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Effect of the Ca^{2+}-ATPase inhibitor, cyclopiazonic acid, on electromechanical coupling in the guinea-pig ureter

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1 We have investigated the effect of the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase inhibitor, cyclopiazonic acid (CPA), on electromechanical coupling in the guinea-pig ureter. All experiments were performed in capsaicin-pretreated (10 μM for 15 min) ureters to prevent the release of sensory neuropeptides from afferent nerves.

2 In organ bath experiments, electrical field stimulation (EFS, 10 Hz for 1 s, 5 ms pulse width, 60 V) produced tetrodotoxin- (1 μM) resistant phasic contractions which were enhanced by Bay K 8644 (1 μM) and abolished by nifedipine (10–30 μM).

3 CPA (10 μM) enhanced the EFS-evoked contractions both in the absence and presence of Bay K 8644. The effect of CPA was concentration-dependent between 1 and 30 μM. The response to 10 μM CPA was biphasic: the maximal enhancement (58 ± 3% increase) was observed within 10–20 min from CPA administration, followed by a decline to a new steady state (25 ± 5% increase over baseline) at 50–60 min. The effect of CPA was reversed by washout.

4 Ryanodine (100 μM) produced a prompt enhancement of the EFS-evoked contractions of the guinea-pig ureter, which peaked at 42 ± 3% increase over baseline; the co-administration of CPA (10 μM) and ryanodine (100 μM) produced a peak effect (60 ± 8% enhancement) which was not different from that produced by CPA alone. With either ryanodine alone *or* ryanodine plus CPA, the enhancement of the EFS-induced contractions was biphasic, showing a time-course similar to that observed with CPA alone. Tetraethylammonium (10 mM) produced a significantly larger effect (93 ± 13% increase over baseline) and its effect was sustained throughout the 60 min observation period.

5 In the presence of Bay K 8644, superfusion for 30 min with a low Na^+ medium (60% of extracellular Na^+ replaced by Li^+ or choline) reduced the amplitude of EFS-evoked contractions by 20–35%. In both Li^+- and choline-substituted media, spontaneous activity developed during superfusion with low Na^+ Krebs solution which was suppressed by 10 μM nifedipine. CPA (10 μM) produced a marked enhancement of the EFS-evoked contractions in low-Na^+ medium (both Li^+- and choline-substituted) and this effect was sustained throughout the 60 min observation period.

6 In the absence of Bay K 8644, the response of the ureter smooth muscle to EFS is characterized by a refractory period: an interval of about 30 s was required between two applied stimuli to produce a second response comparable in size to that elicited by the first stimulus. CPA (10 μM, 10–20 min before) markedly reduced the refractory period of the guinea-pig ureter to EFS.

7 CPA (10 μM, 30–60 min before) increased the phasic component of contraction produced by 80 mM KCl. The tonic component of the response to KCl was slightly but not significantly reduced by CPA, and a 'hump' in the tonic contraction was observed at 1–2 min from addition of KCl.

8 In sucrose gap experiments, 10 μM CPA produced a sustained depolarization of the membrane and reduced the latency between application of electrical stimuli and onset of the action potential; these effects were maintained throughout the 60 min superfusion with CPA. CPA also transiently prolonged the plateau phase of the action potential and increased the peak amplitude of contraction: these effects peaked at about 10–20 min from start of superfusion with CPA and then declined. At the peak of its enhancing effect on contraction amplitude, CPA prolonged the contractile phase of the contraction-relaxation cycle.

9 Superfusion with a low-Na, choline-substituted Krebs solution produced a reversible membrane depolarization. In the presence of Bay K 8644 (1 μM), action potentials and phasic contractions were superimposed on this depolarization which were abolished by nifedipine (10 μM).

10 These findings indicate that CPA augments the excitability and affects the contraction-relaxation cycle of the smooth muscle of the guinea-pig ureter, implying a role for sarcoplasmic reticulum Ca^{2+}-ATPase in the regulation of electromechanical coupling. The effects of CPA resemble those produced by ryanodine and the effect of the two agents on the amplitude of contractions is non-additive. It appears that following blockade of the CPA-sensitive SR Ca^{2+} pump, other mechanism(s) may come into action to reduce intracellular Ca^{2+}. The Na^+/Ca^{2+} exchanger could be involved in the compensatory changes responsible for the fading of the response to CPA.

Keywords: Guinea-pig ureter; electromechanical coupling; cyclopiazonic acid; sarcoplasmic reticulum Ca^{2+} pump; ryanodine; smooth muscle

Introduction

The mechanisms determining contraction of the ureter in response to direct electrical stimulation represent an excellent example of electromechanical coupling in smooth muscle. From both the electrical and mechanical point of view, the ureter smooth muscle is quiescent but when subjected to depolarization, it produces an action potential and accompanying phasic contraction: L-type voltage-sensitive calcium (Ca^{2+}) channels play a fundamental role in excitation-

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contraction coupling at this level, since the action potential and contraction are both powerfully altered by administration of drugs with antagonist or agonist action on these Ca^{2+} channels (Shuba, 1977; Brading *et al.*, 1983; Maggi *et al.*, 1994a). As expected from the pivotal role of voltage-sensitive Ca^{2+} channels, the manipulation of potassium (K^+) permeability also exerts remarkable effects on the action potential and contractility of the guinea-pig ureter (Shuba, 1977; Imaizumi *et al.*, 1989b; Lang, 1989; Maggi *et al.*, 1994b). The contribution of intracellular Ca^{2+} handling mechanisms to electromechanical coupling in the ureter smooth muscle has been little investigated.

In a previous study (Maggi *et al.*, 1994a), we found that ryanodine, a plant alkaloid which blocks Ca^{2+} handling by the sarcoplasmic reticulum (SR) by acting on the calcium-induced calcium release channel (CICR), increases the excitability of the guinea-pig ureter by producing membrane depolarization and reducing the refractory period. Interestingly, ryanodine does not reduce the amplitude of depolarization-induced phasic contraction but, especially after addition of the L-type Ca^{2+} channel agonist, Bay K 8644, it prolongs the contractile cycle of the ureter; these observations suggest that Ca^{2+} handling by the SR regulates excitability and the contractile cycle of the ureter smooth muscle especially to enable removal of cytoplasmic Ca^{2+} while the SR may not be essential as a source of activator Ca^{2+} for contraction.

In this study, we have determined the effect of cyclopiazonic acid (CPA) a mycotoxin from *Aspergillus* and *Penicillum*, on the electrical and mechanical responses of the guinea-pig ureter to depolarizing stimuli. CPA is a specific inhibitor of the SR Ca^{2+}-ATPase and is increasingly used as a tool for studying excitation-contraction coupling in smooth muscles (Deng & Kwan, 1991; Low *et al.*, 1992; Uyama *et al.*, 1992; 1993; Bourreau *et al.*, 1992). CPA acts by preventing the binding of Ca^{2+} to the high affinity sites on SR, thereby impeding the Ca^{2+}-dependent phosphorylation of SR proteins (Goeger & Riley, 1989). A comparison of the effect of CPA and ryanodine on electromechanical coupling of the ureter appears of interest since the two drugs would affect the same structure (SR) by acting on different targets. Since the response to CPA was found to have a biphasic time course, the possible involvement of Na^+/Ca^{2+} exchanger (Aickin *et al.*, 1984; 1987; Aickin, 1987; Aaronson & Benham, 1989) in this phenomenon was assessed by studying the effect of CPA following reduction of extracellular Na^+ which was replaced with lithium (Li^+) or choline chloride.

Methods

General

Male albino guinea-pigs weighing 250–300 g were stunned and bled. The ureters (from the inferior renal pole to their entry into the bladder) were excised, cleaned of adhering fat and connective tissue and placed in oxygenated and warmed (95% O_2 and 5% CO_2, pH 7.4 at 37°C) Krebs solution of the following composition (mmol/l): NaCl 119, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.5, KCl 47, $CaCl_2$ 2.5 and glucose 11. In some experiments (see below) a low-Na^+ Krebs solution was prepared by replacing 86.4 mM NaCl with an equimolar amount of LiCl or choline Cl. Considering the overall Na^+ content of the normal Krebs solution (144 mM) this replacement corresponds to 60% reduction of extracellular Na^+. Because of the existence of a capsaicin-sensitive inhibitory innervation in the guinea-pig ureter, all experiments were performed in capsaicin-pretreated (10 μM for 15 min) ureters to eliminate this neural inhibitory influence on ureteral excitability (Maggi & Giuliani, 1991).

Organ bath experiments

The ureter was placed in a Petri dish, a segment about 2 cm long was excised from its middle portion and the specimen mounted in a 5 ml organ bath for isotonic recording (load 2 mN) of mechanical activity along the longitudinal axis, as described previously (Maggi & Giuliani, 1991; Maggi *et al.*, 1994b).

In a first series of experiments, we investigated the effect of CPA on the phasic contractions produced by electrical depolarization of the ureter. With this aim, trains of pulses (10 Hz for 1 s, 60 V) of long width (5 ms) were automatically delivered at 1 min intervals by means of platinum wire electrodes placed at the top and bottom of the organ bath (electrical field stimulation, EFS) by means of a Grass S88 stimulator. In most experiments, the effects of CPA on EFS-evoked contractions were investigated in the presence of Bay K 8644 (1 μM) which provides reproducible responses to EFS over prolonged periods of time by reducing the refractory period of the guinea-pig ureter (Maggi *et al.*, 1994a; and see Results, Figure 1).

In some experiments we studied the effect of Low-Na^+ Krebs solution (Li^+- or choline-substituted, see above) on the response to EFS and on the effect produced by CPA: two ureters from the same animal were studied in parallel, one of which was exposed to the low-Na^+ Krebs solution and the other served as control. Both ureters were initially exposed to capsaicin in Krebs solution and then 1 μM Bay K 8644 was added, which was present throughout the experiments. After having recorded a steady response to EFS, the ureters were perfused with low-Na^+ Krebs solution or normal Krebs solution for 30 min at a rate of 5 ml min^{-1}. At this stage, the superfusion was stopped and the response to EFS was allowed to reach a new steady state: CPA (10 μM) was added to the bath and its effects observed for 60 min.

In a second series of experiments, we determined the effect of CPA on the refractory period of the capsaicin-pretreated guinea-pig ureter to EFS, as described previously (Maggi *et al.*, 1994a): a conditioning stimulus (10 Hz for 1 s, 5 ms pulse width, 60 V) was applied either in the absence or the presence of 10 μM CPA and a test stimulus of the same intensity was delivered at 3, 5, 10 or 30 s interval from the conditioning stimulus. The amplitude of the phasic contraction to the test stimulus was expressed as a % of that evoked by the conditioning stimulus. These experiments were performed at 10–20 min from administration of CPA, corresponding to the period of maximal enhancement of the evoked responses by the drug.

In a third series of experiments we studied the effect of CPA on the biphasic contractile response produced by application of 80 mM KCl to the capsaicin-pretreated guinea-pig ureter; both the phasic and tonic components of this response are produced through the activation of L-type, nifedipine-sensitive, calcium channels (Maggi & Giuliani, unpublished; Maggi *et al.*, 1994c). Two reproducible control responses to KCl were obtained at 30 or 60 min intervals and the effect of CPA (10 μM) determined after 30 or 60 min contact time.

Sucrose gap experiments

A single sucrose-gap, modified as described in details by Artemenko *et al.* (1982) and Hoyle (1987) was used to investigate changes in membrane potential and mechanical activity in response to electrical stimulation. The ureters were superfused with oxygenated Krebs solution at a rate of 1 ml min^{-1}. The temperature of the solution was kept constant at 35 ± 0.5°C. The experiments were performed in ureters pre-exposed to capsaicin (10 μM for 15 min) and in the presence of 1 μM Bay K 8644 (Maggi *et al.*, 1994a,b,c).

EFS with single pulses using parameters sufficient to produce direct excitation of smooth muscle (20–40 V, 0.5–2 ms pulse width) evoked an action potential and accompanying contraction of the guinea-pig ureter (Shuba *et al.*, 1977;

Brading *et al.*, 1983). To study the effects of CPA, action potentials and contractions of the ureters were produced at 2.5 min intervals: when steady state responses had been obtained, superfusion with CPA (10 μM) were started and maintained for 60 min. In other experiments the ureters were superfused with the low-Na^+ choline-substituted medium for 3 min at 15–20 intervals. The effect of Bay K 8644 (1 μM) and nifedipine (10 μM) on changes in membrane potential produced by the low-Na^+ choline-substituted medium were assessed after 15 min contact time.

Statistical analysis

Each value is mean ± s.e. mean. Statistical analysis was performed by means of Student's *t* test for paired or unpaired data or by means of analysis of variance, when appropriate. Linear regression was performed by the least squares method: EC_{50} and 95% confidence limits were calculated accordingly.

Drugs

Drugs used were: cyclopiazonic acid (CPA), capsaicin, nifedipine and tetrodotoxin (Sigma), ryanodine and Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) (Calbiochem), tetraethylammonium (TEA, Serva).

Results

Organ bath experiments

Effect of CPA on the response to EFS EFS (10 Hz for 1 s, 5 ms pulse width, 60 V) induced phasic contractions of the guinea-pig ureter ranging from 20–40% of the maximal response to 80 mM KCl (Figure 1a, b and c): all ureters developed phasic contractions upon EFS application but the responses became irregular during repetitive cycles of

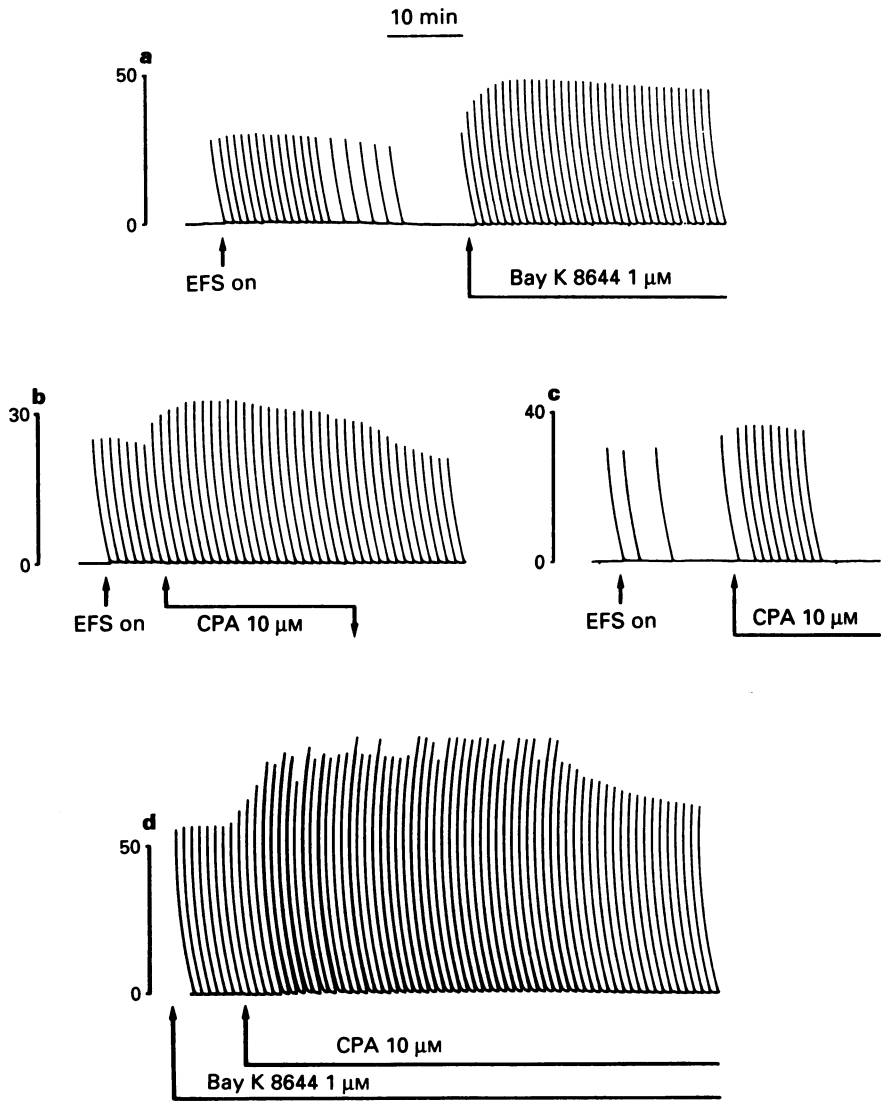

Figure 1 Typical tracings showing the effect of cyclopiazonic acid (CPA) on electrical field stimulation-(EFS) induced contractions of the guinea-pig isolated ureter in the absence and presence of Bay K 8644. Phasic contractions of the ureter were evoked by EFS (train of pulses at a frequency of 10 Hz for 1 s, 60 V, 5 ms pulse width, applied every 60 s). In the absence of Bay K 8644, most ureters responded to EFS for a limited period of time after which the response waned (a and c). Either Bay K 8644 (a) or CPA (c) caused resumption of the response to EFS. CPA added before spontaneous fading of the response enhanced the EFS-induced contractions (b; tracings shown in panels a and b were obtained in paired ureters from the same animal). In the presence of Bay K 8644, CPA produced a prompt enhancement of the response to EFS which showed a spontaneous decline at 40–50 min from CPA administration (d). Vertical bars are percentage response to KCl.

stimulation; the most frequently observed pattern was the failure of some applied stimuli to produce a response (Figure 1a and c). In 5 experiments CPA (10 μM) was applied to one ureter while the other served as control (Figure 1a and b): CPA enhanced the amplitude of the evoked contractions by 36 ± 6% and prevented the decline observed in untreated ureters (cf. Figure 1a and b). If added when the response had become irregular or had disappeared, CPA (10 μM) produced a transient excitatory effect on the responsiveness of the ureter (Figure 1c). As noted in previous studies (e.g. Uyama *et al.*, 1992), the effect of CPA was reversed by washout.

As shown in Figure 1a, the addition of the dihydropyridine calcium channel agonist, Bay K 8644 (1 μM) restored the ability of the ureter to respond to each applied stimulus and enhanced the amplitude of the EFS-evoked contractions. In the presence of Bay K 8644, the response to EFS ranged from 40–65% of the maximal response to KCl (80 mM): these responses were unaffected by tetrodotoxin (1 μM) and were suppressed by 10–30 μM nifedipine. In the presence of Bay K 8644, CPA (1–30 μM, $n = 6$) produced a concentration-dependent enhancement of the amplitude of electrically evoked contractions of the ureter (Figure 1d and 2a). The effect of each concentration developed in 10–20 min to a steady state. When the effect of 10 μM CPA, administered as a single concentration to the bath, was compared to that produced by 10 μM CPA during a cumulative concentration-response curve, the two effects averaged 37 ± 6 and 55 ± 8% increase over baseline, respectively ($n = 6$ each, Figure 2a). The enhancement of the evoked contractions produced by CPA was reversed by washing at any time after its application. In the presence of 10–30 μM CPA, an increased excitability of the preparation was also evident because multiphasic responses to EFS and/or spontaneous contractions were observed in some preparations.

Time course of the effect CPA and comparison with ryanodine In these experiments ($n = 11$), the response to 10 μM CPA was studied over a time course of 60 min. As shown in Figure 1d and 2b, the maximal enhancement was produced at 5–20 min from CPA application (Table 1). At 30–40 min from CPA application, a relatively sudden fading of the enhancement of the evoked contractions was observed and their amplitude equilibrated at a new level, significantly lower than the peak effect of CPA (Figure 1d and 2b, Table 1). When multiphasic responses to EFS had been produced at the peak of the enhancing effect of CPA, these usually disappeared at the new steady state.

For comparative purposes, we investigated the effect of ryanodine, on the response to EFS. The concentration of ryanodine (100 μM) used in this study produces maximal effects on excitation-contraction coupling of the guinea-pig ureter (Maggi *et al.*, 1994a); the effect of combined administration of CPA (10 μM) and ryanodine (100 μM) was also determined (Figure 2b).

Either ryanodine (100 μM, $n = 15$) or ryanodine plus CPA (100 and 10 μM, respectively, $n = 7$) produced an enhancement of the EFS-induced contractions of the guinea-pig ureter, qualitatively and quantitatively similar to that described above for CPA alone (Figure 2b, Table 1). In the presence of ryanodine alone *or* CPA plus ryanodine, a fading of the potentiating effect was observed at 30–40 min from drug administration (Figure 2b): at 60 min, the degree of enhancement produced by ryanodine or ryanodine plus CPA

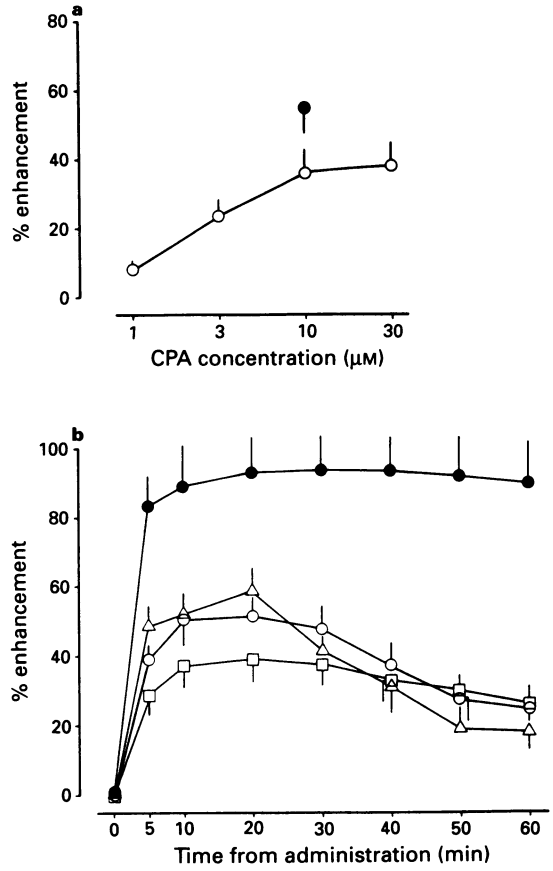

Figure 2 (a) Concentration-dependent enhancement of the response to EFS by cyclopiazonic acid (CPA ○) produced during a cumulative response curve vs. the response produced by 10 μM CPA (●) administered as a single concentration to the bath. All values are mean ± s.e.mean of 6 experiments performed in the presence of 1 μM Bay K 8644. (b) Time course of the potentiation of EFS-induced contractions of the guinea-pig isolated ureter by CPA (10 μM, ○), ryanodine (100 μM, □), CPA plus ryanodine (△) or tetraethylammonium (10 mM) (●). Each value is mean ± s.e.mean of 6–15 experiments performed in the presence of 1 μM Bay K 8644.

Table 1 Enhancement by cyclopiazonic acid (CPA), ryanodine and tetraethylammonium (TEA) of EFS-induced contractions in the guinea-pig ureter.

	n	*Peak effect* (% increase)	*Time to peak* (min)	*Effect at 60 min* (% increase)
CPA 10 μM	11	58 ± 3	15 ± 2	25 ± 5**
Ryanodine 100 μM	15	42 ± 3	18 ± 3	25 ± 3**
CPA 10 μM plus ryanodine 100 μM	7	60 ± 8	20 ± 4	18 ± 5**
TEA 10 mM	6	93 ± 12*	22 ± 3	90 ± 12

All experiments were performed in the presence of Bay K 8644, 1 μM. For each group, the maximal enhancement of EFS-induced contractions and the time required for development of maximal effect have been calculated as well as the enhancement observed at 60 min from drug application.
Each value is mean ± s.e.mean from n experiments.
*Significantly different from CPA or ryanodine, alone or in combination, $P < 0.05$.
**Significantly different from the peak response to the same drug, $P < 0.05$.

was significantly less than the corresponding peak effect (Table 1). Overall, the results indicate that the effect of CPA and ryanodine are non-additive.

The non-additivity of the enhancement produced by CPA and ryanodine is not linked to the attainment of a maximal contractile effect with each drug alone: tetraethylammonium (TEA, 10 mM) produced a rapid enhancement of EFS-evoked contractions which was sustained throughout the 60 min observation period ($n = 6$, Figure 2b, Table 1). Furthermore, when TEA (10 mM) was added 20–30 min after CPA (10 μM) administration (i.e. at the peak of CPA action), it produced a further prompt enhancement of the EFS-evoked contractions by 35 ± 6% ($n = 4$). The amplitude of EFS evoked contractions in the presence of Bay K 8644 and TEA equalled the response produced by a maximally effective concentration of KCl (80 mM, $n = 4$).

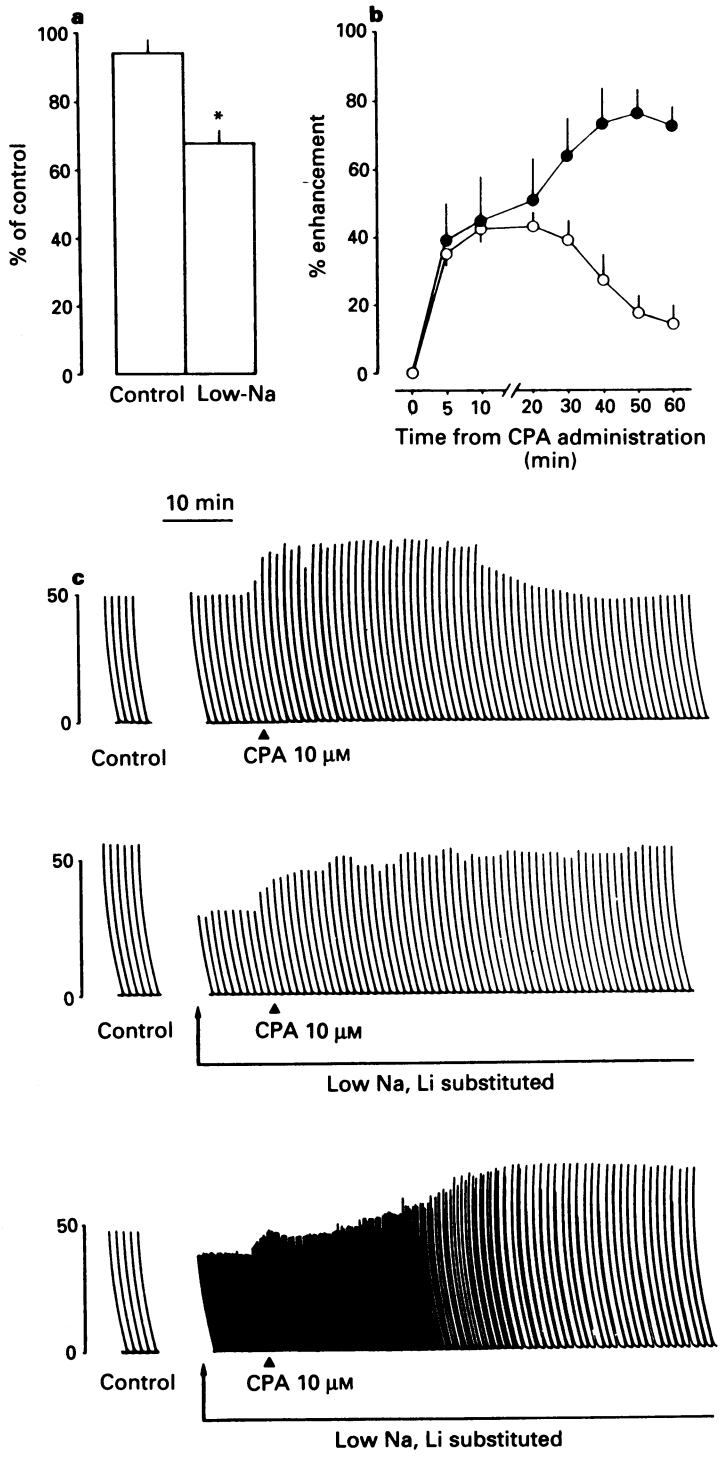

Figure 3 (a) Amplitude of EFS-evoked contractions after superfusion with normal Krebs solution (control) or low Na^+, Li^+-substituted Krebs solution in the presence of 1 μM Bay K 8644. Each value is mean ± s.e.mean of 7 experiments. $^*P<0.05$ vs control. (b) Effect of cyclopiazonic acid (CPA, 10 μM) on EFS-induced contractions in normal Krebs solution (○) or in low Na^+, Li^+-substituted Krebs solution (●) in the presence of 1 μM Bay K 8644. Each value is mean ± s.e.mean of 7 experiments. (c) Typical tracings comparing the effect of CPA on EFS-evoked contractions of the guinea-pig ureter in normal Krebs (upper panel) or in low Na^+, Li^+-substituted Krebs solution (middle and low tracings). All experiments were performed in the presence of 1 μM Bay K 8644. In panel (c), vertical bars are percentage of the response to KCl.

Effect of CPA in a low-Na^+ Krebs solution We postulated that the fading of the response to CPA may involve an increased efficiency of a CPA-insensitive mechanism(s) which removes free Ca^{2+} from the cytoplasm following SR Ca^{2+} ATPase blockade. An involvement of the Na^+/Ca^{2+} exchanger is an interesting possibility, since physiological evidence for the existence of this mechanism has been well documented in the guinea-pig ureter (Aickin *et al.*, 1984; 1987; Aickin, 1987; Aaronson & Benham, 1989). In the absence of selective inhibitors, we studied the effect of reducing extracellular Na^+ on the response to EFS and on the intensity and time course of the effect of CPA.

Superfusion with low-Na^+, Li^+-substituted, Krebs solution (60% reduction of extracellular Na^+ by Li^+) for 30 min reduced the amplitude of the EFS-evoked contractions (in the presence of Bay K 8644) ($n = 7$, Figure 3a). In 5 out of 7 cases tested the low-Na^+, Li^+-substituted medium also induced the appearance of spontaneous phasic contractions and/or biphasic responses to EFS (Figure 3c). The spontaneous contractions had a similar amplitude of those evoked by EFS.

CPA (10 μM) enhanced the amplitude of the EFS-induced contractions in low-Na^+, Li^+-substituted medium as well as the amplitude of spontaneous contractions, when present (Figure 3b and c, Table 2). In the low-Na^+, Li^+-substituted medium, the effect of CPA ensued and developed as observed in controls during the first 20–30 min from CPA administration (Figure 3b): however, while the expected decline in the enhancement of EFS-induced contractions was observed in control ureters, the effect of CPA in low-Na^+, Li^+-

Table 2 Enhancement by cyclopiazonic acid (CPA, 10 μM) of the response to EFS in guinea-pig isolated ureter in normal and low-Na^+Krebs solution

	n	*Peak effect* (% increase)	*Time to peak* (min)	*Effect at 60 min* (% increase)
Control	7	44 ± 4	12 ± 2	15 ± 5*
Low-Na^+, Li^+-substituted	7	73 ± 10	31 ± 5	72 ± 6
Control	6	57 ± 9	17 ± 4	23 ± 4*
Low-Na^+, choline-substituted	6	51 ± 12	22 ± 7	47 ± 8

All experiments were performed in the presence of Bay K 8644, 1 μM. For each group, the maximal enhancement of EFS-induced contractions and the time required for development of maximal effect have been calculated as well as the enhancement observed at 60 min from drug application. Two ureters from the same animal were studied in parallel one of which was superfused with low-Na medium (60% extracellular Na^+ replaced with either Li^+ or choline) and the other served as control. After superfusion, when the amplitude of the response to EFS had reached a steady state, CPA 10 μM was added to the bath and its effect recorded over 60 min Each value is mean ± s.e.mean from n experiments.
*Significantly different from the peak response to CPA, $P<0.05$.

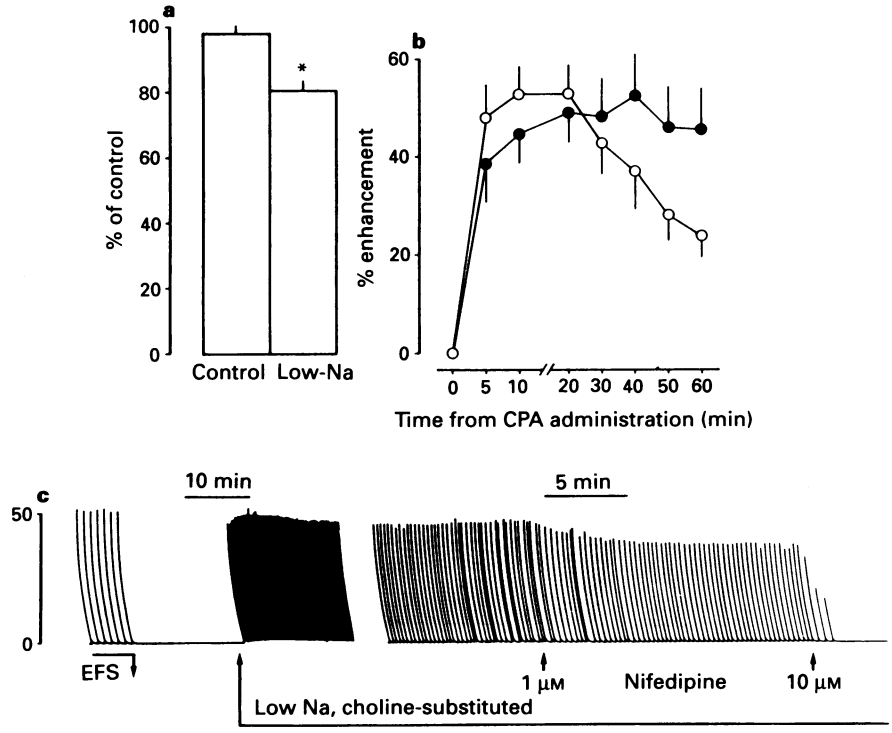

Figure 4 (a) Amplitude of EFS-evoked contractions after superfusion with normal Krebs solution (Control) or low Na^+, choline-substituted Krebs solution in the presence of 1 μM Bay K 8644. Each value is mean ± s.e.mean of 12 experiments. *$P<0.05$ vs control. (b) Effect of cyclopiazonic acid CPA, (10 μM) on EFS-induced contractions in normal Krebs solution (○) or in low Na^+, choline-substituted Krebs solution (●) in the presence of 1 μM Bay K 8644. Each value is mean ± s.e.mean of 6 experiments. (c) Typical tracing showing the appearance of spontaneous contractions after superfusion with low Na^+, choline-substituted Krebs solution and their inhibition/suppression by nifedipine. All experiments were performed in the presence of 1 μM Bay K 8644. In panel (c), the vertical bar is the percentage response to KCl.

substituted medium was sustained throughout the 60 min observation period and in 3 cases the maximal enhancement was produced at 40–50 min (Figure 3b and c).

In the presence of Bay K 8644, superfusion with a low-Na^+, choline-substituted Krebs solution invariably induced the occurrence of a high frequency (see below) spontaneous activity ($n = 12$, Figure 4c). The amplitude of phasic contractions equilibrated, at steady state, at a lower value than that of contractions producted by EFS before reduction of extracellular Na^+ (Figure 4a, $n = 6$). In low-Na^+, choline-substituted medium, CPA (10 μM) produced a sustained increase in the amplitude of contractions which did not show a significant fading during the 60 min observation period (Figure 4b, Table 2).

The characteristics of spontaneous contractions produced by the low-Na^+ choline-substituted medium were investigated in further details (Figure 4c): after having recorded steady responses to EFS, the stimulation was stopped and the ureters were superfused for 30 min with a low-Na^+, choline-substituted medium. Spontaneous contractions appeared in all cases tested ($n = 6$) within 5 min from start of superfusion with the low-Na^+ medium: at a steady state their frequency averaged 4.3 ± 0.6 cycles min^{-1} and their amplitude $86 \pm 1\%$ of that of EFS-evoked contractions. Nifedipine (1 μM) produced $23 \pm 3\%$ inhibition of the amplitude of contractions and abolished the spontaneous activity at 10 μM ($n = 4$, Figure 4c). Washout with normal Krebs solution likewise stopped the spontaneous activity observed during superfusion with low Na^+, choline-substituted Krebs solution and the re-application of low-Na^+, choline substituted medium induced their re-appearance.

Effect of CPA on the refractory period of the guinea-pig ureter The ability of CPA to restore the EFS-induced contractions or prevent their fading in the absence of Bay K 8644 (Figure 1b and c) suggests that CPA increases the excitability of the ureter to depolarizing stimuli. To check this hypothesis and quantitate this effect, we investigated the effect of 10 μM CPA on the refractory period of the guinea-pig ureter, as described previously (Maggi *et al.*, 1994a). In the absence of CPA, only 4 out of 11 preparations developed a response at 3 or 5 s interval between the two stimuli and all ureters responded after a 10 or 30 s interval (Figure 5). In the presence of CPA, 9 out of 11 ureters responded at an interval of 3 s and all ureters responded at 5 s interval between the first and second stimulus. As shown in Figure 5, the amplitude of the response to the test stimulus, expressed as % of the response to the conditioning stimulus was significantly increased by CPA at 3, 5 and 10 s interstimulus intervals.

Effect of CPA on the response to KCl The application of 80 mM KCl produced distinct phasic and tonic components of contraction of the ureter (Figure 6). The amplitude of the phasic contraction ranged between 40 and 90% of that of the tonic contraction (determined at 10 min from application of KCl).

CPA (10 μM, 30–60 min before) enhanced the amplitude of the phasic response to 80 mM KCl and prolonged the duration of phasic contractions (Figure 6); the amplitude of the tonic response, determined at 10 min from addition of KCl, was slightly but not significantly reduced. The influence of the two combined effects was best evident when calculating the ratio between the amplitude of the phasic and tonic components of the response to KCl which was significantly enhanced at both 30 and 60 min after CPA administration. After 30 min incubation with CPA the ratio between the phasic and the tonic response to KCl averaged $100 \pm 7\%$ vs. $63 \pm 4\%$ obtained in the same ureters before addition of CPA ($P < 0.05$, $n = 7$). Likewise, after 60 min incubation with CPA, the ratio averaged $89 \pm 8\%$ vs. $59 \pm 4\%$ of the control response ($n = 8$, $P < 0.05$).

At both incubation times (4 out of 7 cases at 30 min, 8 out of 8 cases at 60 min) CPA also modified the shape of the response to KCl: in the presence of CPA, a 'hump' (indicated by arrows in Figure 6) appeared between the initial phasic and the sustained tonic response, peaking at about 2 min from addition of KCl. This hump could be due to to a faster development of the tonic response to KCl in the presence of CPA since: (a) the hump peaked at about the same tension values measured at 10 min from addition of KCl in the absence of CPA and (b) upon superimposition of tracings, the rate of rise of the sustained response appeared much faster in the presence than in the absence of CPA.

Sucrose gap experiments

Effect of CPA on the action potential of the ureter CPA (10 μM, $n = 5$; Figures 7 and 8) produced a number of changes in the action potential and accompanying phasic contraction of the guinea-pig ureter produced by direct electrical stimulation of smooth muscle (single pulses 30–40 V, 1–2 ms pulse width). All effects ensued within 10–20 min from start of superfusion: some of them were maintained for the whole observation period while others faded within 20–30 min.

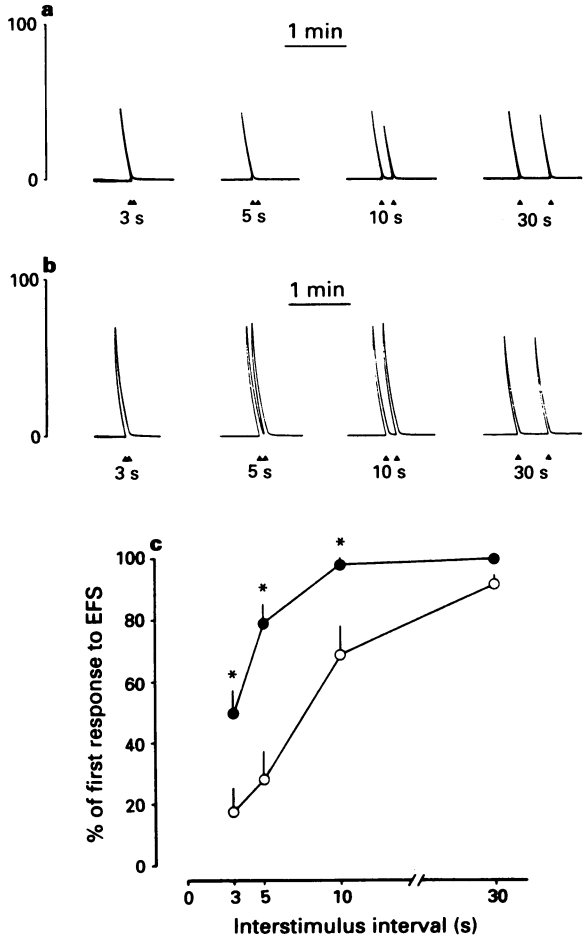

Figure 5 (a and b) Effect of cyclopiazonic acid (CPA) on the refractory period of the guinea-pig ureter. Two consecutive trains of electrical pulses (at arrowheads) were applied at 3–30 s intervals in the absence (a) or presence (b) of 10 μM CPA. Note that CPA markedly enhanced the ability of the ureter to develop a phasic contraction in response to the second stimulus of each pair, thereby reducing the refractory period. (c) Effect of CPA on the refractory period of the guinea-pig ureter. Each value is mean ± s.e.mean of 11 experiments: (●) CPA 10 μM; (○) control. *$P < 0.05$ vs. controls. In panels (a) and (b) the vertical bars are the percentage of the response to KCl.

The amplitude of the first spike of the action potential was not significantly affected by CPA, being 10.4 ± 1.0, 10.7 ± 1.1 and 10.4 ± 1.1 mV before and at 10 or 60 min of CPA action, respectively ($n = 5$).

The time course of CPA action on membrane potential, latency between stimulus application and onset of action potential, action potential duration (at 90% of repolarization) and contraction is shown in Figure 8. Two effects of CPA ensued within 10–20 min and were maintained or developed further during the 60 min observation period: these were membrane depolarization and reduction in the latency between stimulus application and onset of the action potential. Two effects of CPA peaked within 10–20 min and then subsided: these were a prolongation of action potential duration (59 ± 12% increase) and an increase in amplitude of contraction (34 ± 11% increase) (Figure 8).

When expressed as maximal % change from control values observed in each preparation at any time from CPA administration, the latency between stimulus application and onset of action potential was decreased by 39 ± 11%, while action potential duration (at 90% of repolarization) and the amplitude of contraction were increased by 59 ± 12 and 34 ± 11%, respectively ($n = 5$).

CPA also transiently affected the contraction-relaxation cycle of the guinea-pig ureter: these changes peaked at 10–30 min from start of superfusion with CPA. At the peak of its action, CPA increased the duration of the cycle from 1.97 ± 0.1 to 3.1 ± 0.6 s (57% increase). The increase was relatively larger for the contraction time (CT) than for the relaxation time (RT): CT was increased from 0.87 ± 0.06 to 1.67 ± 0.4 s (92% increase), while RT was increased from 1.1 ± 0.03 to 1.57 ± 0.3 s (42% increase). Accordingly the CT/RT ratio was increased from 80 ± 5 to 111 ± 16% ($n = 5$ for each value).

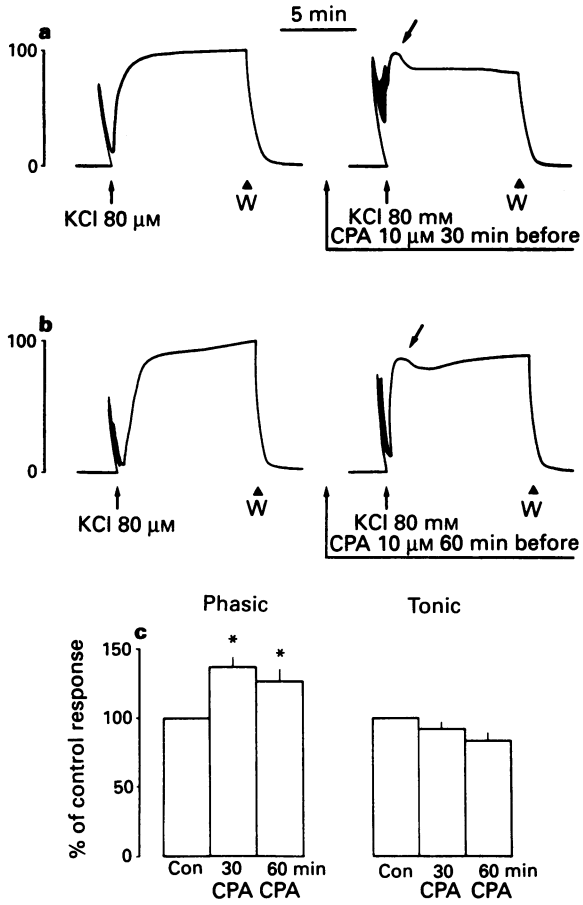

Figure 6 Effect of cyclopiazonic acid (CPA, 10μM) on the biphasic contractile response produced by 80 mM KCl in the guinea-pig isolated ureter: a and b show the effect of CPA at 30 or 60 min from its addition to the bath. Note that CPA enhanced and prolonged the phasic response to KCl and that a 'hump' (indicated by arrows) appears between the phasic and tonic response to KCl. (c) Effect of CPA on phasic and tonic responses to 80 mM KCl. Each value is mean ± s.e.mean of 7–8 experiments. *$P<0.05$ vs control. In panels (a) and (b) the vertical bars are the percentage of the response to KCl.

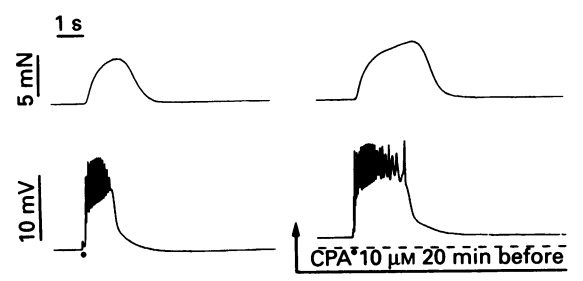

Figure 7 Effect of 10μM cyclopiazonic acid (CPA) on the action potential and contraction of the guinea-pig isolated ureter induced by EFS (applied at dots) recorded by sucrose gap in the presence of 1μM Bay K 8644. Lower tracing shows changes in membrane potential, upper tracing shows tension. Note that CPA depolarized the membrane, reduced the latency between stimulus application and onset of the action potential, prolonged the duration of the action potential and enhanced the amplitude and duration of the accompanying phasic contraction.

Effect of low-Na$^+$, choline-substituted Krebs solution on membrane potential A brief superfusion (3 min) with low-Na$^+$, choline-substituted Krebs solution produced membrane depolarization by 1.96 ± 0.2 mV ($n = 3$, Figure 9) which was reversible upon returning to normal Krebs solution. The repolarization was quite slow and it took >10 min after returning to normal Krebs solution for recovery of resting membrane potential. In 2 cases, the depolarization produced by low-Na$^+$, choline-substituted Krebs solution was accompanied by 1–2 action potentials and phasic contractions upon the first challenge, but this effect disappeared upon repeated applications of low-Na$^+$, choline-substituted Krebs solution to the same preparation.

Addition of Bay K 8644 (1μM) did not change the resting membrane potential but, in its presence, action potentials and phasic contractions were superimposed on the depolarization brought about by the low-Na$^+$, choline-substituted Krebs

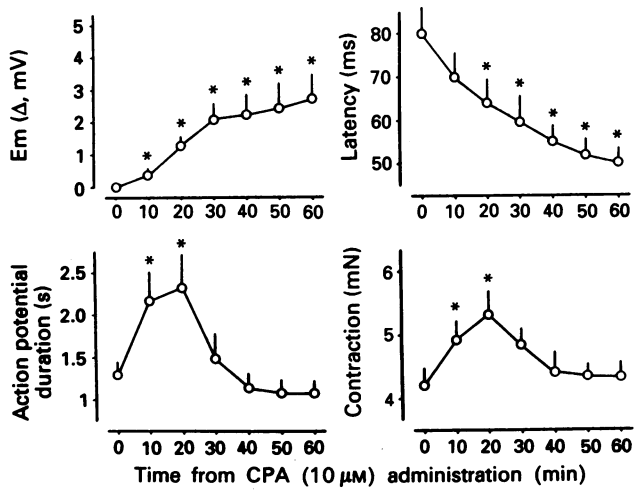

Figure 8 Time course of the effect of cyclopiazonic acid (CPA) on membrane potential (Em), latency between stimulus application and onset of the action potential, duration of the action potential and amplitude of contractions of the guinea-pig ureter in the presence of 1μM Bay K 8644. Each value is mean ± s.e.mean of 5 experiments. *$P<0.05$ vs. controls.

solution. Nifedipine (10 μM) did not affect the resting membrane potential and eliminated the effect of Bay K 8644. In the presence of nifedipine the depolarization observed in response to superfusion with the low-Na^+, choline-substituted Krebs solution averaged 1.48 ± 0.05 mV ($n = 3$).

Both in the absence and presence of nifedipine a further small depolarization was observed when replacing the low-Na^+, choline-substituted Krebs solution with normal Krebs solution (Figure 9).

Discussion

It is widely accepted that SR Ca^{2+}-ATPase plays a role in regulating intracellular Ca^{2+} homeostasis (Grover & Khan, 1992, for review), but the definition of its role in excitation-contraction coupling has been hampered by the lack of selective inhibitors. CPA has been characterized as a highly selective inhibitor of Ca^{2+}-ATPase in skeletal and smooth muscle

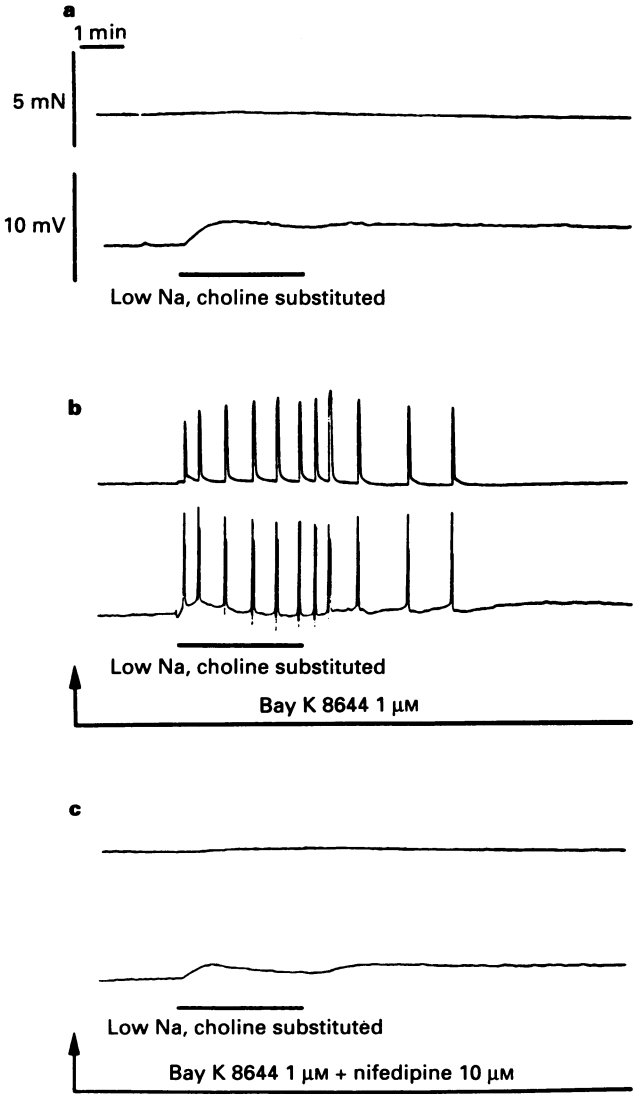

Figure 9 (a) Effect of a brief superfusion with a low-Na^+, choline-substituted Krebs solution on membrane potential (lower tracing in each panel) and tension (upper tracing in each panel) of the guinea-pig ureter recorded by sucrose gap. In the presence of Bay K 8644 (b), action potentials and phasic contraction superimposed on the depolarization induced by superfusion with low Na^+ medium; the effect of Bay K 8644 was abolished by nifedipine (c). Note that, in the absence and presence of nifedipine a small depolarization was evident when changing the bathing solution from low Na^+ to normal Krebs.

SR (Goeger & Riley, 1989; Seidler *et al.*, 1989; Deng & Kwan, 1991). CPA prevents the binding of Ca^{2+} to its high affinity site on SR and inhibits the Ca^{2+}-dependent phosphorylation of the SR proteins (Goeger & Riley, 1989). CPA selectively inhibits the ATP-dependent Ca^{2+} uptake by intracellular storage sites in skinned ileal smooth muscle (Uyama *et al.*, 1992) and blocks contractile responses related to Ca^{2+} mobilization from internal stores by preventing the refilling of the releasable Ca^{2+} pool (Low *et al.*, 1992; Uyama *et al.*, 1992; Bourreau *et al.*, 1993). Furthermore, CPA increases the excitability of smooth muscles and affects Ca^{2+} and K^+ currents (Suzuki *et al.*, 1992; Gagov *et al.*, 1993; Uyama *et al.*, 1993). The latter effects have been interpreted as secondary events to SR Ca^{2+}-pump inhibition by CPA.

The concentrations of CPA producing effects on electromechanical coupling of the guinea-pig ureter (1–30 μM) are comparable to those used in previous studies on other smooth muscles. Since Ca^{2+} movements into and from the internal store(s) were not directly monitored in this study, we cannot totally exclude the possibility that the effects of CPA may involve some as yet undiscovered action of this compound. The discussion of present findings is based on the assumption that CPA selectively affects SR Ca^{2+} ATPase in the guinea-pig ureter smooth muscle. This assumption is indirectly supported by the observation that many effects produced by CPA resemble those produced by ryanodine (Maggi *et al.*, 1994a). CPA and ryanodine affect SR function(s) by acting at different targets: hence, the effects produced by *both* drugs are likely to be consequent to the impairment of SR Ca^{2+} handling, while qualitative/quantitative differences may either reflect their different mode of action on SR Ca^{2+} handling or may involve nonspecific effects. The exact functional relationship(s) between the ryanodine receptor (CICR channel) and the CPA-sensitive Ca^{2+}-ATPase on SR are largely unsettled: replenishment of the SR Ca^{2+} store by Ca^{2+}-ATPase is obviously required for CICR. It may be speculated that the activity of the SR Ca^{2+}-ATPase is indirectly affected following blockade of the CICR ryanodine receptor. This may account for the observed nonadditivity of the effects of CPA and ryanodine (Figure 2, Table 1).

Effect of CPA on electromechanical coupling of the guinea-pig ureter

SR in smooth muscles can influence intracellular Ca^{2+} in two ways: (a) it can increase cytoplasmic Ca^{2+} by releasing Ca^{2+}, resulting in an initiation or augmentation of contraction; (b) it can decrease cytoplasmic Ca^{2+} by uptake system(s) which use ATP to pump Ca^{2+} into the store against a chemical gradient; the second effect can mediate or amplify the relaxation of smooth muscles. Agents which modify intracellular Ca^{2+} handling by the SR can thus be expected to produce either a reduction or an enhancement (or both effects) of the amplitude of evoked contractions, depending on whether the release or storage function of SR predominate. Furthermore, a number of membrane currents can be affected by altering SR Ca^{2+} handling, thereby influencing membrane potential and excitability (see below).

In previous studies, CPA has been used to block the refilling of the internal Ca^{2+} store: this produces a depletion upon consecutive challenges by agents which mobilize Ca^{2+} from the store, thereby eventually abolishing the response under investigation (e.g. Low *et al.*, 1992; Bourreau *et al.*, 1993). In the ureter, CPA did not reduce the amplitude of EFS-evoked contractions produced during repetitive cycles of stimulation, suggesting that release of Ca^{2+} from the internal store does not primarily contribute to the elevation of activator Ca^{2+} which determines electromechanical coupling. This observation is in keeping with our previous results with ryanodine (Maggi *et al.*, 1994a).

On the other hand CPA (and ryanodine) increased the amplitude of EFS-evoked phasic contractions of the ureter:

this effect was more pronounced when tested in the presence of Bay K 8644 but, for CPA, it was also observed without prior addition of the Ca^{2+} channel agonist. This effect was also evident on the phasic response of the ureter to KCl, which is accompanied by the firing of action potentials (Washizu, 1967; Johnishi & Sunano, 1978), and may be explained by assuming that SR Ca^{2+} ATPase plays a role in removing free intracellular Ca^{2+} entering via L-type Ca^{2+} channels activated by depolarization. Blockade of the Ca^{2+} pump would then lead to a more intense/prolonged elevation of free intracellular Ca^{2+} and account for the observed effects on contraction amplitude and duration of the contractile cycle. Clearly, other mechanisms, which can be operationally defined as CPA-insensitive, act to terminate the contractile cycle of the ureter in the presence of CPA; the plasma membrane Ca^{2+} ATPase and the Na^+/Ca^{2+} exchanger are likely candidates for this role (see below).

Both ryanodine (Maggi *et al.*, 1994a) and CPA produced a sustained depolarization of the membrane of the guinea-pig ureter: this effect might be consequent to the blockade of a small spontaneous release of Ca^{2+} from the SR, contributing to the resting membrane potential by activating K^+ channels in the vicinity of the SR. Suzuki *et al.* (1992) showed that 10 μM CPA strongly suppresses Ca^{2+}-dependent K^+-currents in guinea-pig smooth muscles. The prolongation of action potential duration observed with either CPA or ryanodine (Maggi *et al.*, 1994a) in the presence of Bay K 8644 remains unexplained at present. This effect is not consequent to membrane depolarization *per se*, since it is not reproduced by a mild elevation of extracellular K^+ (Maggi *et al.*, 1994a). Assuming that CPA elevates intracellular free Ca^{2+} during the action potential, as suggested by the increased developed tension and prolongation of the contractile cycle, it may be speculated that activation of some Ca^{2+}-dependent conductance(s) is involved in this effect.

An interesting feature of the effect of CPA on the response to KCl was the appearance of a 'hump' between the phasic and the sustained component of the contractile response: triphasic responses of the guinea-pig ureter to elevated extracellular K^+ concentration have been described in previous studies when using higher KCl concentrations than that used here (for e.g. Johnishi & Sunano, 1978). This effect may involve a faster development of KCl depolarization in the presence of CPA which depolarizes the membrane of its own.

Time course and fading of the action of CPA

Previous studies have outlined several aspects in the pharmacology of CPA on smooth muscles which have been confirmed here, including the reversibility of the effect of CPA by washing. In most of the previous studies, the effect of μM concentrations of CPA on smooth muscle excitation-contraction coupling have been studied following a relatively short contact time (e.g. 5–15 min, Suzuki *et al.*, 1992; Janssen & Sims, 1993). We found that some effects of CPA (e.g. depolarization of the membrane) are sustained or anyway develop in full over the 60 min observation period. Other effects, such as the enhancement of the amplitude of contractions showed a peak within 30 min from CPA administration and then faded. We exclude the possibility that this fading involves a degradation of added CPA since: (a) a second addition of CPA (10 μM) at 60 min from the first application in organ bath experiments did not enhance the amplitude of the EFS-evoked contraction (data not shown); (b) a similar biphasic time-course of action of CPA was observed during a prolonged superfusion in sucrose gap experiments. The reason why certain effects of CPA undergo fade is intriguing and may involve different mechanisms, discussed below.

Since CPA has been reported to induce nitric oxide (NO) release from endothelial cells, thereby inducing smooth muscle relaxation (Moritoki *et al.*, 1994), it may be hypothesized that a similar effect is involved in the time-dependent decay of the enhancement of the EFS-evoked contractions. This explanation seems unlikely, however, since we have observed a similar time-course of action of CPA when the ureters had been pre-exposed to the NO-synthase inhibitor, N^G-nitro-L-arginine (L-NOARG, 30 μM for 15 min) (data not shown). L-NOARG by itself does not affect the amplitude of the EFS-evoked contractions (Maggi *et al.*, 1994c) indicating that NO production does not play a major role in regulating the response of the guinea-pig ureter to EFS.

The decay of the action of CPA may theoretically involve a reduction of the Ca^{2+} current by elevated intracellular Ca^{2+} following blockade of the SR Ca^{2+} pump, i.e. a kind of Ca^{2+}-induced inactivation of the Ca^{2+} current: a similar effect has been described to be produced by CPA after the concomitant administration of ryanodine (Gagov *et al.*, 1993). However, neither the amplitude nor the rate of rise (data not shown) of the first spike of the action potential are modified by CPA. Since the present experiments were performed in the presence of Bay K 8644, which counteracts the inactivation of Ca^{2+} channels (Imaizumi *et al.*, 1989a) it is possible that inactivation of Ca^{2+} channels was partially prevented under our experimental conditions.

Third, other mechanisms for Ca^{2+} extrusion may come into action to compensate for the decreased or blocked Ca^{2+} ATPase function by CPA. These may involve the plasma membrane Ca^{2+} pump (Grover & Khan, 1992 for review) and/or the Na^+/Ca^{2+} exchanger, which may be important for Ca^{2+} extrusion and relaxation in guinea-pig ureter smooth muscle under certain circumstances (Aickin *et al.*, 1984; Aaronson & Benham, 1989). CPA does not affect the plasmalemmal Ca^{2+} ATPase (Seidler *et al.*, 1989), while no data are available about its possible effects on the Na^+/Ca^{2+} exchanger.

To address the possible involvement of a compensatory increase in activity of the Na^+/Ca^{2+} exchanger in the fading of the response to CPA, we studied the effect of a low-Na^+ Krebs solution in which extracellular Na^+ had been reduced by 60%. This approach has the limitation that direct effects of the substitute used to replace extracellular Na^+ also occur in parallel. For instance, Li^+ can abolish the response of the ureter to KCl, especially in its tonic component (Washizu *et al.*, 1967). To partially overcome this drawback, we studied two different substitutes for extracellular Na^+, Li^+ and choline: some of the observed effects were, at least qualitatively, observed with both media and may therefore represent a true consequence of Na^+ removal rather than nonspecific effects of the Na^+ substitutes.

We speculated that, if the Na^+/Ca^{2+} exchanger becomes more active after blockade of the CPA-sensitive Ca^{2+} pump, producing the fading of the effect of CPA on the amplitude of EFS-evoked contractions, a reduction of the transmembrane gradient to Na^+ would counteract the fading of the effect of CPA. The results of these experiments showed that the effect of CPA is maintained when tested in low-Na^+ medium. With low-Na^+, Li^+-substituted solution the peak effect of CPA was actually increased; however, the concomitant reduction of baseline amplitude of EFS-evoked contractions prevents a straight quantitative comparison of the data.

Another effect observed with both types of low-Na^+ media was the appearance of spontaneous contractions: this effect was more pronounced when using choline than Li^+ as Na^+-substitute. The spontaneous activity developing under these conditions was suppressed by nifedipine, indicating that L-type Ca^{2+} channels become activated in these conditions. Electrophysiological recordings showed that the depolarization produced by the low-Na^+, choline-substituted medium is the *primum movens* of this response which, in the presence of Bay K 8644, led to development of action potentials and contractions. A depolarization of the ureter smooth muscle upon reduction or total removal of extracellular Na^+ has been noted in previous studies in the guinea-pig ureter (e.g. Kuriyama & Tomita, 1970; Aickin *et al.*, 1984; Aickin, 1987).

Again, the results of these experiments are complicated by the possible direct membrane effects of the Na^+ substitute; furthermore, since endogenous calcitonin gene-related peptide (CGRP) produces hyperpolarization of the ureter smooth muscle (Santicioli & Maggi, 1994), the results of previous studies on this matter may have been influenced by the release of endogenous CGRP from afferent nerves. The nature of the nifedipine-resistant depolarization produced by the low-Na^+, choline-substituted Krebs solution in capsaicin-pretreated ureters needs further investigation.

The present results are in keeping with the hypothesis that the Na^+/Ca^{2+} exchanger becomes more active in removing intracellular free Ca^{2+} after blockade of the CPA-sensitive SR Ca^{2+} pump. Since the Na^+/Ca^{2+} exchanger operates with a 3/1 stoichiometry, this mechanism would be electrogenic and, accordingly, may also participate in the overall membrane depolarization produced by CPA. Clearly, an inhibitor of the Na^+/Ca^{2+} exchanger would be needed for the direct testing of this hypothesis. An abundance of mechanisms operating to lower free intracellular Ca^{2+} and terminate contraction may be expected to be present in the ureter smooth muscle, since any tonic type of contraction would oppose the physiological pattern of motility in this viscus, the phasic pumping of urine to the bladder.

In conclusion, the present findings demonstrate that CPA profoundly affects excitation-contraction coupling in the smooth muscle of the guinea-pig ureter. The striking similarities and the nonadditivity of the effects of CPA and ryanodine (Maggi *et al.*, 1994a) strongly suggest that the effects of CPA are ascribable to an interference of Ca^{2+} handling by the SR Ca^{2+}ATPase. Our present and previous (Maggi *et al.*, 1994a) findings strongly support the idea that SR Ca^{2+} handling is not involved in excitation-contraction coupling of the guinea-pig ureter, but plays an essential role in keeping intracellular Ca^{2+} low. The present findings also suggest that the Na^+/Ca^{2+} exchanger may be involved in regulating excitation-contraction coupling at this level, especially after blockade of the SR Ca^{2+} pump.

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