanical events accompanying contraction are likely to be affected in similar ways by temperature, differences in electrical events lead to subsequent differences in these processes between smooth muscles.

key words: force • electrical activity • ureter • E-C coupling • Ca-activated Cl channels

INTRODUCTION

Moderate cooling (from 37 to 20°C) long has been known to have a strong modulatory influence on the mechanical activity of different types of smooth muscles (for review see Nasu, 1990). However, there is surprisingly little definitive information concerning the fundamental mechanisms underlying the effect of cooling, which vary substantially between different smooth muscles. Investigation of the effects of cooling on smooth muscle is not only of experimental interest; the reduction of blood flow in cutaneous blood vessels in cold environments or inhalation of cold air may affect the tone of blood vessels and trachea and contribute to pathophysiological conditions (e.g., asthma; Deal et al., 1979; Nasu, 1990).

The mechanism of the modulatory effect of cooling on smooth muscle remains controversial. In chemically skinned smooth muscle, it was reported that cooling caused inhibition of the rate of rise and rate of relaxation of force by decreasing the activity of both myosin light chain kinase (MLCK)* and myosin light chain phosphatase (MLCP), but with more potent effect on the latter (Mitsui et al., 1994). Therefore, this was associated with an increased level of MLC phosphorylation and larger levels of force developed by the tissue at low temperatures at the same level of $[Ca^{2+}]_i$ (Mitsui et al., 1994). Thus, these data indicate that cooling can increase the sensitivity of the contractile machinery to Ca^{2+} and increase force development.

However, in intact smooth muscles, cooling was reported to have both potentiating actions (e.g., mesenteric artery [Vanhoutte and Lorenz, 1970], saphenous vein [Webb-peploe and Shepherd, 1968], and airway [Bratton et al., 1987]) and inhibitory actions (e.g., femoral artery [Kawarai and Chiba, 1989]; aorta [Godfraind and Kaba, 1972], and trachea [Stephens et al., 1977]). These different contractile responses were not just dependent upon which smooth muscle was studied, but also on the method of stimulation and the experimental conditions used (Sabeur, 1996; Vanhoutte et al., 1997). In addition, it appears that there is also a

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*Abbreviations used in this paper: EC, excitation-contraction; MCLK, myosin light chain kinase; MCLP, myosin light chain phosphatase; STOC, spontaneous transient outward currents.
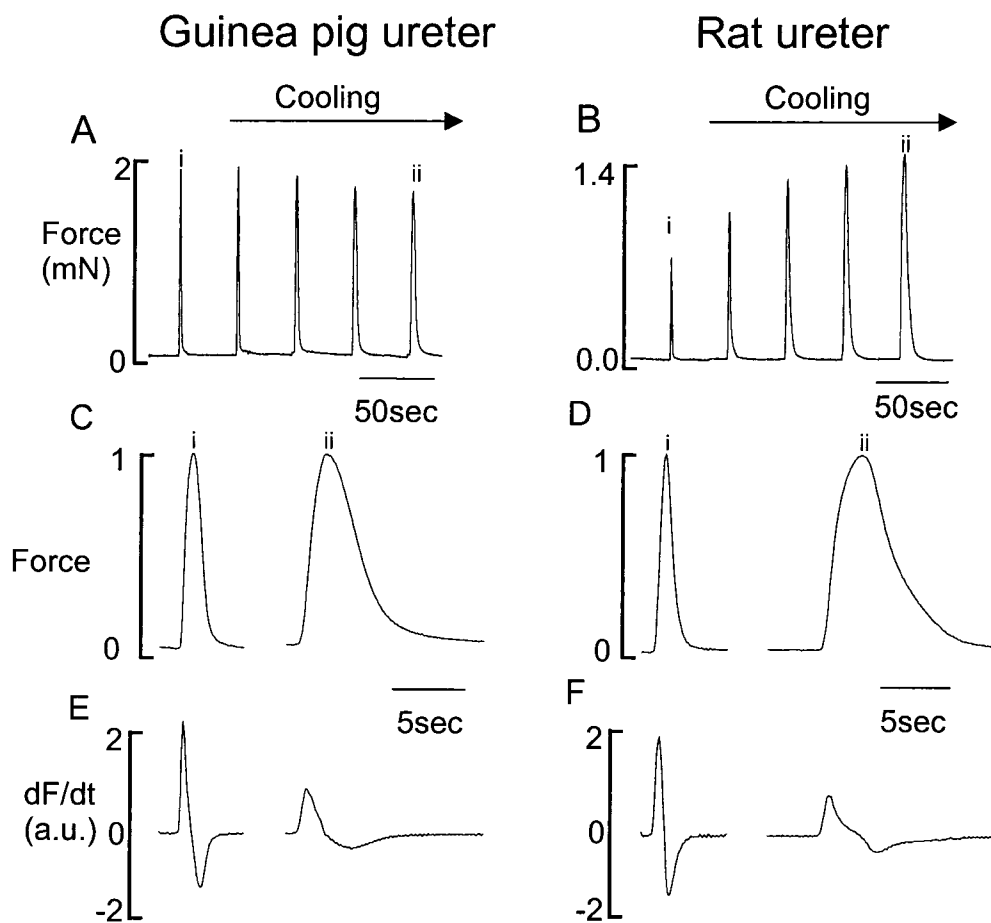
FIGURE 1. Effect of cooling (from 35 to 21°C) on mechanical activity of the guinea pig and rat ureter. (A and B) Effect of cooling on the amplitude of the evoked phasic contraction of the guinea pig and rat ureter, respectively. (C and D) Normalized phasic contractions recorded at 35 and 21°C. (E and F) Effects of cooling on the rate of force development and relaxation. The preparations were excited by electrical field stimulation with the following parameters: amplitude 4–5 V, duration 40–100 ms, and rate 0.025 Hz.

species difference: in guinea pig ureter, cooling decreased the phasic contraction produced by electrical stimulation, whereas in rat ureter, force was increased (Burdyga and Magura, 1986; Burdyga and Kosterin, 1991). The mechanism of this species-dependent effect of cooling on force in ureteric smooth muscle, however, has not been investigated.

As temperature may be expected to affect many of the steps of excitation-contraction (EC) coupling, it is important to measure not only mechanical activity, but also electrical activity and $[Ca^{2+}]_i$ simultaneously with force, if the mechanism is to be understood. Thus, the aim of the present study was to systematically establish which steps in EC coupling were modulated by cooling, and to elucidate how these effects differ in the rat and guinea pig ureter, so that the end effect on force can be explained. We have done this by simultaneously measuring changes in electrical activity $[Ca^{2+}]$ and force in intact ureter and ionic currents in isolated cells.

MATERIALS AND METHODS

Tissue Strips

Guinea pigs (~300 g) or rats (~200 g) were anesthetized with CO_2 and killed by cervical dislocation. The ureters were dis-

sected, cleared of any fat, and cut into strips ~3–4 mm in length. For measurement of Ca^{2+}, the ureters were incubated in the membrane-permeant form of Indo-1 (15 μM; Molecular Probes) for 2–3 h at room temperature. Tissues were rinsed and placed in a 200-μl bath on the stage of an inverted Nikon microscope. One end of the tissue was fixed, and the other was attached to a force transducer. The tissue was stimulated every 20 s by silver electrodes at 3–5 V (duration 50–100 ms). Action potentials were evoked by just suprathreshold depolarizing current pulses of short duration (20–50 ms). For simultaneous force, Ca^{2+}, and electrical measurements, a modified tissue bath was used, as detailed elsewhere (Burdyga and Wray, 1997, 1999a,b). Briefly, the bath became a sucrose-gap chamber with a coverslip at its base to enable the optical measurements to be made. For Ca^{2+} measurement, the tissues were excited at 340 nm, and the Indo-1 fluorescence emitted at 400 and 500 nm was recorded. The ratio of these signals (F_{400}/F_{500}) provides a measure of $[Ca^{2+}]_i$ (Grynkiewicz et al., 1985; Burdyga et al., 1995). Digital imaging also was used to monitor the quality of loading of the muscle strips with fluorescent indicator, i.e., evenness and penetration.

Cell Dispersal

Whole ureters were cut in small pieces and incubated for 60 min in low calcium (40 μM) Hanks' solution. In the case of the guinea pig ureter, 0.2 mg/ml collagenase type II, (Worthington), 0.2 mg/ml protease E, and 1 mg/ml BSA were added at 35°C and the tissue was kept in enzyme containing solution in a shaking water bath for 45 min. For rat cells, the concentration of collagenase type II used was 0.8 mg/ml; for protease E, it was 0.4 mg/
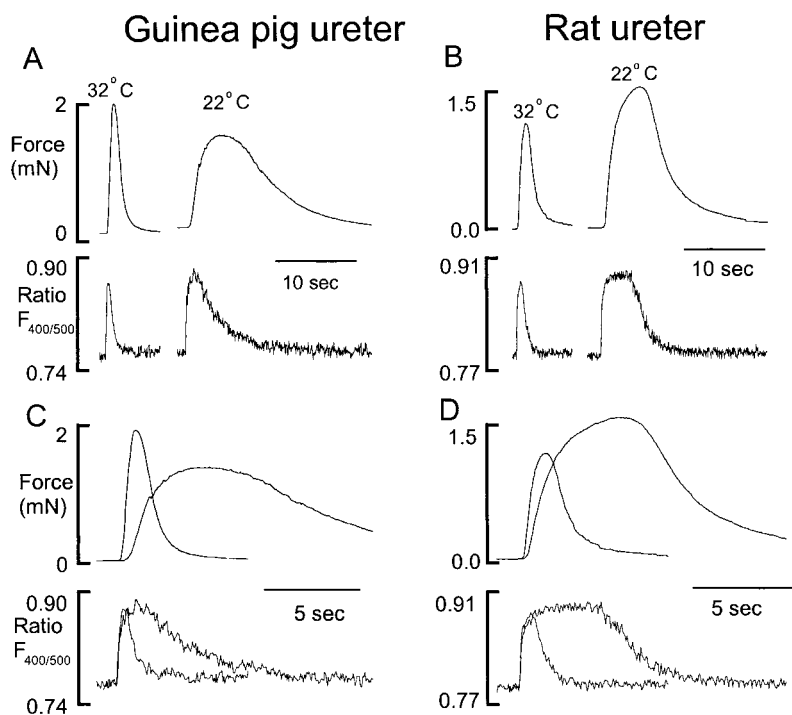
Guinea pig ureter   **Rat ureter**

FIGURE 2. The effects of cooling on force kinetics. (A and B) Effect of 10°C cooling on the parameters of the phasic contractions (top traces) and $[Ca^{2+}]_i$ transients (bottom traces) of guinea pig and rat ureter. (C and D) The above records superimposed and displayed on a faster time scale. The protocol of stimulation was the same as in Fig. 1. Note the difference in the effect of cooling on the rising phase of $[Ca^{2+}]_i$ transient and rate of rise of force.

ml; and for BSA, the concentration was 5 mg/ml. The strips were placed in this enzyme solution and kept in the refrigerator for 6–8 h, and then placed in a water bath at 37°C for 10 min. After enzyme treatment, tissue from both species was placed into enzyme-free solution and triturated using fire-polished Pasteur pipette to release cells. Cells were stored in low-Ca Hanks' solution in the refrigerator and were used on the same day.

Electrophysiology

The whole-cell patch-clamp recording method was applied to the ureteric cells as described previously (Burdyga and Wray, 1999a). The recording system consisted of a patch-clamp amplifier (Axopatch 200B; Axon Instruments, Inc.) and a Digidata 1320 board. Data acquisition was performed using pCLAMP software (Axon Instruments, Inc.) and analyzed using Origin 6.0. Whole-cell currents were recorded online at a sampling rate of 1 kHz and filtered through low-pass filters of 1–2 kHz. Leakage currents positive to −60 mV were subtracted.

Solutions

Tissues and cells were superfused with oxygenated buffered Krebs solution, pH 7.4, of the following composition (in mM): 136 NaCl, 5.9 KCl, 1.2 MgSO$_4$, 2 CaCl$_2$, 11.5 glucose, and 11 HEPES. In some experiments detailed in the text, K$^+$ currents were inhibited with 10 mM TEA, and the myosin light chain phosphatase was inhibited by 1 μM calyculin A. The experimental temperature was changed by altering the temperature of the superfusing solution, except for when a rapid change was required ("temperature jump" experiments; see Fig. 3). For these experiments, a bolus of solution was rapidly injected via a port hole in the side of the bath. The temperature in the bath was monitored throughout via a thermister tip in the bath.

The basic pipette solution contained the following (in mM): 130 CsCl or KCl, 10 HEPES, 5 MgCl$_2$, 10 glucose, 5 ATP, and 0.1 EGTA, pH was adjusted to 7.2 with NaOH. All chemicals were purchased from Sigma-Aldrich, unless stated otherwise. Values are given as means ± SEM, and n is the number of animals. Differences were taken as significant if $P < 0.05$ in the appropriate t test.

R E S U L T S

Effect of Moderate Cooling on the Mechanical Activity

Fig. 1 shows effect of moderate cooling (from 35 to 21°C) on the evoked mechanical activity of guinea pig ($n = 7$) and rat ($n = 6$) ureter. Fig. 1 shows that, in the guinea pig ureter (A), cooling produced a decrease in force, and in the rat ureter (B), it increased. With 14°C cooling (35° to 21°C), the amplitude of force in the guinea pig ureter decreased to 79.5 ± 7.2% (control amplitude 100%), whereas in the rat ureter, the amplitude of force increased to 205 ± 11.5%. The rate of rise and the rate of relaxation of the phasic contractions normalized to their amplitudes (Fig. 1, C and D) in the ureter of both species were, however, both significantly decreased during hypothermia (Fig. 1, E and F).

The temperature coefficient (Q$_{10}$) calculated for the rate of the force development was 2.47 ± 0.12 ($n = 11$) for guinea pig and 2.53 ± 0.12 ($n = 7$) in the rat ureter, whereas the Q$_{10}$ value calculated for the rate of relaxation was 5.1 ± 0.3 for the guinea pig and 3.7 ± 0.2 for the rat ureter. These data clearly show that although cooling had similar effects on the kinetic characteristics of the contractions in both species, it produced opposite effects on the amplitude of force. The higher Q$_{10}$ for relaxation than force development would have been expected to produce an increase in both species if this was the only factor involved. The fact that the Q$_{10}$ values for the rising phase of the contractions were

practically identical suggests that the mechanism controlling the rate of force development may well be the same in the ureter of both species.

Force development in the ureter is dependent upon a rise in intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$). Given that the amplitudes of the phasic contractions were differentially affected by cooling, we next determined the effects of cooling on the relationship between the kinetics and amplitude of the Ca^{2+} transient and contraction in the two species.

Effect of Cooling on the Kinetics of the Ca^{2+} Transient

As previously reported, simultaneous Ca^{2+} and force measurements showed a good correspondence in the ureter (Burdyga and Wray, 1999a,b), with clear Ca^{2+} transients preceding and underlying contractile activity. Despite its clear and opposite effects on force, in the ureter of both species, cooling was found to have little effect on the rate of rise or amplitude of the Ca^{2+} transient (Fig. 2, $n = 5$ [rat] and $n = 7$ [guinea pig]). The Q_{10} for the rate of rise of the Ca^{2+} transient, which was evaluated by taking the first derivative of the normalized transient, was in the range of 1.2–1.4 in the ureter of both species. This indicates that moderate cooling had little effect on the mechanism controlling the rate of rise of $[Ca^{2+}]_i$ (Ca^{2+} entry and/or Ca^{2+} release). This is in contrast to the strong influence of cooling on the rate of force development described above. Cooling also significantly increased the time of delay (t_d) between the rise in $[Ca^{2+}]_i$ and the onset of the force development, which was evaluated by fitting a straight line to the steepest part of the Ca and force traces and measuring the time between the baseline intercepts, from 120 ± 26 ms at 35°C to 300 ± 42 ms at 25°C in the rat ureters ($n = 5$) and from 115 ± 12 ms to 325 ± 33 ms ($n = 7$) in the guinea pig. This can be seen in Fig. 2 (C and D), where the Ca^{2+} transients at the two temperatures have been superimposed, and a clear delay in the rise of force at the lower temperature can be seen in both species.

From Fig. 2, it also can be seen that in rat ureter, cooling produced a significantly greater effect on the duration of the Ca^{2+} transient at its peak level compared with guinea pig ureter (five to eight times versus two to three times, respectively). These data suggest that the effect of cooling on the duration of the Ca^{2+} transient, but not its kinetics, could explain the difference in the mechanical response displayed by the ureter of the two species. Therefore, we further investigated each of these parameters (i.e., kinetics and durations) in more detail.

The above data indicated that force development at lower temperatures is not related to effects of cooling on the kinetics of the Ca^{2+} rise. Thus, if the effects of temperature on force are not accounted for by alteration of the kinetics of the Ca^{2+} transient rise, then one
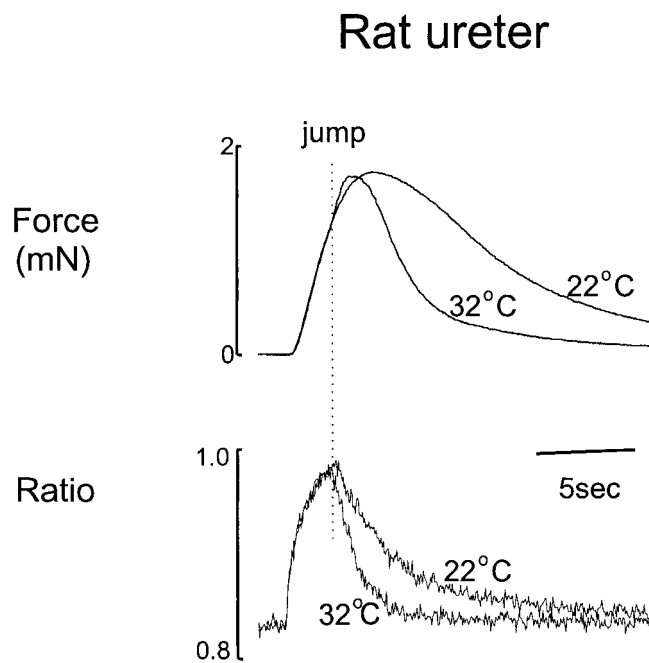

FIGURE 3. The effects of rapid temperature change. A jump from 22 to 32°C on the parameters of the phasic contraction of the rat ureter. The jump was applied during the rising phase of the force development, at a time when $[Ca^{2+}]_i$ had almost reached its maximal value.

would predict that if temperature was altered after the rise in Ca^{2+}, force should still be affected in the same manner as when temperature was altered before the start of the contraction. Thus, in the next set of experiments, we tested this prediction by affecting near-instantaneous rises of temperature (temperature jumps), at or near to when $[Ca^{2+}]$ had reached its peak level, and examining the effects on Ca^{2+} and force.

The data obtained are illustrated in Fig. 3 and are presented in the form of superimposed original records for rat ureter ($n = 7$). There was a fast abbreviation of the Ca transient, which produced a quick relaxation of the contraction. Thus, these data show that in rat ureter, there is a highly temperature-sensitive step that controls the duration of the Ca^{2+} transient, and hence, modulates force amplitude. In guinea pig, a significant increase in the amplitude and the rate of force development, with a prolongation of the duration of the Ca^{2+} transient but no change in its amplitude, had been found previously (Burdyga and Wray, 1999a,b). In the guinea pig, temperature-sensitive steps controlling the rate of force development and Ca^{2+} transient duration appear to modulate the amplitude of the phasic contraction. Previous work (Burdyga and Wray, 1999a,b) has shown that the force-Ca^{2+} relationship recorded during the development of the phasic contraction in the guinea pig ureter was not in steady state, and that under these conditions, the duration of the
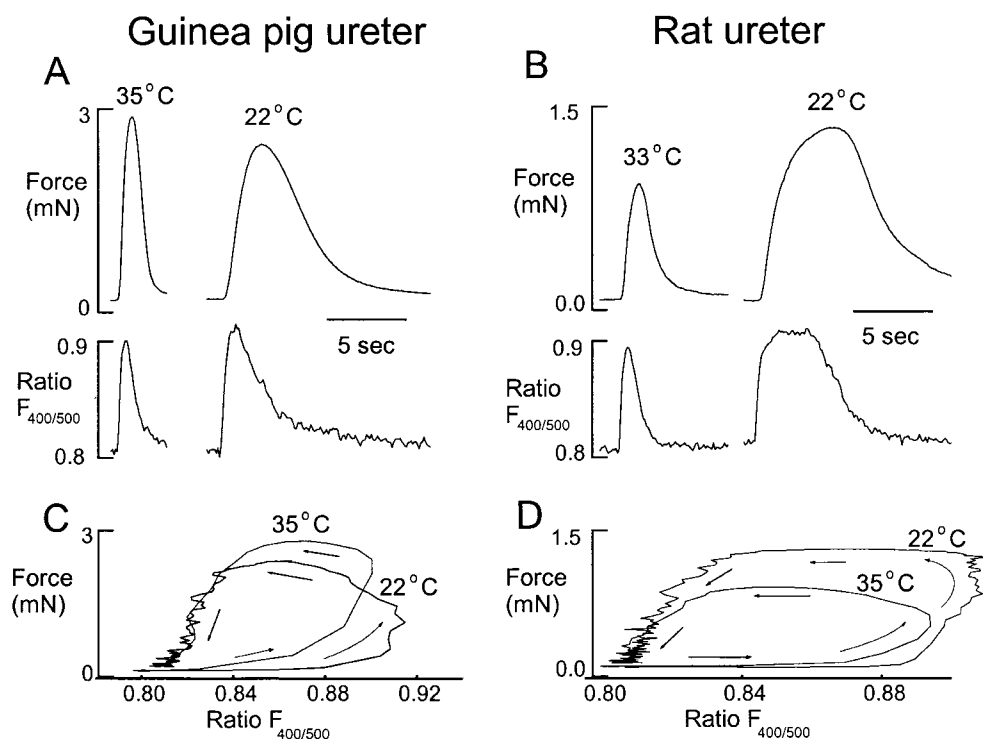
FIGURE 4. Temperature and the force-Ca^{2+} relationship. (A and B) Simultaneous force and calcium records at 35 and 22°C in guinea pig and rat ureter. The data are presented in the form of phase-plane diagram in C and D. The arrows show the time-dependent development of the contraction, which forms a counter clockwise loop. Note the large hysteresis between the force-Ca^{2+} relationship recorded during the rising and relaxation phase of the contraction.

Ca^{2+} transient plays a key role in modulation of the amplitude of the phasic contraction. So, in the next series of experiments, the effect of cooling on the force-Ca^{2+} relationship during phasic contraction in the ureter of both species was investigated.

Effect of Cooling on the Force-Ca Relationship

The force-Ca^{2+} relationship during the phasic contractions in the ureter forms a counter clockwise loop (hysteresis; Fig. 4, $n = 7$ [rat] and $n = 9$ [guinea pig]); i.e., the force-Ca^{2+} relationship recorded during the rising and the relaxation phases of the contraction do not follow the same trajectory. In the ureter of both species, the force begins to rise with a significant lag behind Ca^{2+}. Although caution is needed in interpreting relationships under non–steady-state conditions, we found good agreement between our global Ca signals and those recorded using fast imaging (not shown). At near-physiological temperature in the ureter of both species, force peaked at a time when $[Ca^{2+}]_i$ had fallen to ~50% of its peak level (Fig. 4). Cooling enhanced this hysteresis by increasing the delay and slowing the rate of the force development. From Fig. 4, it also can be seen that in the rat ureter at room temperature, most of the force was developed at a time when $[Ca^{2+}]_i$ was around its peak level. In contrast, in the guinea pig ureter at room temperature, less force was produced at the peak Ca^{2+} level despite the fact that the duration the Ca^{2+} transient was approximately two to three times longer. If the Ca^{2+} transient duration is critical to the development of force in the ureter, then it suggests that in the guinea pig, the reduction in force produced by cooling might be able to be countered by prolongation of the Ca^{2+} transient duration. TEA blocks the major outward currents in the ureter and can significantly prolong the action potential and, hence, the Ca^{2+} transient; and when TEA is added to cooled preparations, we have previously shown that there is a complete restoration of force (Burdyga and Wray, 1999b). Taken together with the data in Fig. 4, it suggests that in the ureter of both species, the slowing of the kinetics of the force development can be compensated for by an increase in the duration of the Ca^{2+} transient, but in the guinea pig ureter, this was not sufficient to compensate for the kinetic lag of force development.

The marked difference in the duration of the Ca^{2+} transient between the rat and the guinea pig ureter is likely to be due to different effects of temperature on the parameters of the action potential and/or the mechanisms controlling the restoration of the Ca^{2+} transient. Thus, to elucidate which mechanism underlies this effect, simultaneous recordings of the electrical activity, Ca^{2+}, and force were made.

Effect of Cooling on the Action Potential, Ca^{2+} Transient, and Force

The action potential of the ureter is species-dependent. It is better characterized in the guinea pig, where it can be described as an initial depolarizing spike followed by further spikes and some repolarization, before a

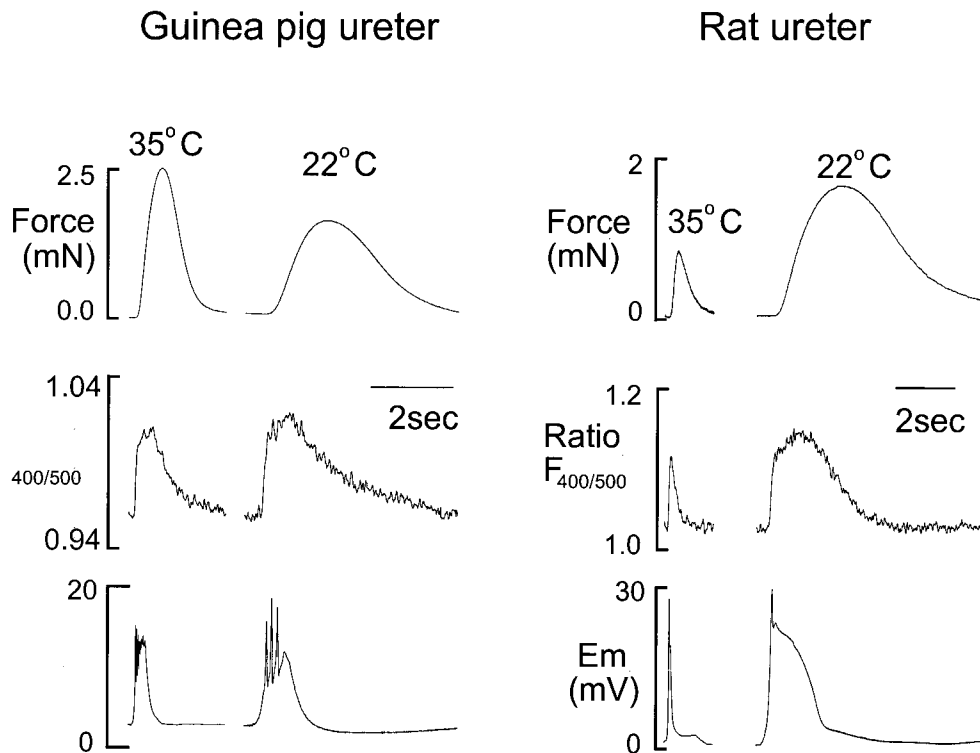# Guinea pig ureter   Rat ureter

FIGURE 5. Simultaneous recording of force (top trace), $[Ca^{2+}]_i$ (middle trace), and action potential (bottom trace) taken at 35 and 22°C. The ureters were stimulated by suprathreshold, short duration (40–50 ms) current pulses. The stimulation artifacts have been removed for clarity. Note the marked difference in the effect of cooling on the duration of the plateau component of the action potential between guinea pig and rat.

long plateau phase, and a final repolarization (Fig. 5, $n = 7$ [rat] and $n = 8$ [guinea pig]). At physiological temperatures, the action potential in the ureter of the guinea pig is particularly long—being between 400–800 ms in duration (Shuba, 1977a,b; Burdyga and Wray, 1999a,b). The rat action potential is less well studied, but it is comprised of an initial single spike and brief plateau before repolarization (Burdyga and Wray, 1997), and the overall duration of the action potential (100–200 ms) is considerably briefer than that of the guinea pig (Fig. 5). The action potentials of both species were markedly altered by cooling, but in different ways. These changes were accompanied by large effects on $[Ca^{2+}]_i$ and force that also differed between the two species, as described above.

Fig. 5 shows that in the rat ureter, there were drastic differences in the duration of the action potentials, Ca^{2+} transients, and the amplitude and duration of the phasic contraction recorded at 35 and 22°C, whereas in the guinea pig ureter, these differences were less pronounced. In rat ureter, cooling produced a significant potentiation of the plateau component to the action potential. A 10°C cooling produced a 4.7 ± 0.9 ($n = 7$) increase in the duration of the action potential measured at its 50% level compared with 2.0 ± 0.3 ($n = 8$) times in the guinea pig. The duration of the individual spikes measured at 50% amplitude in the guinea pig action potential were increased from 21.1 ± 7.2 ms at 35°C to 35.5 ± 6.4 ms at 22°C, but their frequency was decreased from 11.5 Hz at 35°C to 6.1 ± 6.1 Hz at 22°C,

also as noted by others (Mitsui et al., 1992). The rate of rise and fall of the spikes decreased 1.81 ± 0.21 times and 1.75 ± 0.31 times, respectively, when the temperature was reduced to 22°C. There was also a good correlation between the duration of the plateau component of the action potential and duration of the Ca^{2+} transient at its peak level in agreement with previous data (Burdyga and Wray, 1999a,b).

Effect of Cooling on Ionic Currents

To understand the different mechanisms of modulation of the action potential in ureteric smooth muscle, the effect of temperature on the ionic currents was investigated using whole-cell patch clamping in single cells.

Guinea Pig Ureter Cells. Ionic currents have been well characterized in the guinea pig ureter (Imaizumi et al., 1989; Lang, 1989; Sui and Kao, 1997a,b; Burdyga and Wray, 1999a,b). Noninactivating L-type Ca^{2+} channels mediate the inward current (I_{Ca}) to generate both the spike and plateau component of the action potential, and Ca^{2+}-activated K (K_{Ca}) currents, producing spontaneous transient outward currents (STOCs) are the major currents controlling repolarization. Both inward and outward currents were decreased at room temperature (Fig. 6). 10°C cooling decreased I_{Ca} 1.4 ± 0.3, ($n = 4$) times, whereas the peak I_K decreased 1.4 –1.7 times ($n = 5$). At room temperature, the frequency of outward current oscillations (STOCs) measured during depolarizing steps to +10 mV were also reduced (1.75 ± 0.6 times, $n = 5$; Fig. 6, A and C). The effects of cooling

Guinea pig ureter

A

35°C

100msec 200pA

22°C

B

100pA

100msec

22°C

35°C

C

0.6
nA
35° C
0.4
0.2
22° C
mV
-60 -40 -20 0 20 40 60

D

nA 0.1

0.05

mV
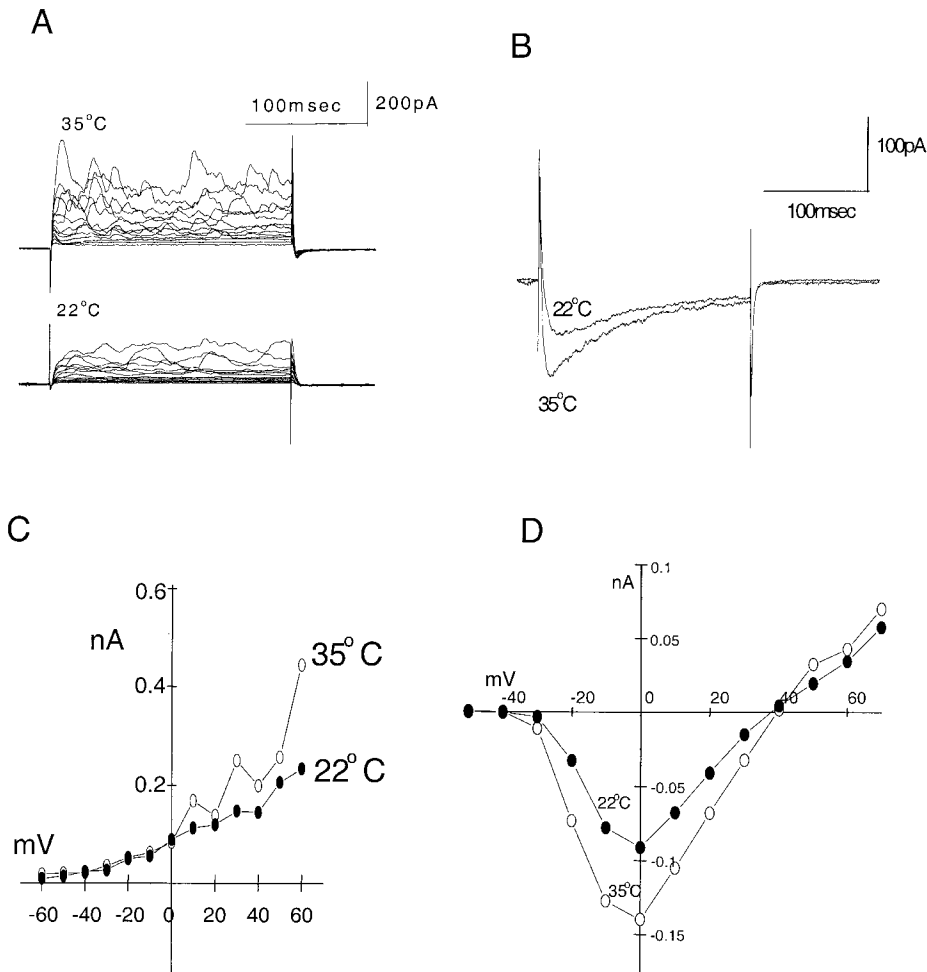-40   -20   0   20   40   60

-0.05

22°C
-0.1

35°C

-0.15

FIGURE 6. Cooling and the ionic currents in guinea pig ureter cells. (A and B) outward and inward currents recorded at two temperatures, during voltage steps from a holding potential of −80 mV to −50 to +50 mV, respectively. (C and D) Current-voltage relationships of the outward and inward currents at 35 and 22°C. The linear leakage current was subtracted from the overall membrane current.

on the peak Ca^{2+} current and I-V curve are shown in Fig. 6 (B and D). The decrease in the peak Ca^{2+} current can explain the decrease in the rate of rise of the spikes and amplitude of the plateau component of the action potential in the guinea pig ureter at room temperature. Decreases in the amplitude of the peak of the early transient outward current and STOCs can explain the increase in the amplitude and duration of the spikes and increase in the duration of the plateau component of the action potential at low temperatures.

Rat Ureter. Ionic currents in rat ureter have not been characterized, but have one outward current, presumably, K^+ current, and two inward currents: a fast one, presumably the Ca^{2+} current, and a late one with a tail current, characteristic of Ca-activated Cl^- (Cl_{Ca}) currents not seen in guinea pig cells (unpublished data). Cooling reduced the outward currents 1.3 ± 0.1 times ($n = 4$), but the most dramatic effect of cooling was seen on the late inward currents (Fig. 7). Despite the decrease in the amplitude of the fast inward current (20–40%, Fig. 7 A and Fig. 7 C, b), the amplitude of the

late inward and tail currents where significantly increased after cooling (Fig. 7 C, b, and Fig. 7, B and D). The rate of decay of the tail current was also significantly reduced upon cooling (Fig. 7 B). Using the integrated form of the Arrhenius equation and values of time constants measured at different temperatures, we calculate the value of energy of activation for the rate of decay of the tail current to be 26.6 kcal/mol.

The increase in the amplitude of the late inward current, and decrease in the rate of the decay of the tail current, can explain the significant increase in the amplitude and duration of the plateau component of the action potential in the rat ureter cells produced by cooling.

Effect of Cooling on the Relaxation of Force

The falling phase of the Ca^{2+} transient in the ureter of both species was significantly affected by cooling, showing high values of Q_{10}: 3.7 ± 0.3 ($n = 6$) for the guinea pig and 3.1 ± 0.2 ($n = 5$) for the rat ureter (see also Figs. 2–5). These high values of Q_{10} indicate that energy dependent Ca^{2+} transporting systems play a key
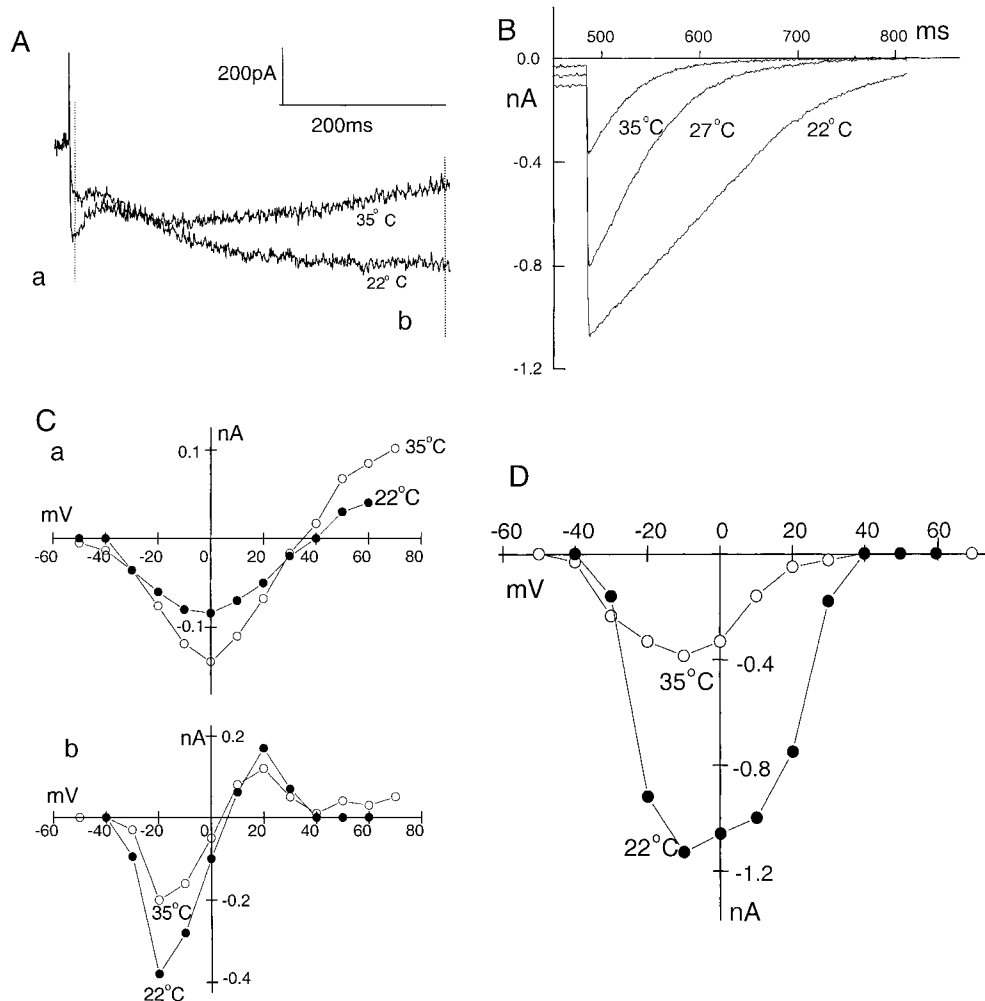
Rat ureter

FIGURE 7. Cooling and the ionic currents in rat ureter cells. (A) Inward currents recorded with cesium in the pipette, at 35 and 22°C, during voltage steps from −80 to 0 mV. (B) Tail currents recorded at 35, 27, and 22°C at −80 mV after depolarizing pulses to 0 mV. (C) Current voltage relationship for the early peak (Ca) current (a) and late (Ca-activated Cl) current measured at the end of the pulse (b) at 35 and 22°C. (D) Plot of the inward tail current amplitude following repolarization to −80 mV versus membrane potential during the depolarizing prepulse. Linear leakage current was subtracted from the overall membrane current.

role in restoration of $[Ca^{2+}]_i$ in the ureter. The slowing of the rate of decay of the late inward current with cooling in the rat ureter may be partly explained by this decrease in the rate of restoration of Ca^{2+} in the cytoplasm; and in both species, the effects of cooling on Ca^{2+} removal systems will contribute to the effects seen on the Ca^{2+} transient duration.

The strongest effect of cooling was seen on the rate of relaxation of the phasic contraction; the Q_{10} in the ureter of both species was in the range of 4–5. This high Q_{10} is in agreement with the previous observations (Mitsui et al., 1994).

MLCP activity, which dephosphorylates myosin light chains and hence promotes relaxation, also has a Q_{10} close to 5 (Mitsui et al., 1994). Thus MLCP could act as the limiting step in relaxation of the contraction in both species. To test this the effect of calyculin A, a cell-permeable blocker of Type I and Type II phosphatases (Sakamoto et al., 1997; Fujiki and Suganuma, 1999), on the force-Ca^{2+} relationship in the ureter of both species was investigated (Fig. 8, $n = 9$ [rat] and $n = 7$

[guinea pig]). It was found that in both species, calyculin A had little or no effect on the Ca^{2+} transient but significantly slowed the rate of relaxation of the phasic contraction. The half time of relaxation of the Ca transient and contraction in the absence and presence of calyculin A increased in the guinea pig ureter from 2.45 ± 0.31 to 3.97 ± 0.33 s and from 2.12 ± 0.23 to 13.90 ± 2.59 s, respectively. In the rat ureter, calyculin A had a stronger inhibitory action on the Ca transient (Fig. 8), but as with the guinea pig, a marked effect on relaxation. Thus, the halftimes of Ca and relaxation, in the absence and presence of calyculin A, were 2.03 ± 0.40 and 3.84 ± 1.97 s and 1.52 ± 0.63 and 25.10 ± 5.41 s, respectively. Fig. 8 B clearly shows the incomplete relaxation in both species, which was not accompanied by an elevation of basal Ca.

DISCUSSION

In this paper, we have shown that a moderate amount of cooling increases phasic contractions associated with
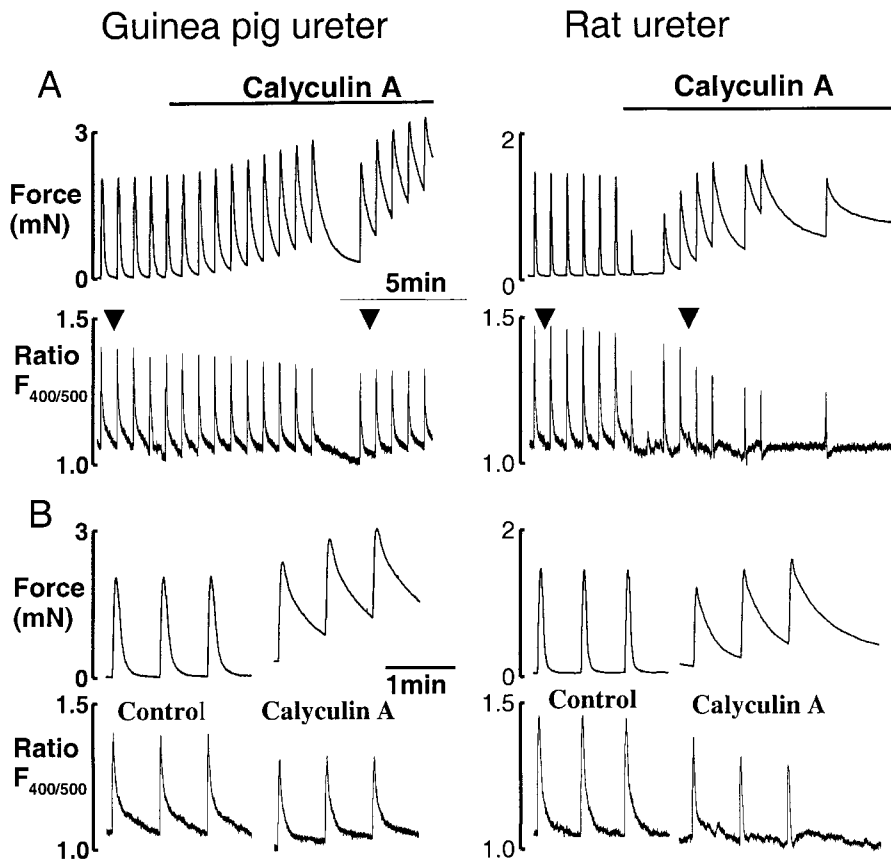
FIGURE 8. The effect of calyculin A (1 μM) on force and Ca in guinea pig and rat ureter at 25°C. In B, three control and calyculin A records (indicated by arrowheads in A) have been enlarged to show the much more marked effects on relaxation than Ca in both species.

the action potential of the rat ureter, but decreases those of the guinea pig ureter. Despite these differences in contraction amplitude, some parameters of force development were affected similarly by cooling in both species, indicating similar mechanisms controlling force development. The rate of rise and fall of force were both significantly slowed by cooling, and the delay between the Ca^{2+} rise and onset of force development was significantly prolonged in both species. These changes did not correlate with the rate of change in the rising phase of the underlying Ca^{2+} transient, which was little affected by cooling, and points instead to effects on Ca-insensitive steps in force production being affected by cooling, such as diffusion of calmodulin and complexing with Ca ions and the formation of active MLCK. With cooling in both species, the action potential was prolonged, in particular the plateau phase and thus, in turn, caused a prolongation of the Ca^{2+} transient, particularly in the rat. From these data, we suggest that in the rat, force is increased by cooling because of the very large prolongation of the action potential and the associated maintenance of Ca^{2+} at near maximal level. This prolonged Ca transient allows the myofilament to come closer to equilibrium with Ca^{2+} and increase force production. This occurs despite the slower kinetics of force development. However, in the guinea pig ureter, the action potential

is not as prolonged by cooling, which, in turn, means that the Ca^{2+} transient duration is not as maintained. This combined with the slower kinetics of force development results in a decreased amount of force. A large effect on the fall of force i.e., relaxation; Q_{10} ~5 in both species is consistent with a marked effect of temperature on MLCP in smooth muscles.

In this study, several of the key parameters in EC have been measured to develop an understanding of the mechanisms whereby temperature modulates force production in smooth muscle. The influence of temperature on smooth muscle force production has been reported for several smooth muscles (Nasu, 1990), and as mentioned above the effects of decreasing temperature have been reported to potentiate or inhibit force. The finding that the effects of temperature on a particular smooth muscle can be species dependent confirms the earlier result of Burdyga and Magura (1986) and Burdyga and Kosterin (1991). As will be discussed in the next section, it seems likely that differences in the characteristics of the action potential between the rat and the guinea pig underlie the different effects.

Electrical Activity

The changes found with cooling on electrical activity, go some way, but not entirely, to explaining the effects

seen on force. As noted in Fig. 5, the action potential shape is very different between the ureter of rats and guinea pigs. These differences must, in turn, be due to differences in the presence or activity of the surface membrane ion channels. One of the most striking differences is that the long plateau phase of the guinea pig ureter is absent from the rat, at normal temperatures; hence, the rat action potential is much briefer. In the guinea pig ureter, the plateau phase has been attributed to a maintained Ca^{2+} entry via voltage-gated type Ca^{2+} channels, with very slow inactivation processes and the major outward current in the guinea pig ureter is a K_{Ca}^+ current (Imaizumi et al., 1989; Lang, 1989; Sui and Kao, 1997a,b). Both inward and outward currents were decreased by cooling and, therefore, can explain the decrease in the rate of rise and fall and an increase in the duration of both spike and plateau components of the action potential. The plateau component of the action potential in rat ureter was extremely temperature-sensitive ($Q_{10} = 4.7$). This prolongation of the plateau component of the action potential can be explained by potentiation of the inward currents observed in rat. Strong potentiation of Cl_{Ca} current by cooling has been found in rabbit portal vein smooth muscle cells (Helliwell and Large, 1995). An increase in the amplitude and decrease in the rate of decay of the tail currents suggests that cooling prolongs the opening times of the Cl_{Ca} channels. The mechanism of this potentiation is unclear, but one possibility is the temperature sensitivity of the rate of decay of Ca^{2+} transient, which is significantly decreased. Another possibility is metabolic control of the gating of Cl_{Ca} channels. Wang and Kotlikoff (1997) have presented evidence that inactivation of these channels in rabbit portal vein cells is caused by CaM kinase II–dependent phosphorylation. Whatever the mechanism of the effect of cooling on Cl_{Ca} channels, the strong temperature sensitivity of this inward current can explain the difference in effect of temperature on the parameters of the action potential between guinea pig and rat ureter. We can clearly see that the prolongation of the rat action potential leads to a much longer Ca^{2+} transient, and that this correlates with the greatly increased amplitude and duration of force at lower temperatures. In the guinea pig ureter, cooling also produced a prolongation of the action potential but this was not as great as in the rat, and hence the period for which peak Ca^{2+} transient was present was little altered. Cooling also produced a significant increase in the size of the spikes on the guinea pig action potential, although their frequency was reduced. This spiking activity has been attributed to cyclic Ca^{2+} release and reuptake by the SR (Imaizumi et al., 1989), and the K_{Ca} current causing repolarization, which is countered by depolarizing Ca^{2+} entry. Therefore, it is suggested that, at lower temperatures,

these spontaneous Ca^{2+} releases occur less frequently, but release more Ca^{2+} when they do occur. Nevertheless, it remains to be addressed why force should actually fall in amplitude in the guinea pig ureter despite a longer action potential and Ca^{2+} transient, and this will be addressed next.

Effects of Calcium on the Amplitude of Contraction

Phasic contractions will depend on the magnitude, duration, and rate of change of $[Ca^{2+}]_i$. The temperature jump data obtained in this paper showed that the rate of change of the upstroke of Ca^{2+} was not responsible for the effects of temperature on force. The small effects of temperature on the Ca^{2+} rise ($Q_{10} \sim 1.2$–1.4), contrast markedly with its effect on Ca^{2+} restoration ($Q_{10} > 3$). This can be related to the different energetics of the two processes. Ca^{2+} removal is achieved against concentration gradients and is largely dependent on the Ca^{2+} ATPase's of both the sarcolemma and the SR. In contrast, the Ca^{2+} rise is due to Ca^{2+} entry across the sarcolemma and Ca^{2+} release from the SR, neither of which require energy. Thus, we conclude from our data that the kinetics of the Ca^{2+} transient rise are little influenced by temperature and do not underlie its effects on force.

There is evidence that both the Ca^{2+} transient amplitude and duration influence contraction amplitude. Although it is perhaps clear that the magnitude of the Ca^{2+} transient will determine the amount of force, the role of duration should not be overlooked in phasic smooth muscles. We have shown that as phasic activity is brief, there is not a steady state between $[Ca^{2+}]$ and force (Burdyga and Wray, 1999a,b). In other words, under control conditions, maximal force at any given level of $[Ca^{2+}]$ is not obtained because the myofilaments do not have sufficient time to enter into equilibrium with the Ca^{2+}. Thus, there is a hysteresis between force and $[Ca^{2+}]$ for phasic smooth muscles. If the Ca^{2+} transient can be maintained at maximal Ca^{2+} amplitude, then more force will be developed. This is clearly what is occurring in the ureter of the rat with cooling. Indeed, the effect of cooling on the rat Ca^{2+} transient duration was found to far exceed its effects on the Ca^{2+} amplitude.

In the guinea pig, as measured above, cooling produces less marked effects on the duration of the Ca^{2+} transient. The fact that force falls obviously suggests that some other parameter is inhibiting force production. This is not the magnitude of the Ca^{2+} transient, as this was not significantly altered by cooling. As mentioned above the kinetics of the Ca^{2+} rise were also little altered by cooling. Thus, in the case of the guinea pig ureter, it is necessary to consider factors other than the Ca^{2+} transient and, in particular, the myofilaments themselves, which will be discussed next.

Effects of Cooling on Contraction

Cooling produced a reduction in the amplitude of the contraction of the guinea pig ureter, but an increase in the rat ureter. However, amplitude was not the only force parameter to be modified with cooling. There was also in both species, a marked slowing in the rate of force production as well as relaxation. Unlike the parameters of the Ca^{2+} transient, which are closely linked to the underlying action potentials, that differ between rat and guinea pig, the kinetics of the phasic contractions will reflect the properties of the myofilaments and the Ca^{2+}-insensitive steps in force production. These are likely to be much more similar between the two species and, indeed, between different smooth muscles of the same species. Clearly if temperature reduction is slowing force development, we may expect this to reflect slower activation of the cross-bridges. In addition, estimates of the temperature dependence of the cross-bridge cycle by measuring of shortening velocity, have indicated Q_{10} values of around 2, (Stephens et al., 1977; Klemt and Peiper, 1978) that we have also found for the ureter (unpublished data). Such values would produce a slowing of contraction at low temperature.

We found a Q_{10} value of ~2.5 for the effect of cooling on the rate of contraction. This is similar to, but a little higher than, the value of 1.7 reported in permeabilized portal vein (Mitsui et al., 1994), perhaps due to differences between intact and permeabilized preparations. In intact preparations, data for force development indicates that the temperature sensitivity of force production is not limited to MLCK activity (Klemt et al., 1981; Jaworowski and Arner, 1998). In both ureteric preparations, however, a much more marked effect on the rate of relaxation than contraction was found; Q_{10}'s of 5.1 in the guinea pig and 3.7 in the rat ureter, and 3.9 was found in the portal vein (17). MLCP has been found to have an extremely high Q_{10} of 5.3 (Mitsui et al., 1994). Thus, the marked effect on relaxation can be attributed to its major role in dephosphorylating the light chains in smooth muscle. The relative activities of MLCK and MLCP plays an extremely important role in governing force production in smooth muscle. Both these enzymes are temperature-sensitive, but MLCP is more sensitive than MLCK (Q_{10}= 5.3 vs. 1.7, respectively; Mitsui et al., 1994). Thus, as the amount of phosphorylated myosin will depend upon the relative activities of these two enzymes, as temperature is decreased, it would be predicted that at any given Ca^{2+}, there would be more phosphorylated myosin. In turn, this would tend to increase force production. In permeabilized vascular smooth muscle, both maximal force and phosphorylated myosin were increased as temperature was lowered, at constant $[Ca^{2+}]$ (Mitsui et al., 1994), which is consistent with the greater effect on MLCP than MLCK. In the intact ureter, under physiological stimulation (i.e., action potentials),

this effect was one of several (already discussed), which contribute to the overall effect on contraction. Furthermore, the stronger effect of temperature on the phosphatase rather than kinase would also be expected to slow the recovery of force.

It was also found that cooling significantly increased the delay between the rise of Ca^{2+} and force development. This delay in phasic smooth muscle, even under control conditions, is substantial (>100 ms) and has been attributed to mobilization of calmodulin and formation of the active Ca-calmodulin MLCK complex (Stull et al., 1997).

In summary, then there are, as predicted, many effects of temperature on EC coupling in smooth muscle. We can use the data obtained in this study to understand the effects of cooling to increase maximal force in the rat and decrease it in the guinea pig, as follows. The most temperature-sensitive step in EC coupling in the rat was the plateau phase of the action potential (Q_{10} = 4.7). This led to a very prolonged Ca^{2+} transient, which gave the myofilaments sufficient time to reach equilibrium with the Ca^{2+} signal which does not occur with the brief Ca^{2+} transient produced under control conditions. This effect was sufficient to overcome the smaller effect of temperature, slowing the kinetics of the rate of rise of force (Q_{10} = 2.5) and Ca^{2+} transient (1.3). Finally, the higher temperature sensitivity of MLCP compared with MLCK would also promote force production and inhibit relaxation. In the guinea pig ureter, similar effects of cooling were observed apart from on the action potential. The prolongation of the plateau phase was much less than in the rat (only around twofold) and this was reflected in the lesser prolongation of the duration of the Ca^{2+} transient in the guinea pig. These stimulatory effects consequently were insufficient to overcome the slowing of the rate of rise of force, and thus force falls. The TEA data obtained previously (Burdyga and Wray, 1999b) in the guinea pig showed that if the plateau phase of the action potential were sufficiently prolonged, then the $[Ca^{2+}]$ duration was further prolonged and this could be sufficient to overcome the inhibitory effects of cooling (i.e., the guinea pig ureter produced more force than under physiological temperatures). We further suggest that the differences in the effects of cooling on force between different phasic smooth muscles also might be accounted for by similar variations in the sensitivity of the steps in EC coupling to temperature.

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