Contractile Properties of the Guinea Pig Vas Deferens

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Declaration

The work presented in this thesis was undertaken in the Department of Cell Physiology. This thesis is my composition and to the best of my knowledge it contains no material previously published or written by another person, except when due reference is made in the text of the thesis.

Kenichi Kato
January 1995

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Chapter 1

General Introduction

The central aim of this thesis is to investigate and characterize the contractile properties of the smooth muscle in the guinea pig vas deferens. It is known that regional differences in contractile responses to field stimulation or direct application of several neurotransmitters have been found to exist along the vas deferens. This thesis presents some of the mechanisms which contributed to the difference in; innervation, receptor types, channel populations and intracellular mechanisms. Since differences in innervation, receptor type and receptor distribution are well established, the role of intracellular calcium regulation in the regional contractile differences was investigated. Secondly, the effects of the well known second messenger, cyclic adenosine 5'-monophosphate (cAMP), thought to be a relaxant agent of smooth muscles, on the noradrenaline-induced contraction were investigated.

Smooth muscle cells, which are an important component of the walls of many tubular organs, assume not only the role of transporting, mixing or excreting the luminal contents, but also of supporting tissue. Generally, smooth muscles are innervated by autonomic nerves and regulated by neurotransmitters and co-released peptides. For example; acetylcholine (ACh), adrenaline, noradrenaline (NA), adenosine triphosphate (ATP), 5-hydroxytryptamine (5-HT), histamine, neuropeptide Y (NPY), somatostatin and vasoactive intestinal peptide (VIP) (Kupfermann, 1991). A number of receptor subtypes have been reported in smooth muscles or innervating nerve terminals.

In contrast to the neurotransmitters and receptors of smooth muscle, the basic mechanism of contraction is virtually the same amongst various mammalian smooth muscle tissues. It is generally thought that the trigger of muscle contraction is an increase in intracellular Ca^{2+} concentration, and that a decrease in the intracellular Ca^{2+} concentration results in relaxation (Ebashi, 1991). The dependency of contraction and relaxation on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is widely accepted. After the rise in $[Ca^{2+}]_i$, Ca^{2+} binds to specific proteins, troponin in striated muscle (Ebashi, 1964) and calmodulin in smooth muscle (Somlyo and Somlyo, 1994), which, by activation of myosin light-chain kinase (MLCK), phosphorylates the regulatory light chain of myosin (Somlyo & Somlyo, 1994). This active form of myosin increases the actin-activated Mg^{2+} ATPase activity of myosin, and interaction of myosin and actin resulting in contraction (Somlyo & Somlyo, 1994). A decrease in $[Ca^{2+}]_i$ inactivates MLCK, and the phosphorylated MLC is dephosphorylated by MLC phosphatase, consequently the actomyosin ATPase is deactivated, and then the muscle relaxes. Excitation-contraction (E-C) coupling of smooth muscle is principally mediated by $[Ca^{2+}]_i$ in this way.

Generally E-C coupling is controled by $[Ca^{2+}]_i$, however, Ca^{2+}-independent contraction and relaxation have been recently reported in the smooth muscle. These studies have been developed since the establishment of the direct measurement of $[Ca^{2+}]_i$ using fluorescent dyes (Grynkiewicz et al., 1985). Simultaneous measurements of both contractile responses and $[Ca^{2+}]_i$ reveal a non-linear correlation in certain phases of contraction. Carbachol evoked long-lasting contraction without $[Ca^{2+}]_i$ increase in guinea pig mesotubarium smooth muscle (Lydrup et al., 1992). In permeabilized smooth muscles, on the other hand, cyclic nucleotides elicited muscle relaxation even in the presence of levels of

intracellular Ca^{2+} sufficient to sustain contraction (Nishimura & Breemen, 1989). The $[Ca^{2+}]_i$-independent relaxation will be demonstrated in Chapters 3 and 4.

The vas deferens is a tubular organ the main role of which is the transport of spermatozoa and seminal emission from the testis to the urethra. This duct consists of an external areolar coat surrounding a muscular coat with an internal mucous coat lining the lumen. The thickest portion of the wall is the muscular coat arranged in three layers: outer and inner (often very thin) longitudinal layers with a circular layer sandwiched between them. The organization of the muscle layers is usually looser and less distinct in the epididymal end and around the ampullary parts of the organ in those species which have a distinct ampulla (Sjöstrand, 1981). Histological differences in the guinea pig vas deferens have been reported: the average ratios of the diameter of the epididymal, middle and prostatic portions were 1 : 1.7 : 1.6, respectively, and those of the total cross-sectional areas of the muscle layer were 1 : 3.4 : 3.0 (Sunano & Shimodan, 1981).

Hukovic (1961) introduced the isolated hypogastric nerve-vas deferens preparation as a model organ for mechanical studies of adrenergic neurotransmission. Since then, the vas deferens has been used widely in physiological and pharmacological experiments investigating the innervation by autonomic nerves, the co-transmission systems and the electrophysiological properties of smooth muscle cells in these tissues (Sneddon and Westfall, 1984; Allcorn et al., 1986; Avellar et al., 1993). These studies revealed that adenosine triphosphate (ATP) and noradrenaline (NA) are essential co-transmitters at the presynaptic terminal of the sympathetic neuron in the vas deferens. The roles of the two transmitters in the biphasic contractions evoked by field stimulation

are quite distinct; a phasic component mainly mediated by ATP with a tonic phase mediated by NA (Sneddon and Westfall, 1984; Allcorn et al., 1986). Regional contractile responses to field stimulation were investigated by McGrath (1978), and found to be biphasic in the epididymal portion and monophasic in the prostatic portion.

In summary, the contractile responses of the vas deferens to field stimulation show considerable regional differences. A large part of previous studies have been focused on the nerve innervation and neurotransmitter regulation at nerve terminals. The focus of this thesis is on the intracellular responses during stimulation with neurotransmitters and excess external K^+. In Chapter 2, the basic contractile character of the guinea pig vas deferens will be summarized in terms of experimental conditions, definition of receptor subtypes and extracellular Ca^{2+} dependency. In Chapter 3, the regional contractile difference in the vas deferens will be discussed. Finally, because the relxation mechanism is unknown in this organ, the relxation effects of cAMP-increase agents on NA-induced responses will be demonstrated in Chapter 4.

Chapter 2

Basic characterization of contractile properties of the guinea pig vas deferens

Introduction

The innate contractile properties of the guinea pig vas deferens are considered in this Chapter. As described in Chapter 1, the properties of smooth muscles vary from organ to organ. Therefore, it is important to obtain information on the basic characters of the preparations being used. The main purpose of this Chapter is to define the basic characters on which subsequent experiments on the guinea pig vas deferens will be based.

Experimental conditions are known to affect smooth muscle contractile responses induced by field stimulation and application of agonists application. Reduction of experimental temperature from 35 °C to 25 °C markedly alters (maximum responses observed 5 times larger) the contractile responses induced by field stimulation (Ambache & Zar, 1971). However, studies on the contractile responses or whole cell current responses to NA and ATP in single smooth muscle cells have routinely been conducted at room temperature (Nakazawa et al., 1987; Imaizumi et al., 1991). Species differences have been reported to exist in the contractile responses of vas deferens to ACh in rabbit, rat and guinea pig. ACh activates prostatic neural nicotinic receptors, and epididymal muscular muscarinic acetylcholine receptors thereby eliciting contraction of the rat and guinea pig vas deferens (Wakui & Fukushi, 1986; Carneiro & Markus, 1990). In the rabbit vas deferens, however, activation of M_1-muscarinic acetylcholine receptors inhibited neurogenic twitch contraction,

whereas activation of M_2-muscarinic acetylcholine receptors led to potentiation of the electrically induced twitch contractions (Eltze, 1988; Grimm et al., 1994).

This Chapter simply describes the basic properties of the guinea pig vas deferens, particularly the contractile responses to noradrenaline (NA), adenosine triphosphate (ATP), acetylcholine (ACh) and excess external K^+. The results of this Chapter will be helpful following experiments in subsequent Chapters.

Methods

Animals

Albino male guinea pigs were obtained from Japan SLC Inc. (Shizuoka) at a body weight of 250 to 300 g (4 weeks old). Guinea pigs were allowed unrestricted access to standard chow (CG-7; Nihon CLEA Co. Ltd.) and filtrated water without special diet and bred in a stainless cage. The day-night cycle was 12 hours. Daytime started at 8:00 hrs. and ended at 20:00 hrs. by automatic control of the light switch. Room temperature and humidity were controlled to 24 ± 2 °C and 50 ± 10 %, respectively (Ogiso, 1989).

Isolation of vas deferens

Guinea pigs (300 - 600 g; Slc: Hartley) were killed by a blow to the head and subsequently bled out. After laparotomy, both ends of the vas deferens were dissected as soon as possible. Connective tissues and adventitia of the vas deferens were removed carefully under a stereo-microscope (×10) in nominally Ca^{2+}-free solution (mM) : NaCl, 122.5;

KCl, 5.8; $MgCl_2$, 3.5; $NaHCO_3$, 5.8; glucose, 11.7 and HEPES, 5.8 (pH was adjusted to 7.4 with NaOH). The lumen of the vas deferens tube was also washed with the Ca^{2+}-free solution using a syringe. The vas deferens was then divided into three equal segments, epididymal, middle and prostatic portions from epididymis side (upper part) to prostatic side (lower part). These preparations were stored in refrigerator at 4 °C until use.

Perfusion system and experimental chamber

The experimental chamber used throughout these experiments is shown in Fig. 2-1. The temperature in the chamber was constantly regulated at 37 ± 0.5 °C with circulated warm water. The influx solution was also heated by passing the solution tube through the warm water. The bath was aerated with 95 % O_2- 5 % CO_2 all the time. Overflowing solution was removed by suction, and the bath volume was adjusted to 5 ml (Sazi & Ozaki, 1992). In some experiments, the temperature of bath solution was adjusted at 25 °C by mixing 4 °C solution and 37 °C solution.

Measurement of contraction

The preparations were suspended horizontally under 300 mg tension in an experimental bath filled with a physiological salt solution (PSS) that was prepared by replacing $MgCl_2$ (2.3 mM) in Ca^{2+}-free solution with equimolar $CaCl_2$, for at least 30 minutes prior to experiments. The bath was continuously perfused by a peristaltic pump at a rate of approximately 2 ml/min. The isotonic contractions were recorded by a transducer (Sanei 45347, Japan) connected to an amplifier (Sanei 1829, Japan), and sampled via a MacLab Interface (sampling rate of 4 Hz) to a Macintosh computer

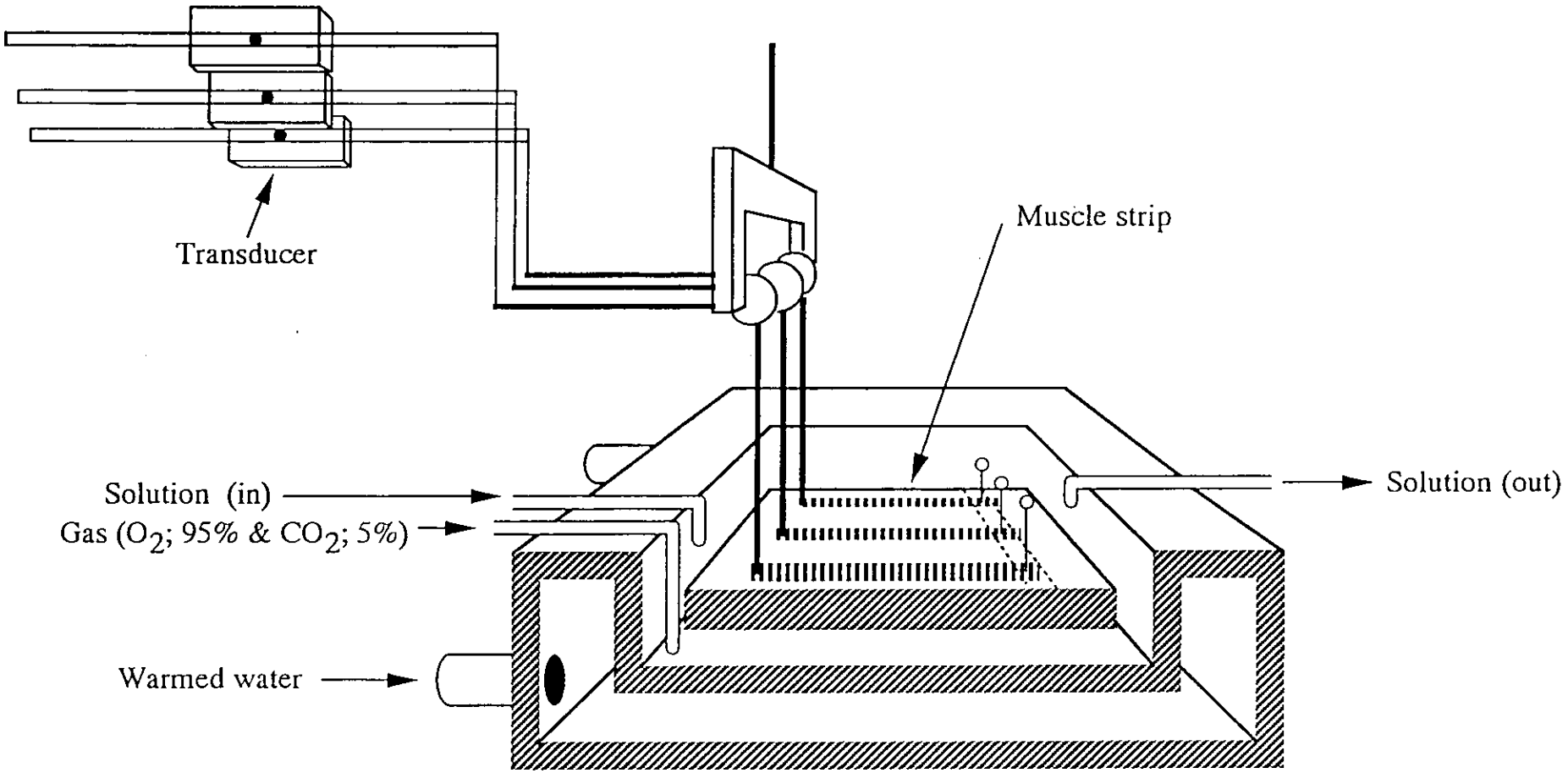


Fig. 2-1 Experimental acrylic chamber for measurement of contractile activity (5 ml).

(SE-30). Isotonic measurements were performed to observe the dynamic muscle contractile activity. The initial length of the preparations was adjusted to 10 ± 1 mm by using a prop to take up the slack in the thread by which the preparation was attached to the transducer. All drugs were applied to the bath directly, and bath perfusion was stopped during drug application (7 to 9 min). The maximum volume of applied drug solution was 50 μl. Homogenous distribution of the drugs applied in the bath was confirmed by dye diffusion. The preparations were washed by PSS for at least 20 min. In most muscles, contractile responses to NA were not noticeably reduced after repeated application and washout of the neurotransmitter 10 times. Experiments in which the final response to NA had decreased from the initial response by more than 20% were discarded. Desensitization of contractile responses to ATP and ACh were occasionally observed on repeated application of these neurotransmitters; these data were also omitted from the analysis.

Data storage and analysis

The MacLab program was used to analyze the data. All contractile responses, including concentration-response curves, were evaluated as % shortening of the initial length. Data are expressed as mean ± s.e. (n: number of observations) or mean alone. Statistical significance was evaluated by Student's two tailed paired and unpaired *t*-tests.

Drugs

Noradrenaline (NA), pirenzepine, prazosin and propranolol were obtained from Sigma Chemical Co (St. Louis, MO, USA). Acetylcholine (ACh), ATP and TTX were obtained from Wako (Osaka, Japan). Suramin

was obtained from Funakoshi (Tokyo, Japan). Metprolol was obtained from Fujisawa Co. (Osaka, Japan). AFDX116 (11[[2-[(diethylamino) methl]-1-piperidinyl]-acetyle]-5, 11-dihydro-6H-pyrido[2,3-b][1,4]benzo-diazepine-6-one) and p-FHHSiD (p-fluorohexa-hydrosiladifenidol) were supplied by Dr. A. Kuwahara (NIPS) who obtained them as a gift from Fujisawa Co.

Results

Effects of temperature on contractile responses

The influence of low temperature (25 °C) on the contractile responses to NA, ATP or ACh (each at 100 μM), and excess external K^+ (54 mM) is shown in Fig. 2-2. Control responses to these stimulants were obtained at 37 °C. Lowered bath temperature (25 °C) produced a marked potentiation and prolongation of the responses induced by neurotransmitters compared with the responses at 37 °C. The time to reach the initial transient peaks in response to all stimulants had a tendency to be shorter at 37 °C than at 25 °C.

As summarized in Fig. 2-3 the mean of the transient peaks of the responses of epididymal, middle and prostatic portions to NA were 1.2, 1.9 and 1.4 times larger, respectively, at 25 °C than at 37 °C , and those of the tonic phases (3 min after drug application) were 1.8 and 10.3 times larger in the middle and prostatic, but there was no temperature-dependent difference in the epididymal portion. The time to reach the initial transient peaks were 63.8 ± 11.2, 79.6 ± 11.3 and 85.4 ± 15.8 at 25 °C, and 49.1 ± 12.5, 53.1 ± 13.7 and 58.6 ± 8.5 (sec, n = 5) at 37 °C for epididymal, middle and prostatic portions, respectively.

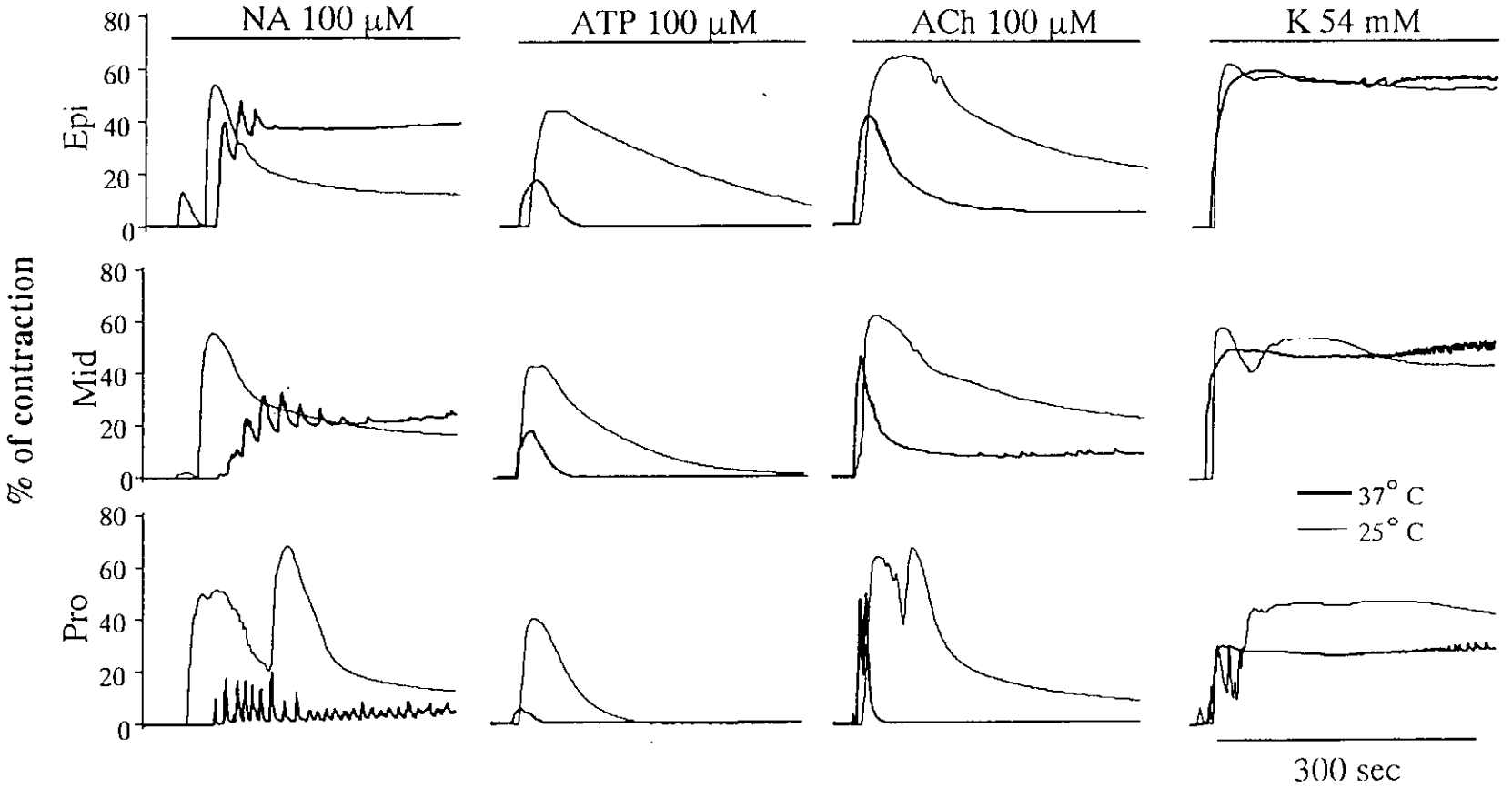


Fig. 2-2 Effects of low temperature on contractile responses. Representative responses to 54 mM K^+, NA, ATP and ACh (100 μM) at 25 °C (thin line) and 37 °C (thick line) in the epididymal (Epi), middle (Mid) and prostatic (Pro) portions are presented. Bars indicate the duration of exposure to each stimulation.

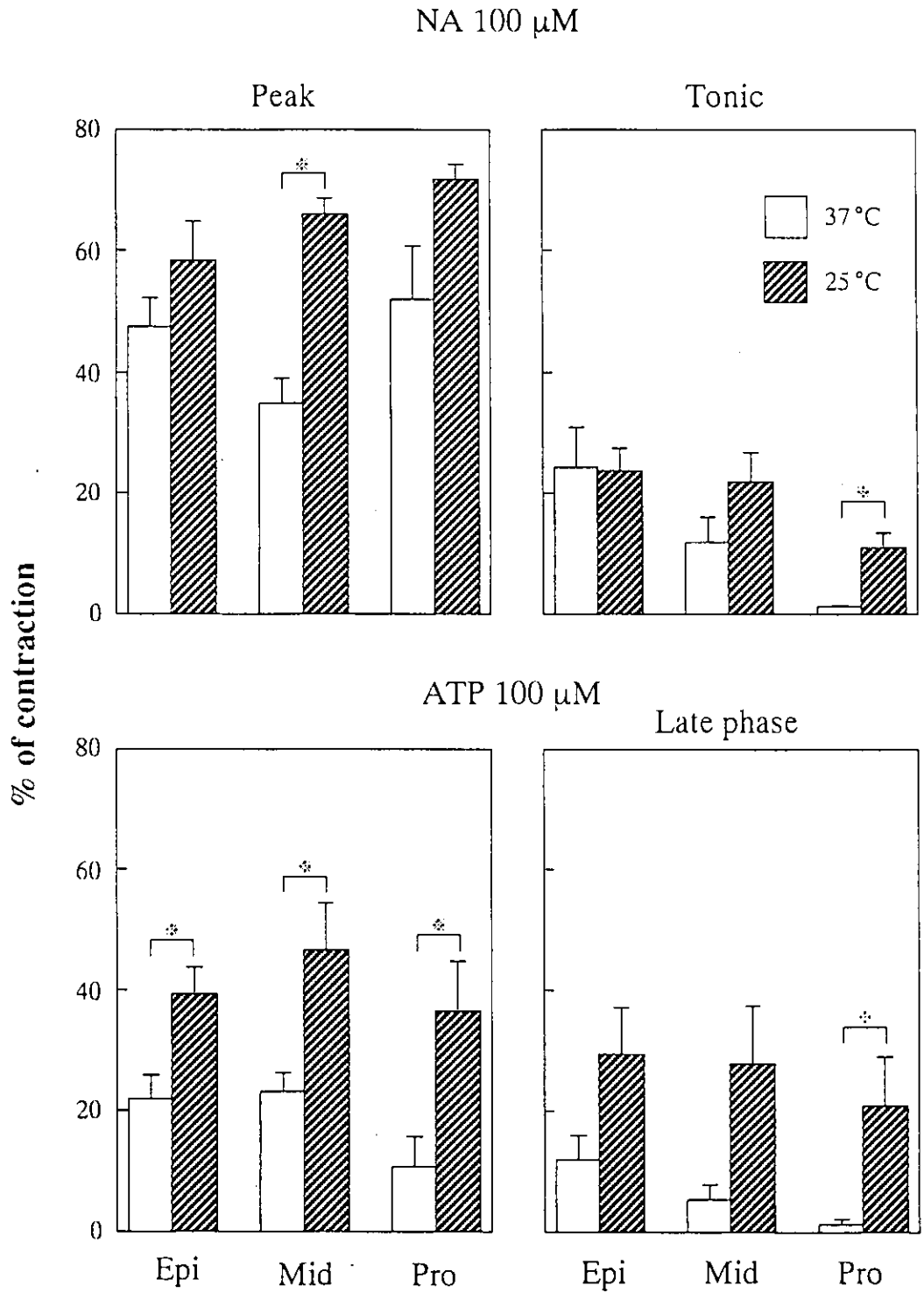


Fig. 2-3 Effects of temperature on the transient peak and tonic (3 min after application of NA) or late phase (at 30 sec after peak) of the responses to NA and ATP (100 μM). Open bars indicate mean values (mean ± s.e.) of the responses at 37 °C and shadowed bars at 25 °C. *$P < 0.05$, $n = 5$.

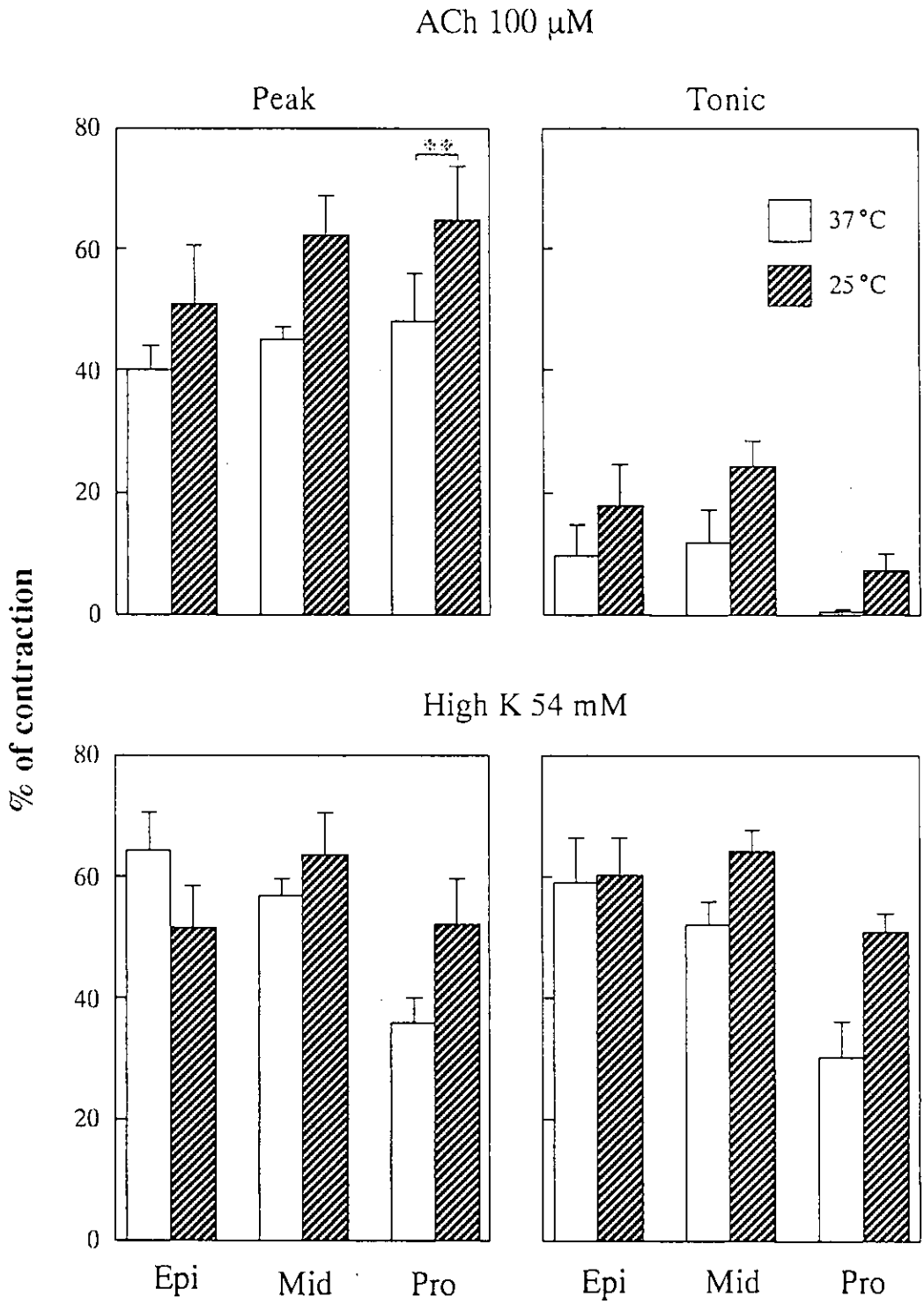


Fig. 2-4 Effects of temperature on transient peak and tonic (3 min after application) phase of the responses to ACh (100 μM) and excess external K^+ (54 mM). Open bars indicate mean values (mean ± s.e.) of the responses at 37 °C and shadowed bars at 25 °C. *P < 0.05, **P < 0.01, n = 5.

The cooling effect on the ATP-induced contractions was more marked (Figs 2-2, 2-3). The mean of the transient peaks were 1.8, 1.0 and 3.5 times larger at 25 °C than 37 °C, and the late phases (30 sec after initial peak) were 2.5, 5.3 and 17.2 times larger in epididymal, middle and prostatic portions, respectively. The time to initial transient peaks were 38.9 ± 5.4, 30.8 ± 4.1 and 28.5 ± 3.7 at 25 °C, and 26.3 ± 3.9, 21.6 ± 0.5 and 20.0 ± 2.9 (sec, n = 5) at 37 °C, respectively. The ACh-induced contraction was also affected by cooling in a similar manner (Figs. 2-2, 2-4). The mean of the transient peaks were 1.3, 1.9 and 1.4 times larger at 25 °C than 37 °C in epididymal, middle and prostatic portions, and in tonic phases 1.8, 2.0 and 17.2, respectively. The time to initial transient peaks were 94.8 ± 31.9, 72.9 ± 38.0 and 80.8 ± 41.1 at 25 °C, and 36.5 ± 12.5, 41.8 ± 20.7 and 46.4 ± 19.1 (sec, n = 5) at 37 °C.

The contractile responses to excess external K^+ showed little temperature dependency (Figs. 2-2 and 2-3). In the epididymal portion, the magnitude of the transient peak at 25 °C was 0.8 times as large as that at 37 °C, whereas the tonic phase was almost the same under both conditions. In the middle portion, the transient peak and tonic phase were slightly larger at 25 °C than 37 °C. In the prostatic portion, however, those both peak and tonic responses at 25 °C were 1.4 and 1.7 times larger than those of at 37 °C. The time to initial transient peaks were 43.6 ± 15.7, 36.0 ± 7.8 and 33.2 ± 2.3 at 25 °C, and 39.0 ± 10.4, 30.1 ± 4.2 and 26.2 ± 1.8 (sec, n = 5) at 37 °C for epididymal, middle and prostatic portions, respectively.

Effects of TTX on contractile responses to neurotransmitters

The contractile responses to each of the neurotransmitters were not affected by pre-treatment of the smooth muscle with 1 μM TTX (Table 2-

Table 2-1 Effects of TTX on the nurotransmitter-induced contractions

	Epididymal portion		Middle portion		Prostatic portion	
	peak	tonic or late p.	peak	tonic or late p.	peak	tonic or late p.
NA	55.0 ± 2.1	37.2 ± 4.7	49.2 ± 2.3	22.3 ± 3.3	46.1 ± 2.0	2.3 ± 0.7
with TTX	53.7 ± 1.9	35.3 ± 5.29	46.1 ± 2.0	21.4 ± 2.7	54.5 ± 1.7	0.9 ± 0.4
ATP	38.6 ± 2.6	9.9 ± 2.0	40.5 ± 2.2	1.3 ± 0.7	27.7 ± 0.8	0.1 ± 0.1
with TTX	40.0 ± 2.6	9.6 ± 2.3	41.3 ± 2.7	2.4 ± 1.2	27.9 ± 1.5	0.0 ± 0.0
ACh	48.9 ± 2.9	11.6 ± 3.1	47.3 ± 3.7	9.4 ± 2.5	38.0 ± 5.5	0.1 ± 0.1
with TTX	47.8 ± 2.7	9.6 ± 3.5	47.4 ± 4.5	7.6 ± 3.0	34.7 ± 6.0	0.0 ± 0.0

There is no statistical significance between control and TTX-pretreated muscle.

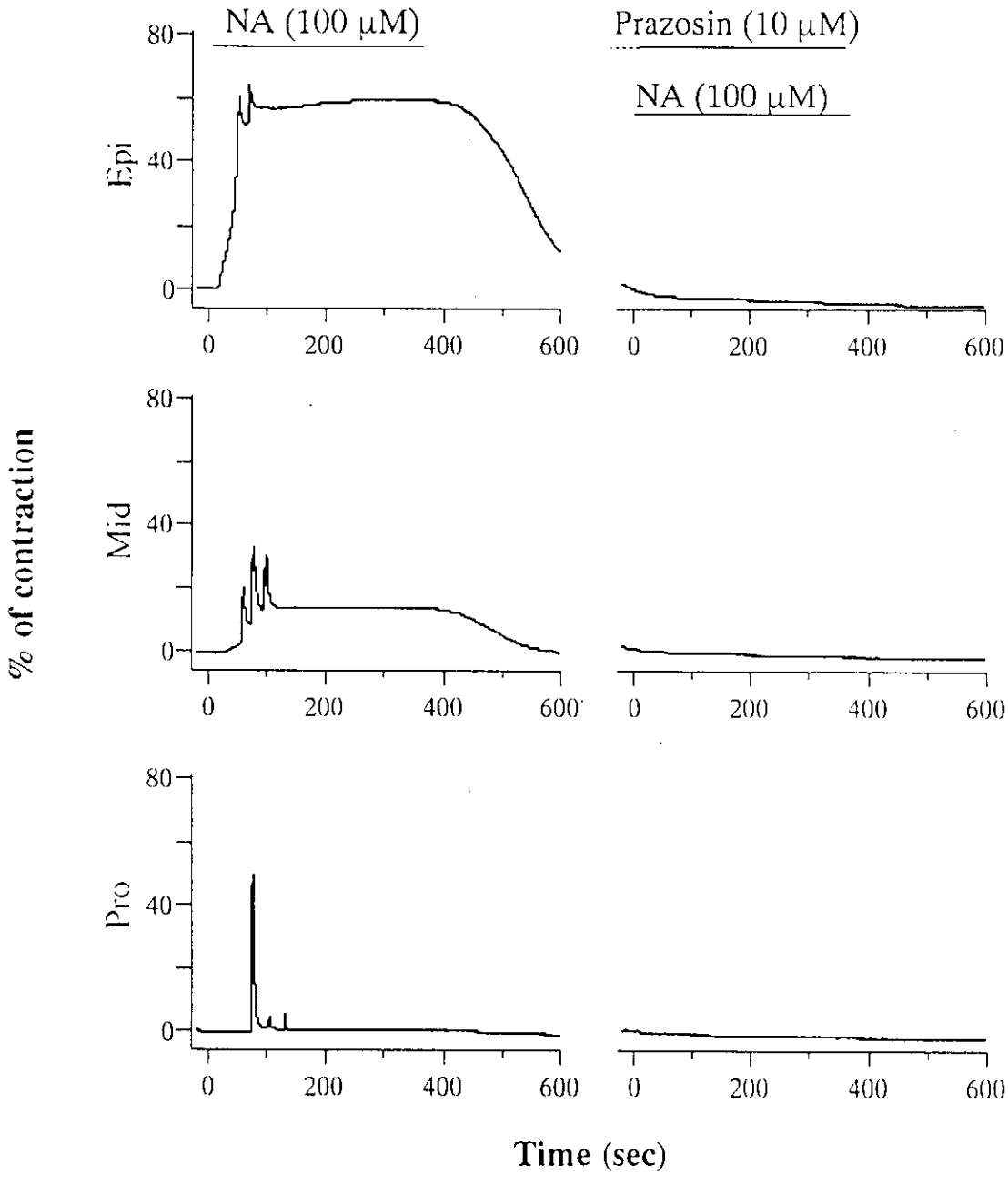


Fig. 2-5 Effects of prazosin (10 μM) on the contractile responses to NA. Control responses to NA (100 μM, left) were completely blocked by α_1-receptor antagonist prazosin (right). n = 3

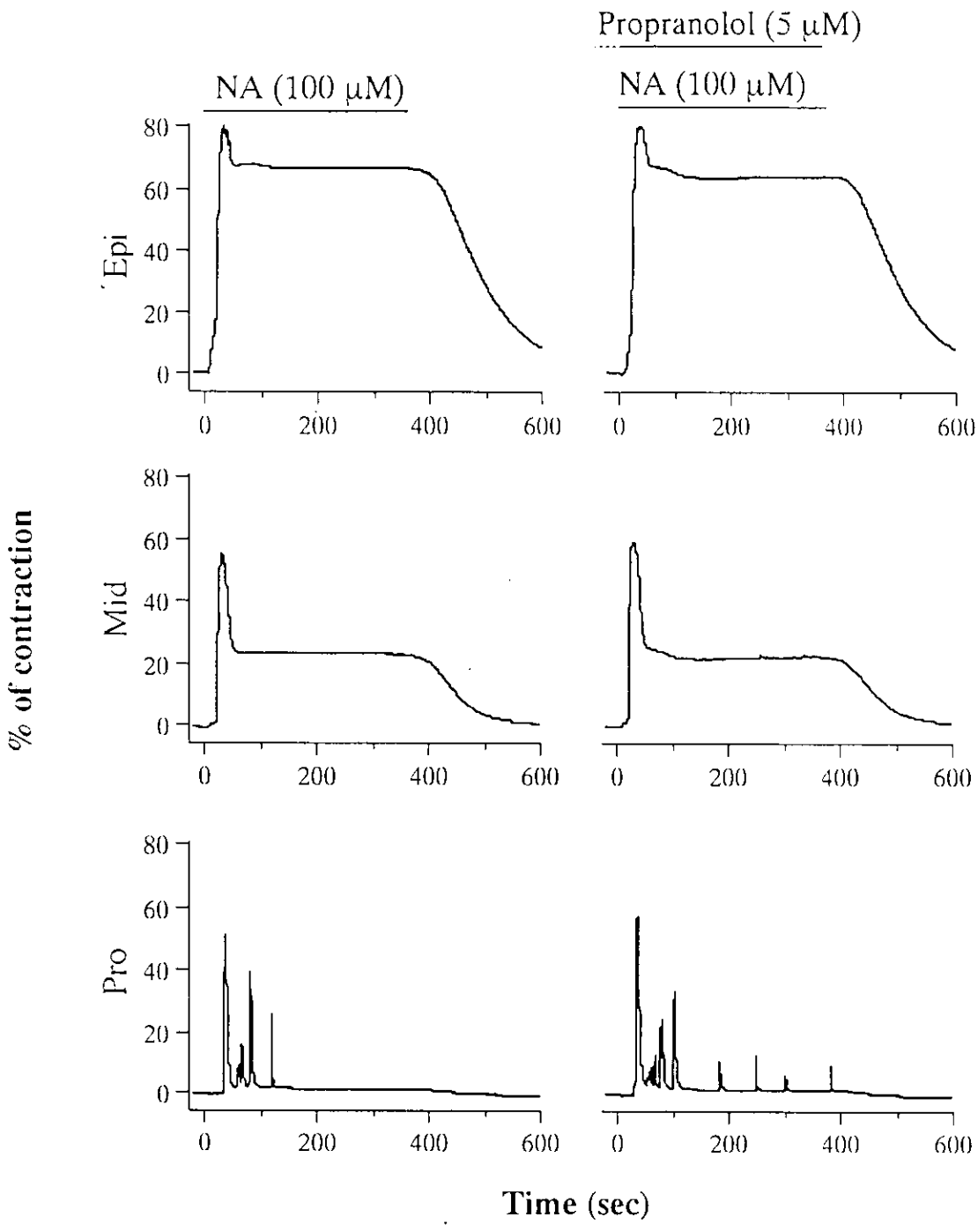


Fig. 2-6 Effects of propranolol (5 μM) on the contractile responses to NA. Control responses to NA (100 μM, left) were not affected by the β-receptor antagonist propranolol (right). n = 2

1), which had been found to completely block the contraction evoked by field stimulation to the nerve (data not shown).

Effects of α- and β- adrenoceptor antagonists on the contractile responses to noradrenaline (NA)

Adrenoceptors can be classified broadly into two types of receptors; α - and β-adrenoceptors. Alpha-adrenoceptors are further sub-classified into α_1- and α_2-adrenoceptors. In vas deferens, α_2-adrenoceptors exist in nerve terminal, whereas α_1-adrenoceptors exist in smooth muscle (see Chapter 1). The α_1- and β-adrenoceptors are completely different in characters from each other (Bülbring et al., 1981). Figure 2-5 shows the effect of the α_1-adrenoceptor antagonist prazosin (10 μM) on the contraction induced by NA (100 μM). In all three portions of the vas deferens, the control responses to NA were completely inhibited by the pretreatment with prazosin. Prazosin was irreversible. In contrast, yohimbin (10 μM) which exerts an antagonistic action against α_2-adrenoceptors at low concentrations was without effect on the NA-induced contraction (data not shown). The β-adrenoceptor antagonist, propranolol (5 μM), had no effect on the contractile responses to NA (Fig. 2-6). Another β-adrenoceptor antagonist, metoprolol (10 μM), also had no effect on the contraction (data not shown). Thus, it appears that the NA-induced response of the guinea pig vas deferens is due to activation of α_1-adrenoceptors.

Effects of P_2-purinergic receptor antagonists on the contractile responses to adenosine triphosphate (ATP)

Recently, P_2-purinergic receptors have been classified into 6 subtypes; P_{2X}, P_{2Y}, P_{2Z}, P_{2D}, P_{2T} and P_{2U} (Fredholm et al., 1994). The receptor

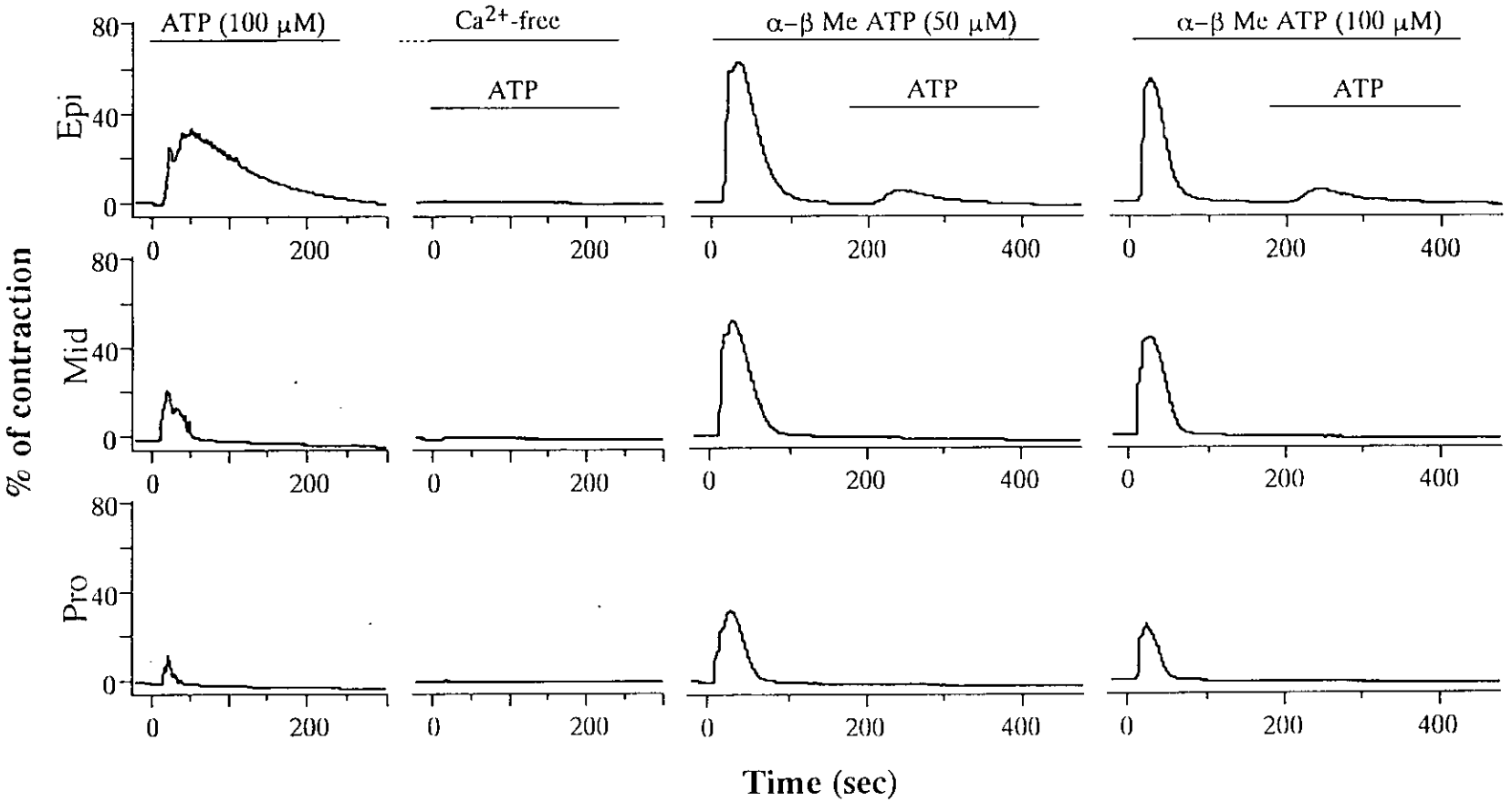


Fig. 2-7 Effects of α,β-methylene ATP (α,β-Me ATP) and contribution of external Ca^{2+} on the contractile responses to ATP (100 μM). Bars show the exposure time to ATP, Ca^{2+}-free (EGTA, 1 mM) and α,β-Me ATP (50 and 100 μM). n = 4

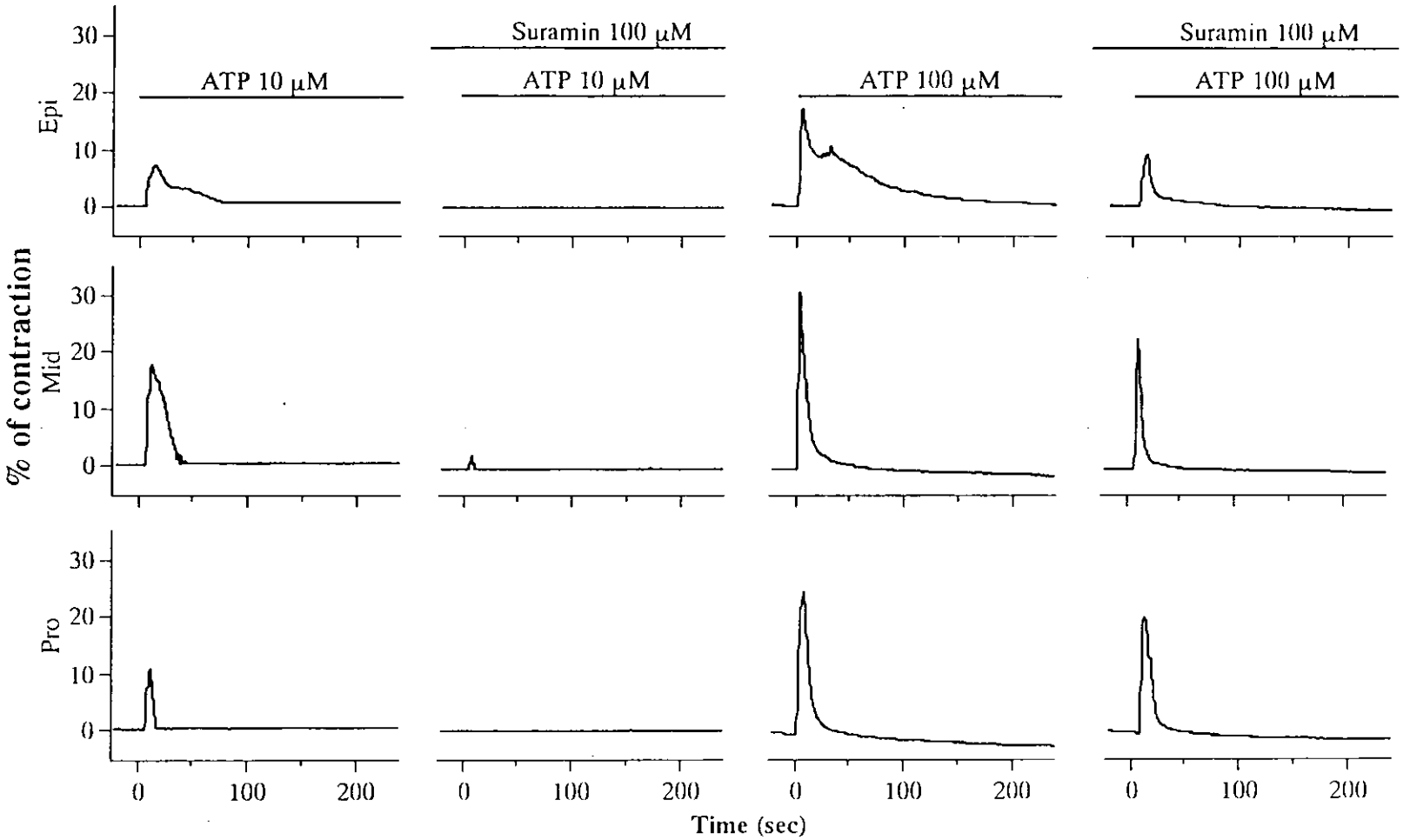


Fig. 2-8 Effects of suramin (100 μM) on the contractile responses to ATP (100 μM). n = 3.

subtype which mediates the main part of the ATP-induced contraction was investigated. The novel P_{2X} purinergic receptor specific agonist α,β-methylene ATP (α,β-Me ATP, 100 μM) stimulated the P_{2X} purinergic receptor and rapidly caused tachypylaxis presumably due to desensitization of the receptor. α,β-Me ATP also evoked contractile responses, and thereafter almost completely blocked the contractile responses to ATP in all three portions of 4 muscle strips out of 4 (Fig. 2-7). Blocking effect of α,β-Me ATP was reversible. In all portions of the vas deferens, suramin, a P_{2X} purinergic receptor antagonist (Brake et al., 1994), attenuated the ATP-induced contractions. Suramin (100 μM) almost completely abolished the contractile response to 10 μM ATP, but only partially inhibited that to 100 μM (Fig. 2-8). The reduction from control was 46.4 ± 24.5, 28.0 ± 11.9 and 28.5 ± 30.3% (n = 3), in the epididymal, middle and prostatic portions, respectively. Late phases (see Fig. 2-3) were inhibited by 94.9 ± 1.1% (n = 3) and were completely abolished in the epididymal and middle portions. Blocking effect of suramin was reversible. On the other hand, UTP (100 μM), which is known to be a agonist of the P_{2U} purinergic receptor, failed to evoke contraction. Thus, it appears that the ATP-induced response of the guinea pig vas deferens is predominantly due to activation of P_{2X}-purinergic receptor.

Effects of muscarinic acetylcholine receptor antagonists on the contractile responses to acetylcholine (ACh)

Four subtypes of muscarinic acetylcholine receptors (M_1 - M_4) have been reported. The subtype predominantly involved in the contractile responses to ACh in the guinea pig vas deferens was investigated. The non-specific muscarinic acetylcholine receptor antagonist, atropine

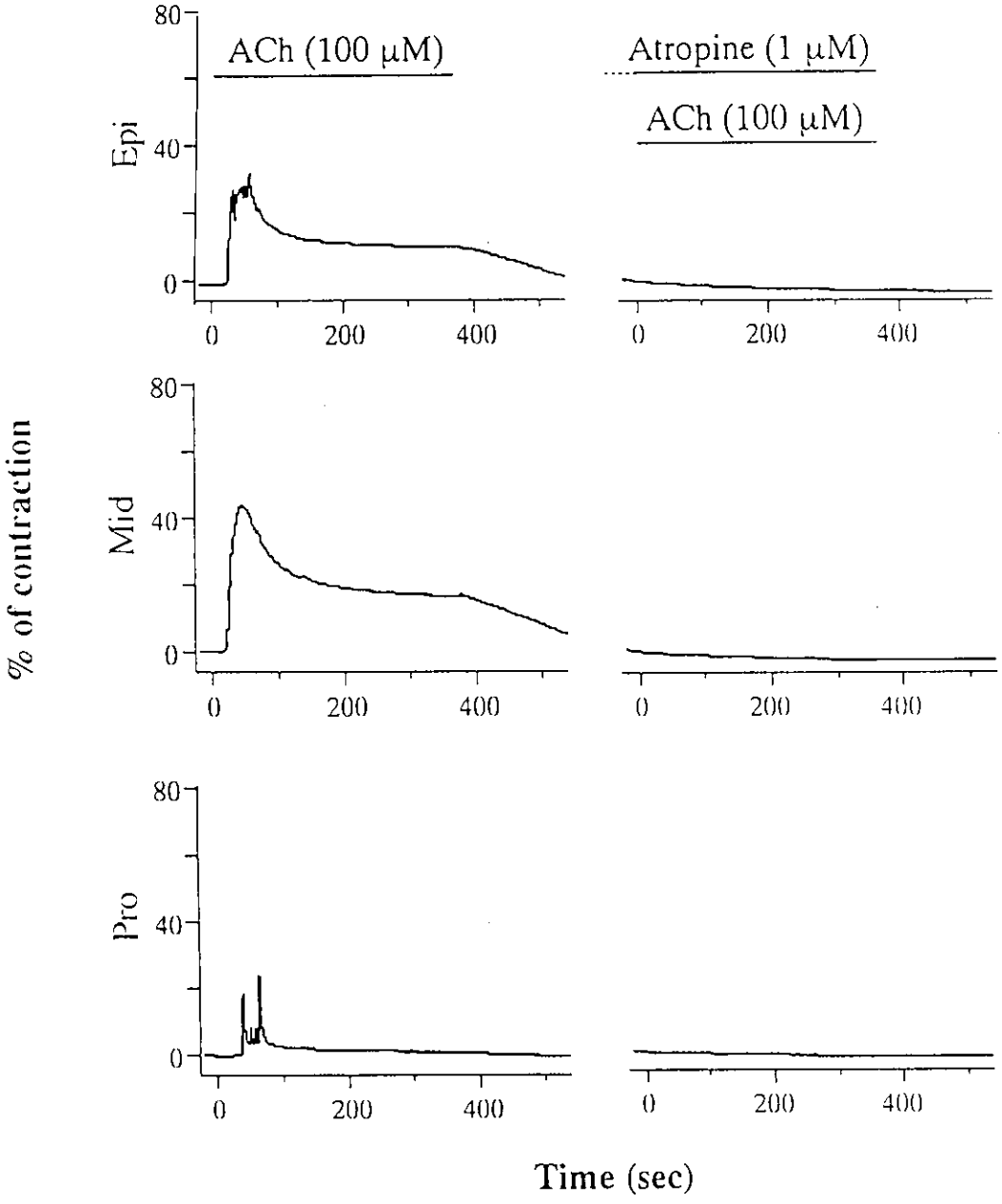


Fig. 2-9 Effects of atropine (1 μM) on the contractile responses to ACh (100 μM). Control responses to ACh (left) were completely blocked by non-selective muscarinic receptor antagonist atropine (right). n = 3.

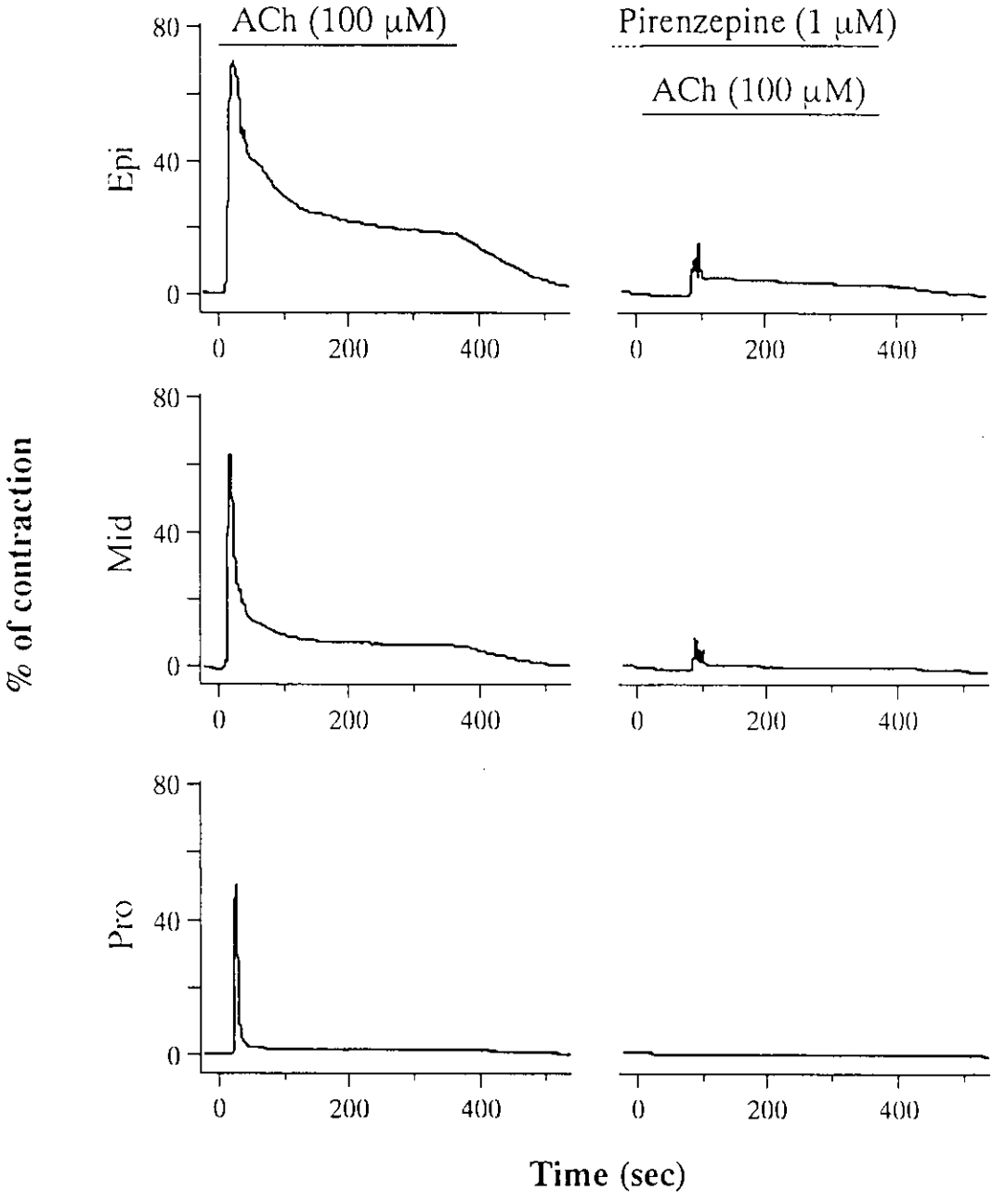


Fig. 2-10 Effects of pirenzepine (M_1-receptor antagonist, 1 μM) on the contractile responses to ACh (100 μM). Control responses to ACh (left) were partially blocked by the M_1-muscarinic receptor antagonist, pirenzepine (right). n = 2.

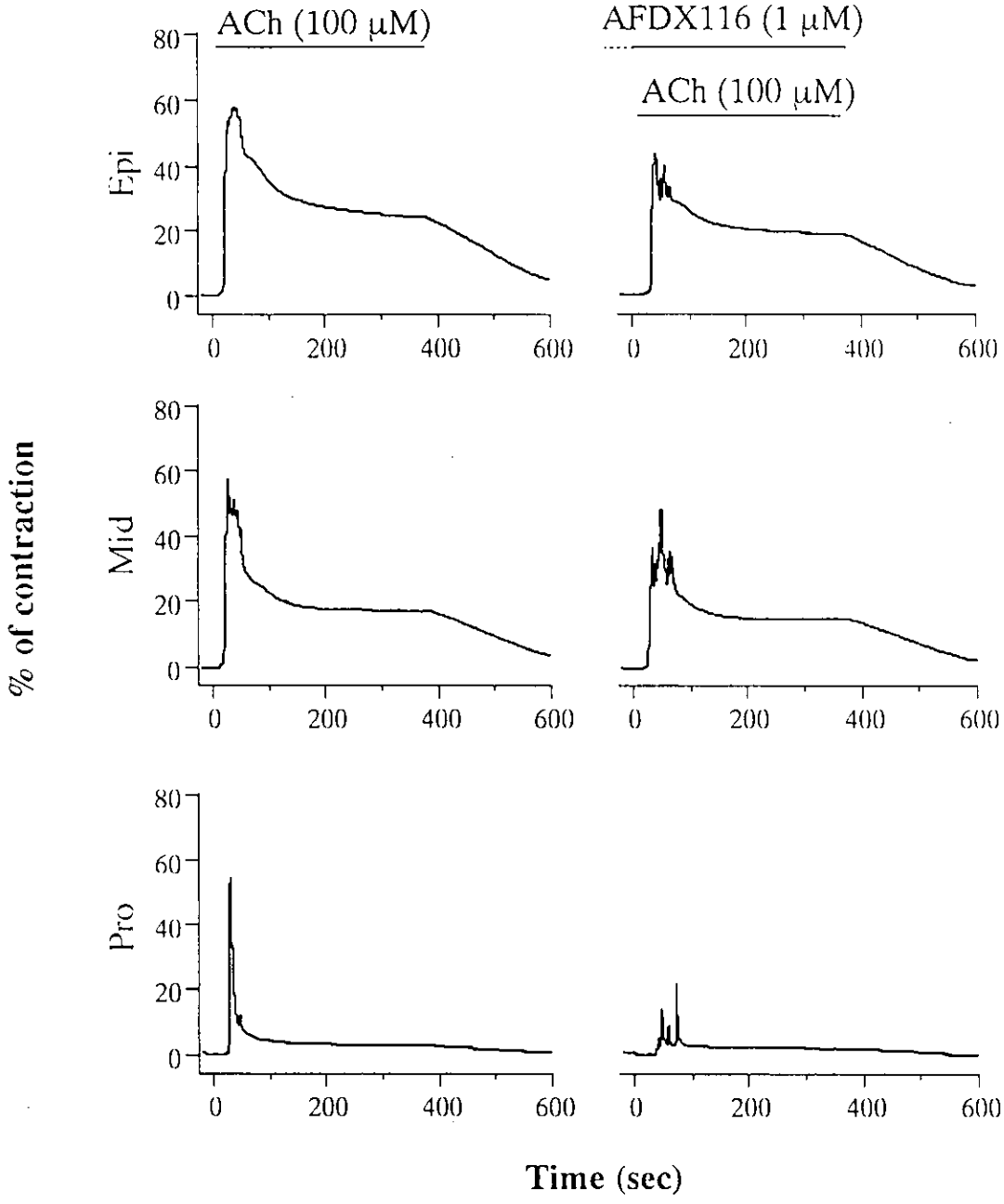


Fig. 2-11 Effects of AFDX116 (1 μM) on the contractile responses to ACh (100 μM). Control responses to ACh (left) were partially blocked by M_2-muscarinic receptor antagonist AFDX116 (right). n = 2.

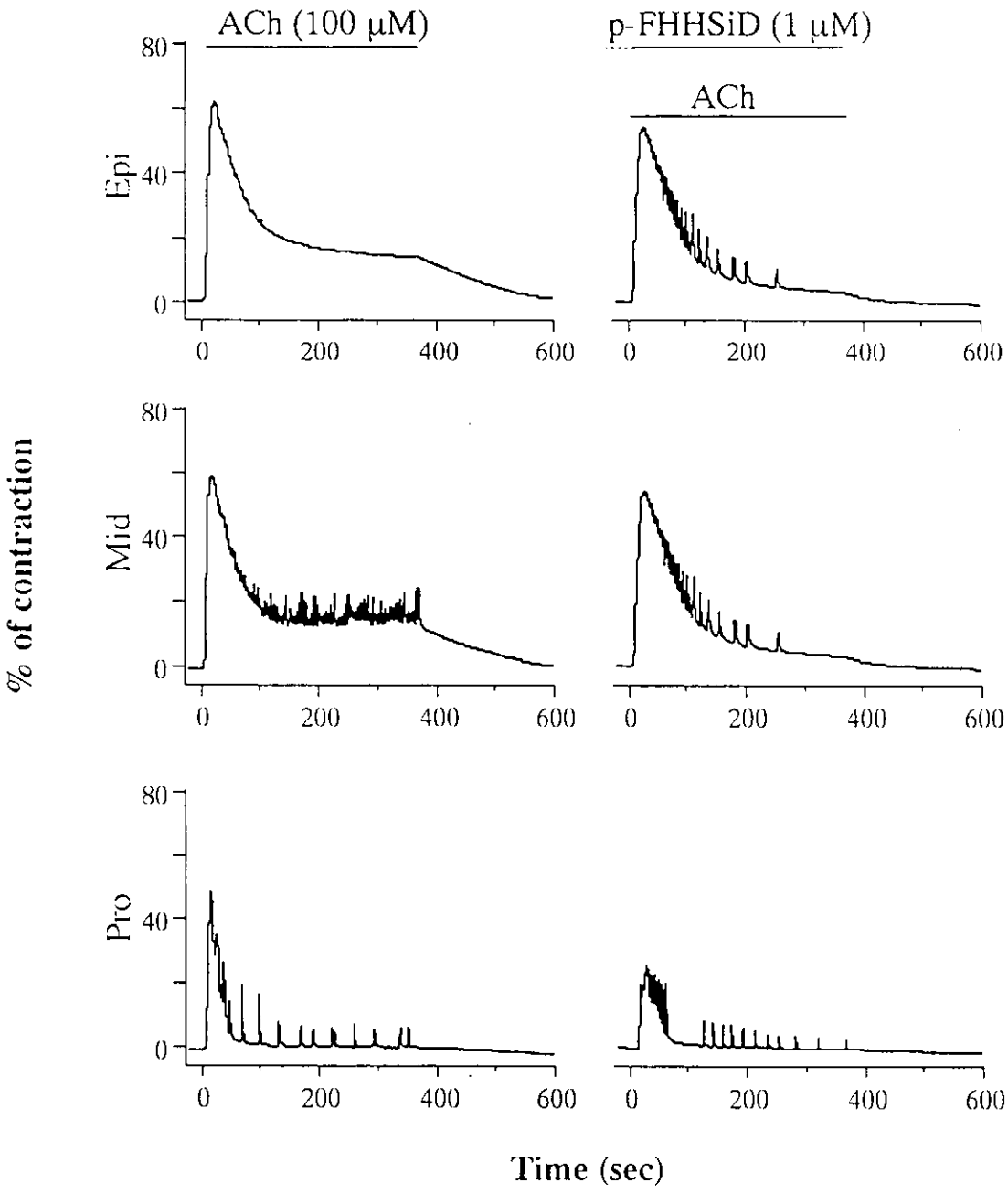


Fig. 2-12 Effects of p-FHHSiD (1 μM) on the contractile responses to ACh (100 μM). Control responses to ACh (left) were partially blocked by M_3-muscarinic receptor antagonist p-FHHSiD (right). n = 3.

(1 μM), completely inhibited the contractile responses to ACh (100 μM, Fig. 2-9), and this effect was irreversible. The M_1-muscarinic acetylcholine receptor antagonist, pirenzepine (1 μM), partially inhibited the contraction in the epididymal and middle, and completely blocked in the prostatic portions (Fig. 2-10), and effect was partially reversible. The magnitude of the transient peak was reduced from control by 89.0, 76.8 and 100% (n = 2), in the epididymal, middle and prostatic portions, respectively. The tonic phases were reduced from control by 79.6, 90.3 and 100% (n = 2). The M_2-muscarinic acetylcholine receptor antagonist, AFDX116 (1 μM), had a very small effect on the contraction (Fig. 2-11), and this effect was reversible. The magnitude of transient peaks were reduced from control by 24.8, 15.6 and 74.0% (n = 2) in the epididymal, middle and prostatic portions, respectively. The tonic phases were reduced from control only by 26.6, 7.9 and 42.1%, respectively (n = 2). Finally, the M_3-muscarinic acetylcholine receptor antagonist p-FHHSiD, 1 μM) was almost without effect on the contraction (Fig. 2-12). The transient peaks were reduced from control by 13.2 ± 1.0, 13.8 ± 1.9 and 72.5 ± 12.0% (n = 3), in the epididymal, middle and prostatic portions, respectively. The tonic phases were reduced from control by 54.4 ± 12.4, 36.3 ± 16.2 and 91.3 ± 5.5% (n = 3). Because the antagonists of each subtype are not entirely specific, and because the contribution of the M_4 subtype to contraction was not investigated, it is not possible to define unequivocally which receptor subtypes contributed to the ACh-mediated contraction of the guinea pig vas deferens. However, it can be concluded that M_1-muscarinic acetylcholine receptor is predominant in mediating the response.

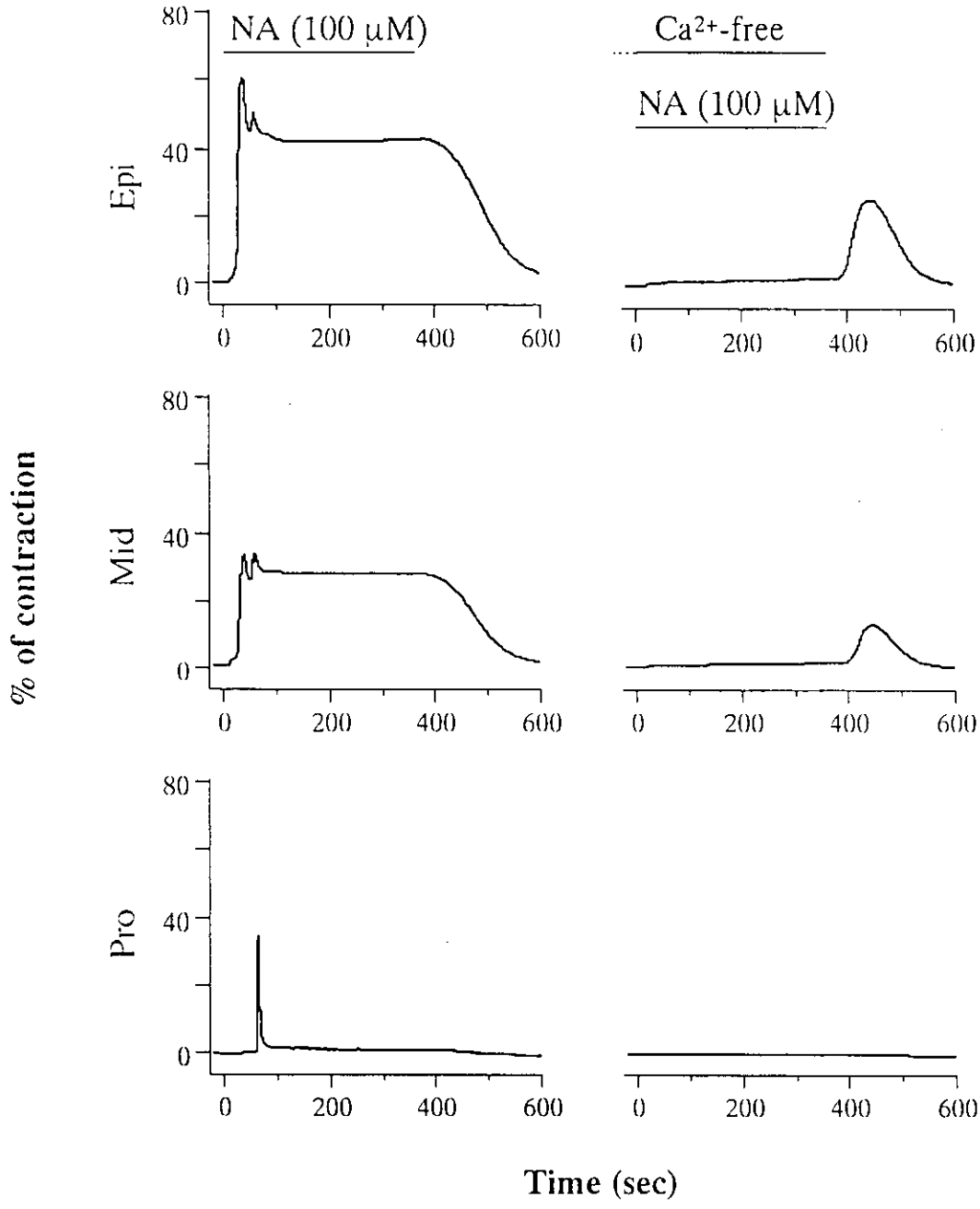


Fig. 2-13 Contribution of external Ca^{2+} to the contractile responses to NA (100 μM). Traces on the left show control responses to NA in the PSS, and traces on the right show responses in Ca^{2+}-free (EGTA 1 mM) PSS. PSS was changed to Ca^{2+}-free PSS 3 min before application of NA to the bath.

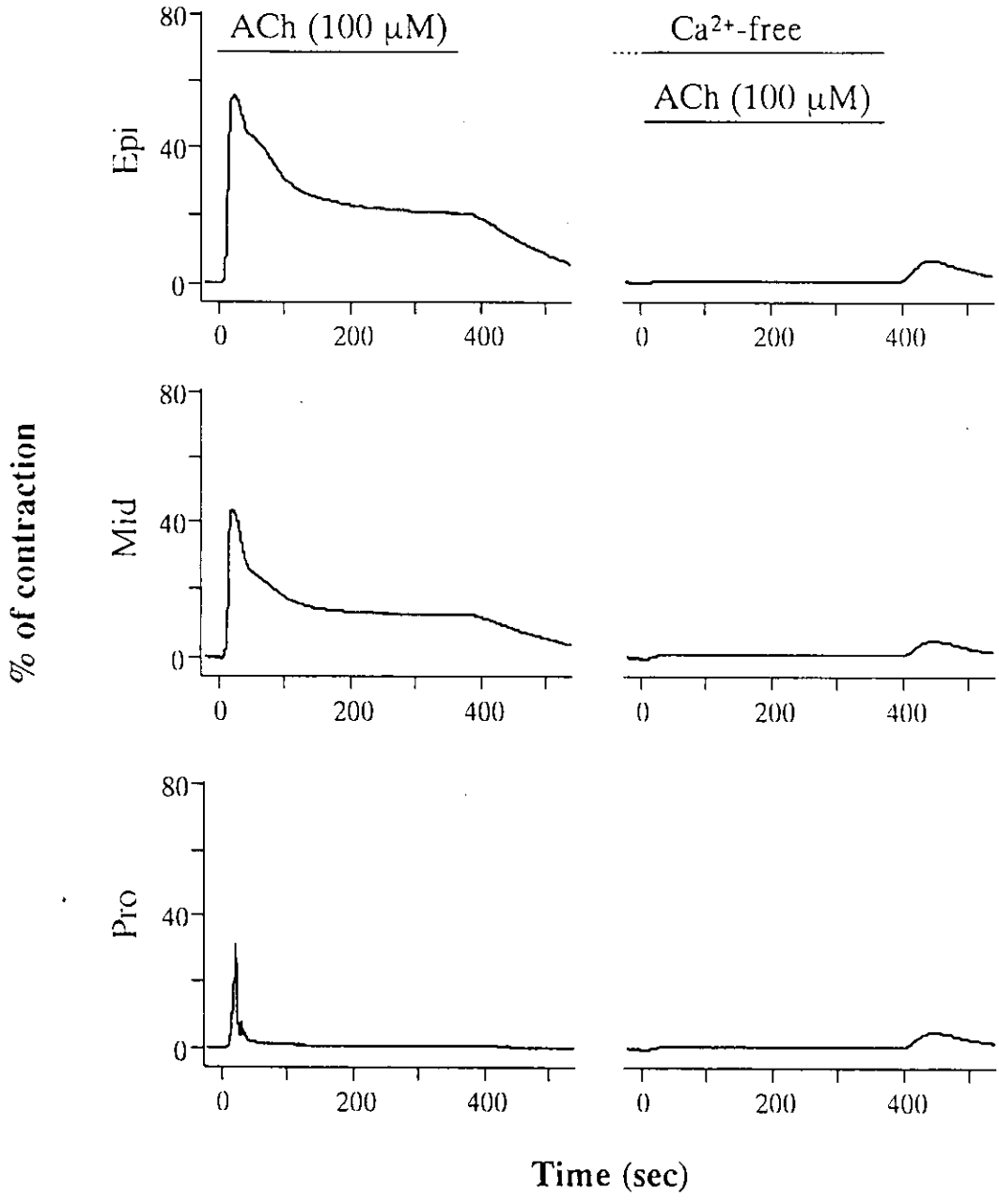


Fig. 2-14 Contribution of external Ca^{2+} to the contractile responses to ACh (100 μM). Left traces show control responses to ACh in the PSS, and right traces show responses in Ca^{2+}-free (EGTA 1 mM) PSS. PSS was changed to Ca^{2+}-free PSS 3 min before application of ACH to the bath.

Effects of deprivation of external Ca^{2+} on the contractile responses to neurotransmitters

An increase in the intracellular free Ca^{2+} ($[Ca^{2+}]_i$) is known to be the principal mechanism initiating smooth muscle contraction (Somlyo & Somlyo, 1994). Both Ca^{2+} influx from the extracellular space and Ca^{2+} release from intracellular Ca^{2+} stores contribute to the increases in $[Ca^{2+}]_i$. The bath was continuously perfused with PSS and then with Ca^{2+}-free PSS containing 1 mM EGTA for at least 3 min before administration of neurotransmitters. The contraction induced by NA (100 μM) was found to almost disappear from all portions in the Ca^{2+}-free PSS (Fig. 2-13). Immediately after changing the bath solution from Ca^{2+}-free PSS to normal PSS, contractile responses occurred in the epididymal and middle portions. As shown in Fig. 2-14, the ACh-induced responses disappeared in Ca^{2+}-free PSS. Reduced contractions were evoked in all portions upon restoring external Ca^{2+}. Similar Ca^{2+} dependency was also observed for the contractile responses induced by ATP (Fig. 2-7: second traces). Therefore, it appears that the extracellular Ca^{2+} is a prerequisite to the contractile responses to NA, ACh and ATP in vas deferens of the guinea pig.

Effects of nifedipine and deprivation of external Ca^{2+} on the contractile responses to excess external K^+

It is well known that excess external K^+ causes membrane depolarization, eliciting increased Ca^{2+} influx in turn leading to contraction. An increase in external K^+ up to 54 mM caused contractile responses among all portions, as shown in Fig. 2-15. This depolarization-induced Ca^{2+} influx is thought to be through voltage-dependent Ca^{2+} channels. Nifedipine was used to define the L-type Ca^{2+} channels activity

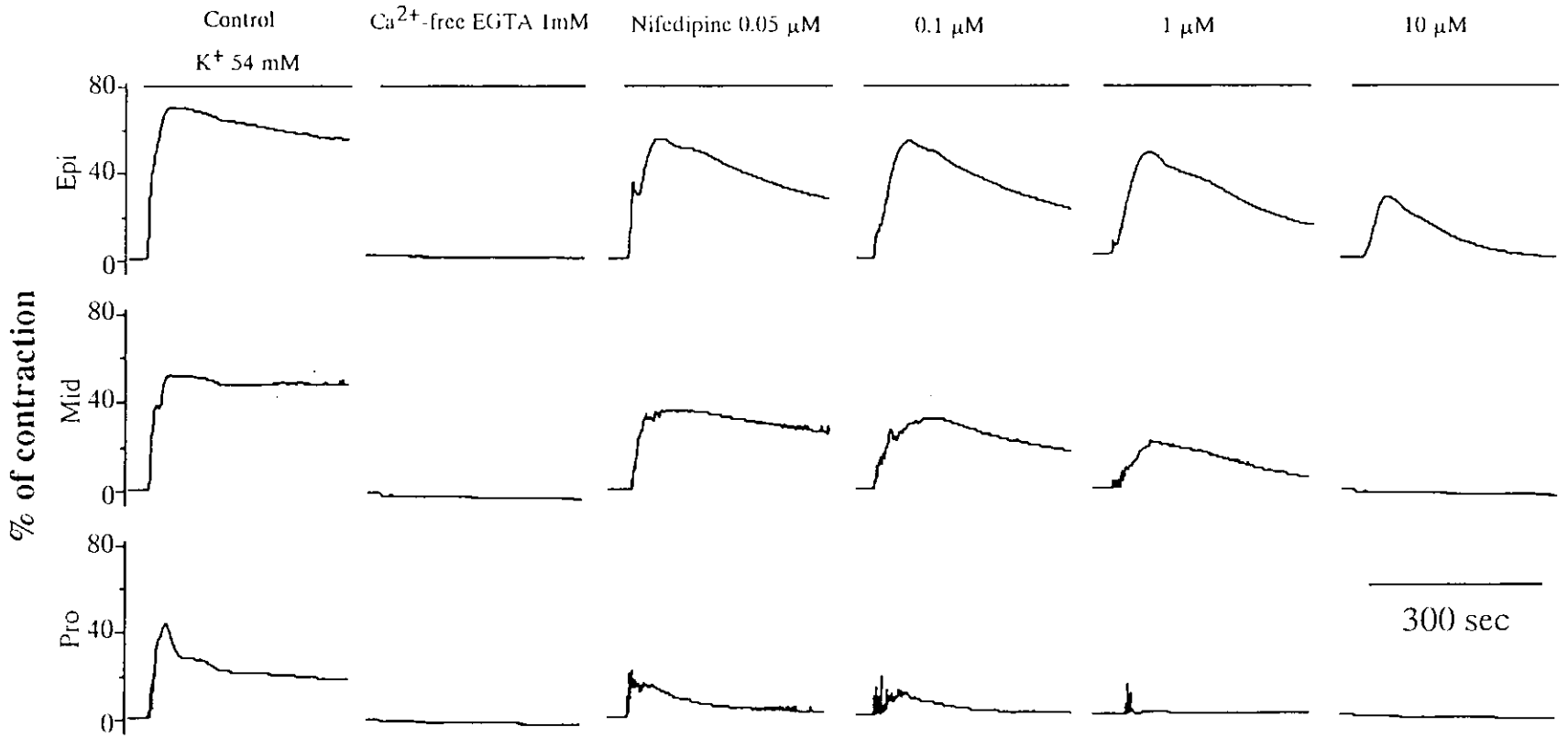


Fig. 2-15 Effects of removing external Ca^{2+} and of nifedipine on excess external K^+-induced contraction. The control contraction induced by excess external K^+ (54 mM) disappeared in Ca^{2+}-free PSS (EGTA 1 mM). Nifedipine was applied to the bath 3 min before external K^+ stimulation. Sensitivity to nifedipine was greater in the prostatic than epididymal portions. n = 2.

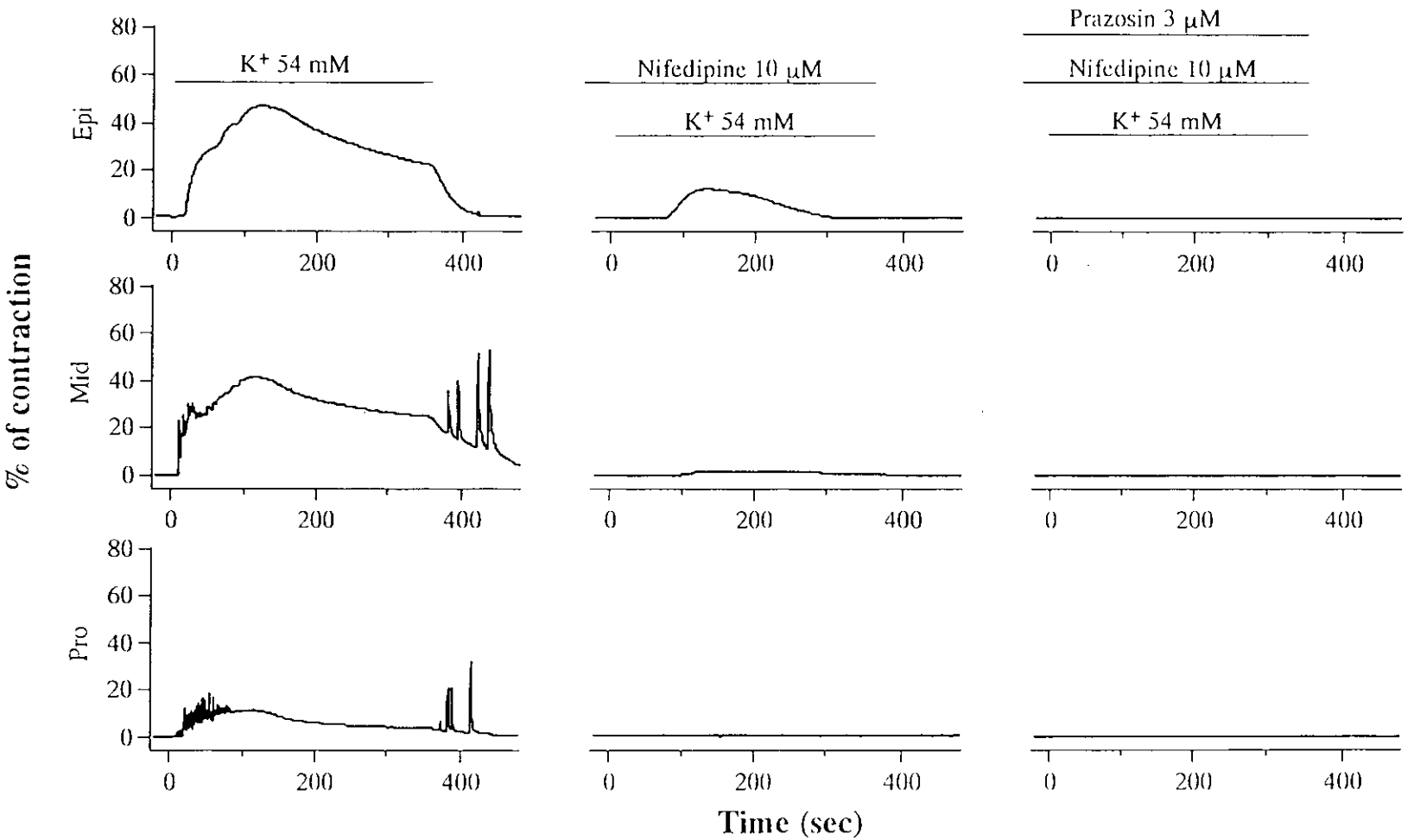


Fig. 2-16 Effects of prazosin (3 μM) on the nifedipine-insensitive contraction. Nifedipine and prazosin were applied to the bath 3 min before excess K^+ stimulation. Nifedipine-insensitive contractions (middle trace in epididymal) were blocked by prazosin (right trace). n=3.

in contractile responses to the stimulation of external K^+. The contractile responses induced by excess K^+ were inhibited by nifedipine in a concentration dependent manner (Fig. 2-15). The sensitivity to nifedipine was higher in the prostatic than in the middle and epididymal portions. The epididymal portion was least sensitive to nifedipine; concentrations of nifedipine as high as 10 μM failed to block the excess K^+-induced contraction completely. The nifedipine-insensitive contraction could be blocked by prazosin (3 μM, Fig. 2-16).

Effects of nifedipine on the contractile responses to NA, ATP and ACh

The nifedipine-insensitive contraction (Fig. 2-16) implies that the contraction induced by neurotransmitters may also be insensitive to nifedipine. It was shown above that extracellular Ca^{2+} is necessary for the NA-, ATP- and ACh-induced contractions. The contribution of the L-type Ca^{2+} channel to the neurotransmitter-induced contraction was investigated. The transient of the NA-induced contraction was suppressed by nifedipine (10 μM) in the epididymal and prostatic portions, whereas the tonic contraction was resistant to nifedipine in the epididymal and middle portions (Fig. 2-17a). The relative insensitivity to nifedipine was reflected in the average of inhibition (Fig. 2-7b). In contrast, the ATP-induced contractions were almost completely inhibited by nifedipine (Fig. 2-18a,b). The sensitivity to nifedipine of the ACh-induced responses was similar to the NA-induced responses. Transient contractions were sensitive to nifedipine in all portions, but the tonic phases were resistant to nifedipine in the epididymal and middle portions (Fig. 2-19a,b). These data suggest that nifedipine-insensitive Ca^{2+} influx is predominantly involved in NA- and ACh-induced contraction, whereas nifedipine-

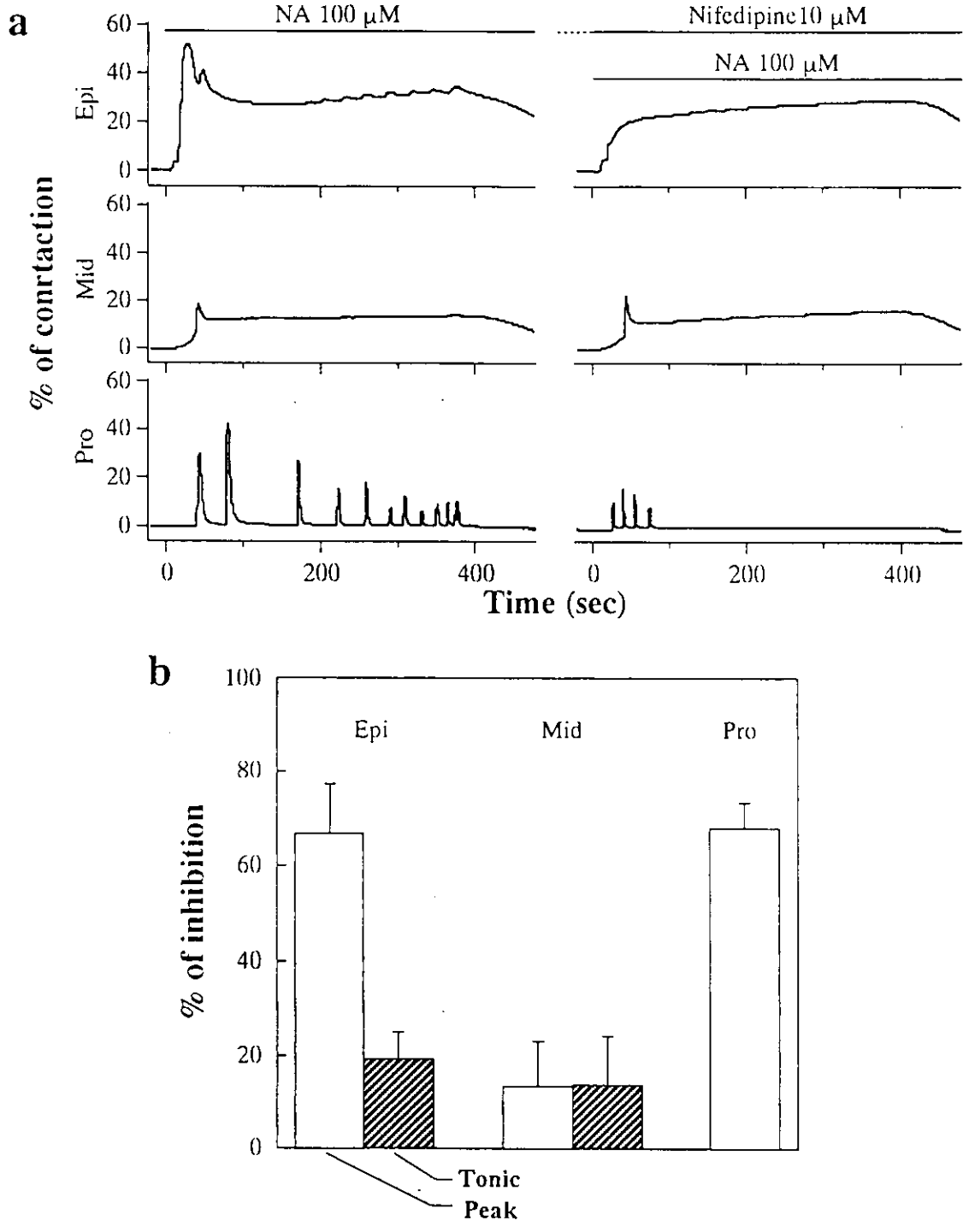


Fig. 2-17 Effects of nifedipine (10 μM) on NA-induced contractions. a, Control responses to NA 100 μM (left) were attenuated by pretreatment with nifedipine (10 μM, right). b, Inhibitory effects of nifedipine were described by % inhibition. Average of inhibitory effects of nifedipine from 5 preparation. The bars show mean ± s.e.

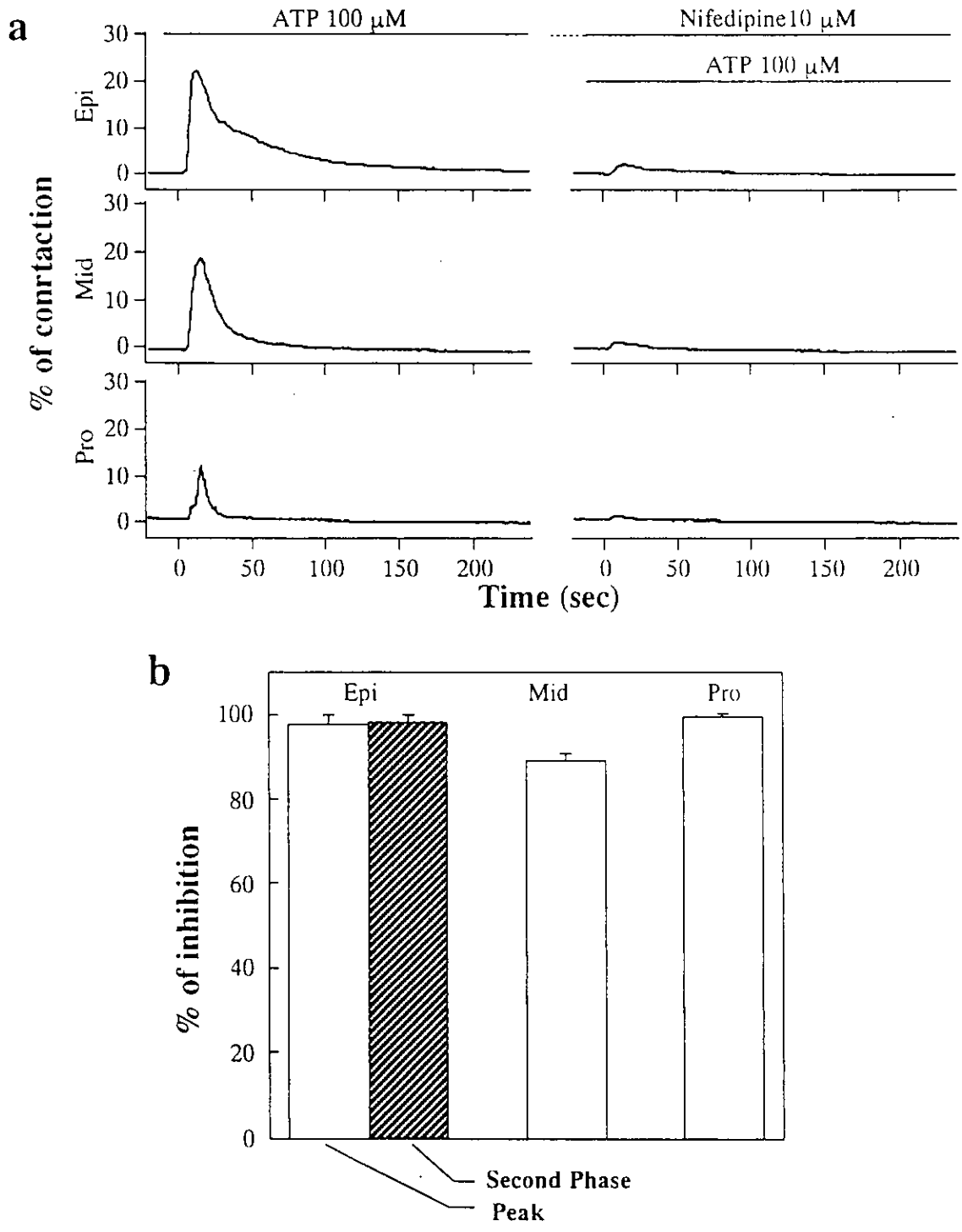


Fig. 2-18 Effects of nifedipine (10 μM) on ATP-induced contractions. a, Control responses to ATP 100 μM (left) were almost completely abolished by pretreatment with nifedipine (10 μM, right). b, Average of inhibitory effects of nifedipine from 4 preparations. The bars show mean ± s.e.

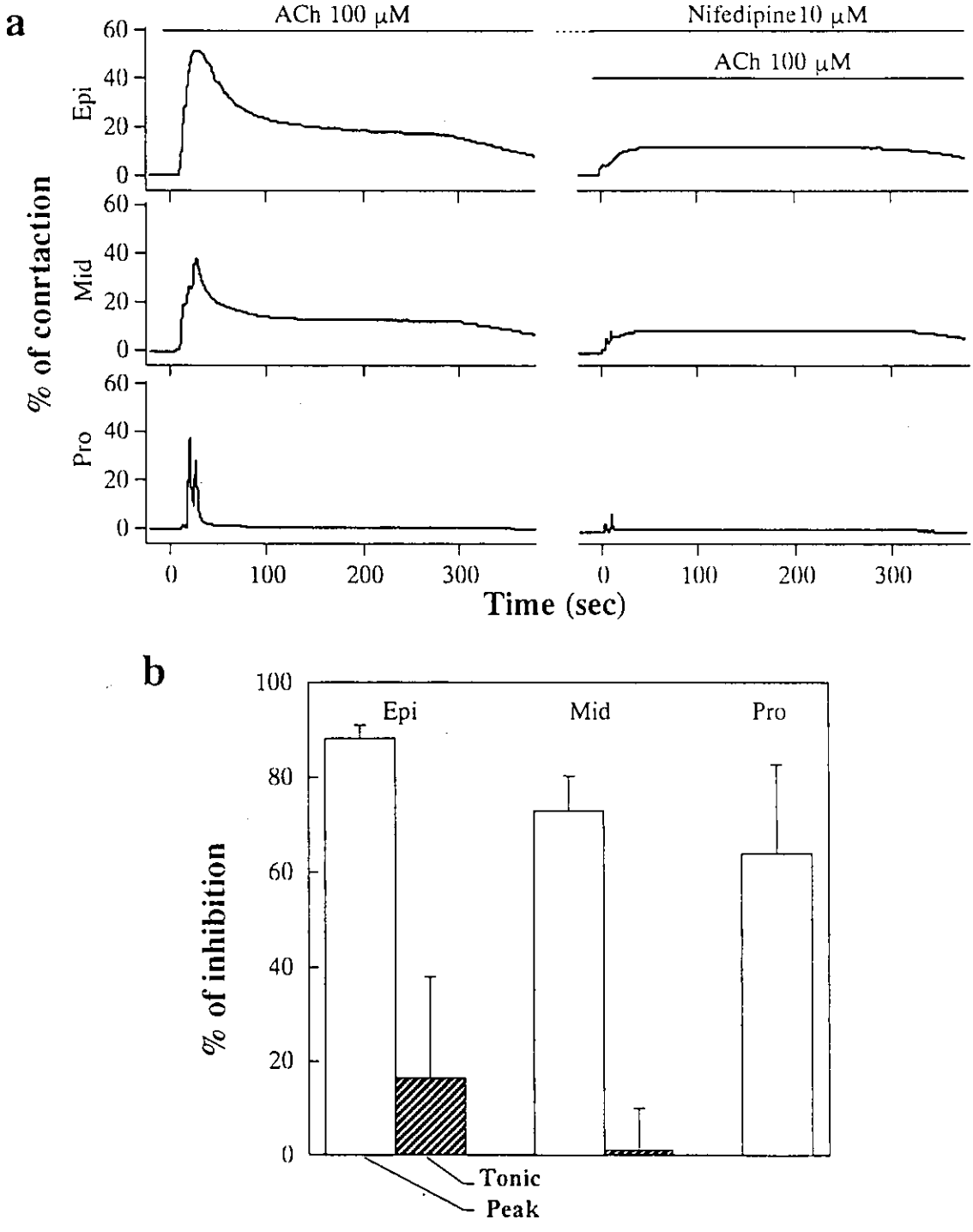


Fig. 2-19 Effects of nifedipine (10 μM) on ACh-induced contraction. a, Control responses to ACh 100 μM (left), particularly transient peaks, were inhibited by pretreatment with nifedipine (10 μM, right). b, Average of inhibitory effects of nifedipine from 4 preparation. The bars show mean ± s.e.

sensitive Ca^{2+} influx may play an important role in the ATP-induced contraction.

Discussion

Basic experimental conditions

The diurnal variation of the contractile responses is one of important factor of experimental conditions. Carneiro et al. (1993) reported that the diurnal variation of the contractile responses to ACh exists in the rat vas deferens. Although the diurnal change of the contractile responses in the guinea pig was not investigated, operations were started at almost the same time (8:30 to 9:30) in every experiment. Age-related changes in neuronal activity involved in contraction in rat vas deferens have also been reported by Carneiro et al. (1993) and Avellar & Markus (1993). In this study, however, there was no critical change in the contractile properties evoked by administration of neurotransmitters or excess external K^+ stimulation in the range of animal weight (300 - 600 g) used for all experiments.

The temperature of the perfusate was an important factor in the muscle contraction. The contractile responses to neurotransmitters or excess external K^+ were affected by a decrease in the bath temperature to 25 °C from 37 °C. Reduction of the temperature of the perfusate produced a marked potentiation in the peak amplitude of the initial transient of the contractile responses induced by neurotransmitters. For the tonic phase, this effect was also observed except the NA response in epididymal portion. Upon the excess external K^+ stimulation, however, the cooling effect was more complicated. The influence of temperature on contraction has been reported by Ambache & Zar (1971) and McGrath (1978).

Recently, Lee & Earm (1994) reported that cytosolic Ca^{2+} is pumped out or taken up more rapidly at higher temperature in rabbit pulmonary artery smooth muscle cells. Hypothermic inotropy of rabbit, rat and sheep cardiac ventricular muscle may be caused by activation of Ca^{2+} release from the sarcoplasmic reticulum (SR) and other un-identified mechanisms (Shattock & Bers, 1987; Sitsapesan et al., 1991). These possibilities are not entirely applicable to the tonic contractile responses to NA in the epididymal portion (Figs. 2-2 and 2-3). If the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was increased by these mechanisms, the contractile responses to all stimulants should be increased upon cooling. However, in several preparations, responses at 37 °C were not smaller than those at 25 °C. Therefore, it is likely that other unknown mechanisms, in addition to Ca^{2+}-homeostasis, may also underlie the hypothermic potentiation in the guinea pig vas deferens. All experiments thereafter were performed at 37 ± 0.5 °C.

In order to determine the involvement of neuronal activities in the neurotransmitter-induced contractions, TTX (1 μM) was used to block the generation of action potential in the nerve. The amplitude of first maximum peaks, tonic phases and late phases in all responses were not affected by pretreatment with TTX. This concentration of TTX completely and reversibly blocked the contraction evoked by field stimulation (1 msec duration, 5 Hz, supramaximal voltage, data not shown).

Receptor subtypes involved in responses to NA, ATP and ACh

α_1- and β-adrenoceptors have quite distinct actions; α_1-adrenoceptors link to the turnover of phosphatidylinositol-4,5-bisphosphate (PIP_2) which results in Ca^{2+} influx inducing muscle contraction, whereas β-

adrenoceptors link to a G protein-activated adenylate cyclase which causes increases in cAMP, a relaxing agent of smooth muscle (Hardie, 1991). The presynaptic α_2-adrenoceptors that mediate the auto-inhibition system of neurotransmitter release have been reported to exist in the guinea pig vas deferens and rabbit ileocolic artery (Sneddon & Westfall, 1984; Bulloch & Starke, 1990; Driessen et al., 1993). The NA-induced contraction was inhibited only by prazosin but not by α_2- and β-adrenoceptor antagonists. These results indicate that α_1-adrenoceptors are dominant in the guinea pig vas deferens.

There is no antagonist of purinergic receptors sufficiently selective to identify the receptor subtype unequivocally. In this study, the contraction evoked by ATP was found to be sensitive to α,β-Me ATP and suramin. Thus, it appears that the P_{2X} type of purinergic receptors is dominantly involved in the ATP responses of vas deferens.

Finally, an involvement of the muscarinic acetylcholine receptor was investigated using atropine (non-selective muscarinic acetylcholine receptor antagonist), pirenzepine (M_1 antagonist), AFDX-116 (M_2 antagonist) and p-FHHSiD (M_3 antagonist). The sequence of inhibiting potency was atropine > pirenzepine > AFDX-116 > p-FHHSiD. These results suggest that M_1-muscarinic acetylcholine receptors are predominant in the vas deferens of guinea pig.

Extracellular Ca^{2+} dependency of stimulant-induced contraction

In smooth muscles, an increase in Ca^{2+} is the essential trigger of contraction. This $[Ca^{2+}]_i$ increase is mediated both by Ca^{2+} influx from the extracellular space and by Ca^{2+} release from intracellular stores. It is important to define which is the predominant Ca^{2+} source for stimulant-induced contraction. Neurotransmitters and excess external K^+ were

found not to evoke the muscle contraction in Ca^{2+}-free PSS containing EGTA (1 mM). It is well known that the α_1-adrenoceptors stimulate Ca^{2+} release from the IP_3-dependent intracellular Ca^{2+} store (Berridge & Irvine, 1984; Han et al., 1987; Minneman, 1988; Itoh et al., 1992). In experiments using $^{45}Ca^{2+}$, NA-induced Ca^{2+} influx was not observed in either the rat or guinea pig vas deferens (Khoyi et al., 1987; Khoyi et al., 1988). However, extracellular Ca^{2+} influx was found to be necessary for NA-induced contraction in the guinea pig vas deferens in the present study. These results are supported by a previous paper which suggested that extracellular and intracellular Ca^{2+}-dependencies were different from cell to cell (Han et al., 1987) and that α_{1a}-adrenoceptor subtypes largely depended on extracellular Ca^{2+} to increase the IP_3 whereas α_{1b}-adrenoceptor subtypes were almost insensitive to extracellular Ca^{2+} to increase the IP_3 (Wilson & Minneman, 1990). Recently, the P_{2X}-purinergic receptor was defined as a member of the ligand-gated ion channel family (Valera et al., 1994; Brake et al., 1994). The results of this Chapter are not inconsistent with this finding. M_1-muscarinic acetylcholine receptors are also thought to be related to IP_3 generation, whereas in the present study, extracellular Ca^{2+} is essential for the ACh-induced contraction. In conclusion, several receptors eliciting contraction of the guinea pig vas deferens may be coupled to release of intracellular Ca^{2+} stores, though extracellular Ca^{2+} is the main source of Ca^{2+} regulating the contraction.

Contribution of L-type Ca^{2+}-channels to the external K^+-induced contraction

The L-type Ca^{2+} channel is one of the voltage-dependent channels and is thought to be the main Ca^{2+} influx pathway in smooth muscle cells

(Han et al., 1987; Nakazawa et al., 1987). This channel is blocked by the dihydropyridines (DHP), such as nitrendipine, nicardipine and nifedipine (Hille, 1992). Nifedipine inhibited the contractile responses to excess external K^+ (54 mM) in a concentration-dependent manner. In the epididymal portion, however, nifedipine failed to block completely the contraction. This nifedipine-insensitive contraction was blocked by prazosin. Figures 2-17 and 2-19 show that NA- and ACh-induced contractions, particularly the tonic contractions were, resistant to nifedipine. It is suggested that Ca^{2+} entry is largely mediated by a pathway distinct from the L-type Ca^{2+} channels in NA- and ACh-induced responses. Boot (1994) reported that the contraction evoked by field stimulation is inhibited concentration-dependently by ω-conotoxin GVIA (an N-type channel blocker) in rat vas deferens. In the guinea pig vas deferens, there is no report of the existence of other Ca^{2+} channels. As shown in Figs. 2-17 and 2-19, NA- and ACh-induced contractions were abolished by the external Ca^{2+} deprivation. It is thus suggested that NA- and ACh-induced responses involved nifedipine-insensitive Ca^{2+} channels or Ca^{2+}-permeable non-selective cation channels.

Chapter 3

Characterization of the responses to noradrenaline, ATP and acetylecholine

Introduction

The mechanisms underlying smooth muscle contraction and diversity in the development and duration of contraction are currently the focus of much attention. Diversity in the contractile responses to agonists exists between regions of certain smooth muscles. In the whole vas deferens, biphasic and triphasic contractions have been observed by field stimulation and drug application (Sneddon et al., 1982; Sneddon & Westfall, 1984; Allcorn et al., 1986; Tsunobuchi & Gomi, 1990; Avellar & Markus, 1993). The contractions of the bisected vas deferens were biphasic in the epididymal portion but monophasic in the prostatic portion (McGrath, 1978; Brown et al., 1983; Rohde et al., 1986).

Regional differences in the shape of contractions were initially thought to be due to differences in innervation; predominantly purinergic in the prostatic portion and adrenergic in the epididymal portion (McGrath, 1978; Brown et al., 1983; Rohde et al., 1986). Regional differences in the cholinergic contribution have also been observed in the vas deferens, being predominant in the epididymal portion (Sjöstrand, 1981). It was later noted that acetylcholine (ACh) activates prostatic neural nicotinic receptors, in addition to epididymal muscular muscarinic acetylcholine receptors (Wakui & Fukushi, 1986; Carneiro & Markus, 1990).

Further investigations of the muscle membranes showed a heterogeneous distribution of α_1-adrenoceptor subtype along the vas

deferens (Ohmura et al., 1992), the existence of a non-linear relationship between occupancy and response in each portion (Sallés & Badia, 1991; Sallés & Badia, 1993) and a higher sensitivity in the epididymal portion than in the prostatic portion (Nakazawa et al., 1987). These results suggested that the regional differences of contractile responses to NA may be due to heterogeneous receptor distribution. In addition, there is a possibility that the differences of intracellular mechanisms are involved in the regional variations in contractile responses.

The aim of present study was to investigate the regional differences in contractile responses to NA, ATP and ACh, known to be neurotransmitters in the vas deferens. The regional differences in intracellular Ca^{2+} responses to the neurotransmitters were investigated using fura-2. Excess external K^+ was used to investigate the regional differences in response to direct voltage-dependent Ca^{2+} influx. My data suggest that regional variation in the mechanisms of Ca^{2+} homeostasis and sensitivity of the contractile apparatus to intracellular Ca^{2+} ions may be involved in the regional contractile differences of the vas deferens.

Methods

Measurement of contraction and data analysis

Guinea pig vas deferens were isolated and trisected by the methods described in Chapter 2. Perfusing condition was also same. All contractile responses, including dose-response curves, were expressed as % shortening from the initial length. Dose-response curves were fitted by the Michaelis-Menten equation. The EC50, Emax and T1/2 (half decay time for ATP-induced responses) were calculated from a series of experiment of a preparation and averaged for all experiments. In prostatic portion of NA and ACh, and all ATP experiments, the measurement values at the highest concentration of agonists were regarded as Emax, since I could not get the satisfactory fitting. Data are expressed as mean ± s.e. and statistical significance was evaluated by Student's two tailed paired *t*-test.

Measurement of intracellular Ca^{2+} concentration

The intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured with an image analysis system (ARGUS-50/CA, Hamamatsu Photonics) using the fluorescent dyes, fura-2 and indo-1 (Grynkiewicz et al., 1985), as described previously (Furuya et al., 1994). Preparations were incubated with 5 μM fura-2 acetoxymethyl ester (AM) or indo-1 AM (1 mM stock solution in dimethylsulfoxide) for 1 hour at room temperature (23 - 24 °C). Preliminary experiments using a confocal laser microscope indicated that most of longitudinal muscle layer was well loaded with fura-2. A transmit image and a F360 image are shown in color plate figure (p 86). After loading with the fluorescence dyes, preparations were washed with PSS

and stored at 4 °C. $[Ca^{2+}]_i$ was measured under conditions of isometric contraction. A portion of the vas deferens was placed in a chamber (volume of 1 ml, Fig. 3-1) by fixing both sides, and the chamber was mounted on the stage of an inverted epifluorescence microscope (×20, Olympus IMT2-RFL, with a xenon lamp). The solution (37 ± 5 °C) was perfused continuously at the rate of 2 ml/min. The fluorescence intensity upon 360-nm excitation was monitored to check for muscle movement which could not be completely eliminated in spite of the isometric conditions, and area showing little movement during the contractile responses were selected for measurement.

Fura-2 fluorescence was excited at 340 nm and 360 nm alternately using a filter exchanger (C4312, Hamamatsu Photonics). Emission images of both wavelengths were detected with an ICCD camera (C2400-87, Hamamatsu Photonics) and stored to computer (PC/AT-compatible) at intervals 5 - 30 sec after background correction. In the case of experiments using indo-1, the preparations were excited at 360 nm, and the emitted fluorescence was divided to 420 nm and 480 nm images with W-view optics (A4313, Hamamatsu Photonics). Both images were combined in a frame and were stored on computer after background subtraction. The geometric correction of both images in the frame was done before each experiment.

In experiments using fura-2, the ratio images were occasionally observed with abnormal non-uniformity that was caused by muscle movement during the time lag between excitation at the two wavelengths (340 and 360 nm). These images were discarded from my analysis. Further, to avoid the effects of muscle movement on the ratio images, indo-1 was also used for $[Ca^{2+}]_i$ imaging. Similar NA-induced $[Ca^{2+}]_i$ oscillations to those recorded using fura-2 were observed by the method using indo-1.

Calibration converting the fluorescence ratio to Ca^{2+} concentration was done by a cell-free calibration method (Furuya et al., 1994). The fluorescence ratio of each solution, containing a known concentration of free Ca^{2+}, was measured under the same conditions as those for the experiment. The solution consisted of (in mM) KCl 150, $MgCl_2$ 0·8 and MOPS 10 (pH 7.2). Free Ca^{2+} concentrations were controlled by Ca^{2+}/EGTA buffers assuming apparent dissociation constants for the Ca^{2+}-EGTA complex of 145 nM and for the Mg^{2+}-EGTA complex of 15·8 mM (25 °C, pH 7.2). A series of solutions with different free Ca^{2+} concentrations can be achieved from the mixing of two types of the solutions, which contained 10 mM EGTA and 10 mM Ca^{2+}-EGTA respectively, in various proportions (Grynkiewicz et al., 1985). The concentration of fura-2 in each solution was 3 μM. The concentration curve was fitted with the equation of Grynkiewicz et al. (1985).

The onset of $[Ca^{2+}]_i$ responses to neurotransmitters was frequently found to be slower than that of the contractile responses. This may be caused by the difference between experimental condition for measurements of contraction (isotonic) and of $[Ca^{2+}]_i$ (isometric) and/or by the difference between the drug application procedures in measurements of contraction (bath application) and of $[Ca^{2+}]_i$ (perfusion).

Drugs

Noradrenaline (NA) and prazosin were obtained from Sigma chemical Co. (St. Louis, MO, USA). Acetylcholine (ACh), ATP, TTX and atropine were obtained from Wako (Osaka, Japan). Suramin was obtained from Funakoshi (Tokyo, Japan). Fura-2 and Indo-1 were obtained from Dojindo (Kumamoto, Japan).

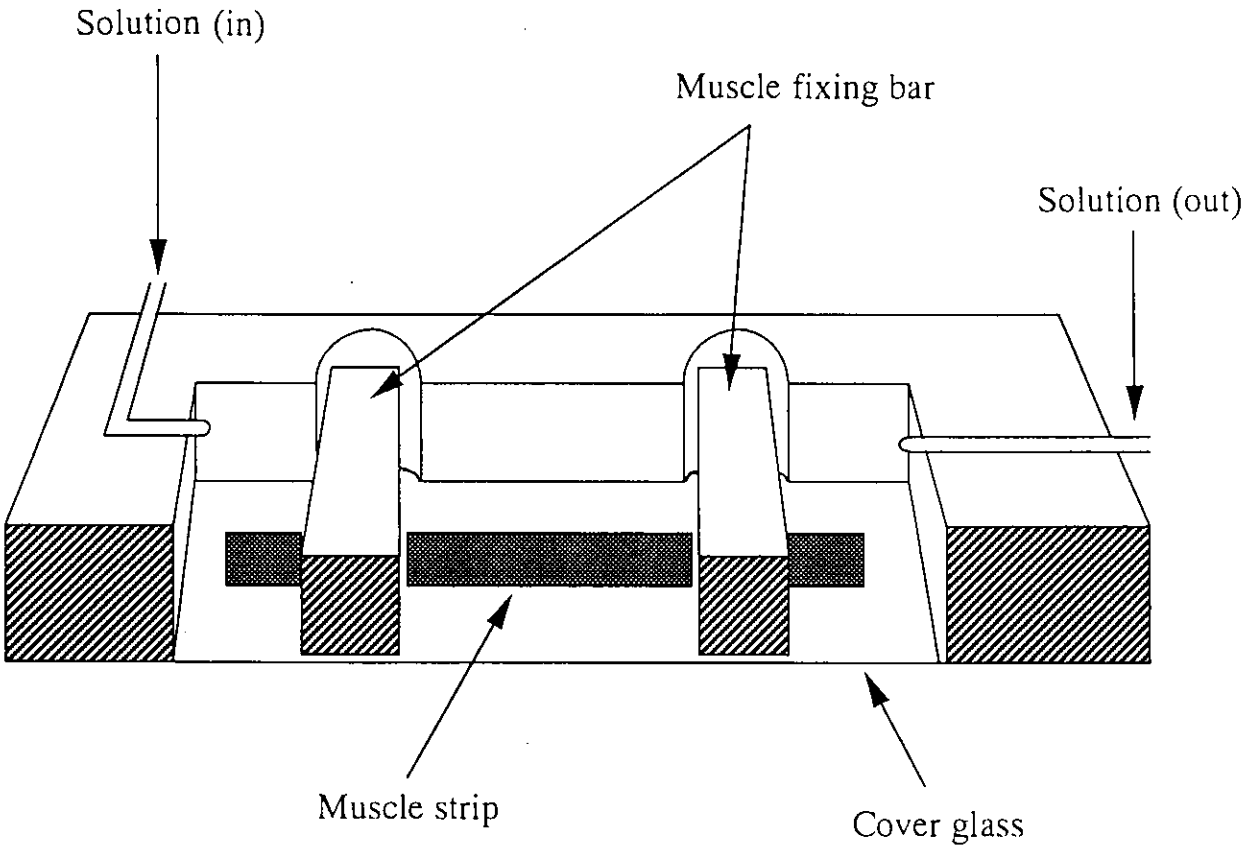


Fig. 3-1 Experimental chamber for measurement of intracellular Ca^{2+} concentration (1 ml).

Results

Contractile responses to noradrenaline (NA)

Contractile responses of the epididymal, middle and prostatic portions of the guinea pig vas deferens to NA are shown in Fig. 3-2. Each portion of the preparation exhibited different contractile responses to various concentrations of NA (Fig. 3-2a). The responses consisted of two contractile components; a transient contraction (time to peak, 27.7 ± 2.4 sec, 31.4 ± 5.4 sec and 39.3 ± 6.3 sec, for epididymal, middle and prostatic portions, respectively, at 100 μM) followed by a sustained tonic contraction (Sneddon et al., 1984; McGrath, 1978). The maximal amplitude (Emax) of the peak transient responses was almost the same in all three portions, although the EC50 markedly decreased from the prostatic to epididymal portion (Fig. 3-2b, Table 3-1). The tonic contraction was sustained or gradually increased during exposure to NA. Although the transient contractions were observed in all portions, tonic contractions were largest in the epididymal portion, relatively smaller in the middle portion and virtually non-existent in the prostatic portion. The ratio of the Emax of the tonic phase to that of the transient peak was 67% in the epididymal portion and 55% in the middle portion. The Emax of the tonic contraction increased by 22.1% from the prostatic to the middle, and by 10.1% from the middle to the epididymal portion. As shown in Fig. 3-2a, repetitive transient contractions superimposed on the tonic contraction were observed at high agonist concentrations. The diversity of the patterns of NA-induced contractile responses is summarized in Fig. 3-3. The typical contractile patterns were as follows; the transient peak followed by tonic contraction in epididymal and middle portions, and the transient peak followed by oscillatory contractions in the prostatic portion. The tonic

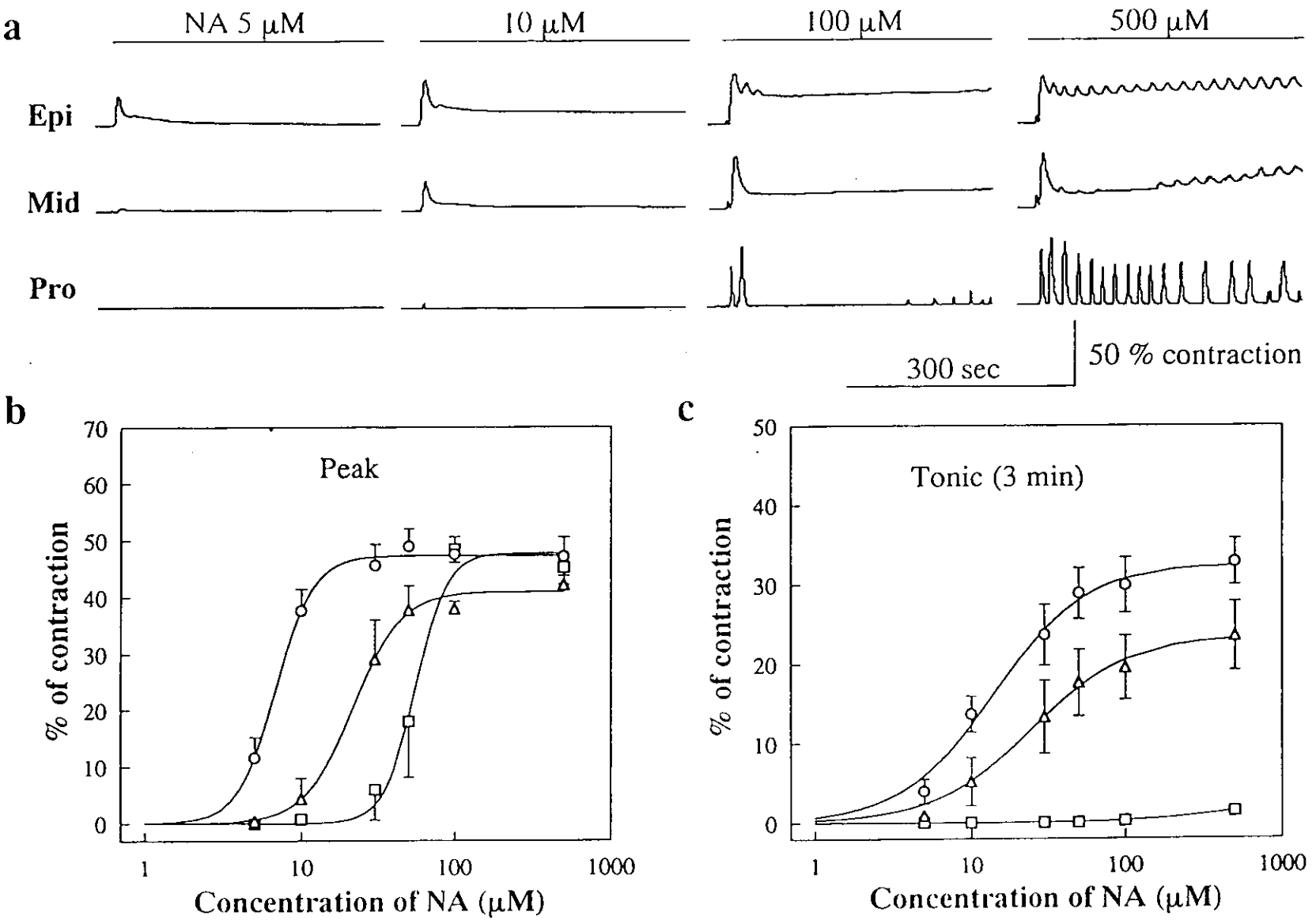


Fig. 3-2 Contractile responses to NA in the epididymal (Epi, O), middle (Mid, Δ) and prostatic portions (Pro, □). a, Typical responses of three parts of vas deferens to various concentrations of NA. Horizontal bars above the traces indicate the exposure time to NA. b and c, Concentration-response curves of NA, for first maximum transient contraction (b; peak) and 3 min after application (c; tonic). Points and vertical bars are mean ± s.e. (n = 5), and vertical bars are the s.e. where those exceeded to the symbol size.

Table 3-1 EC_{50} (μM), E_{max} (%) and half decay time ($T_{1/2}$, sec, in ATP)

	Epididymis		Middle		Prostata	
	EC_{50}	E_{max} or $T_{1/2}$	EC_{50}	E_{max} or $T_{1/2}$	EC_{50}	E_{max} or $T_{1/2}$
NA peak	8.0 ± 2.1	48.4 ± 3.7	23.4 ± 5.7*	40.6 ± 9.8	53.8 ± 9.5**◆	48.6 ± 3.0
tonic	17.7 ± 3.2	32.5 ± 3.5	29.4 ± 5.1*	22.4 ± 4.3	———	(0.2 ± 3.0)**◆
ATP peak	———	(52.4 ± 6.8)	———	(43.7 ± 5.1)	———	(31.3 ± 6.0)*
half decay	———	55.7 ± 8.6	———	24.6 ± 3.5**	———	16.9 ± 7.5**
ACh peak	2.3 ± 0.2	56.4 ± 1.9	3.9 ± 0.9	54.2 ± 4.0	9.0 ± 4.4	44.8 ± 5.6
tonic	19.6 ± 9.1	9.3 ± 2.5	13.8 ± 5.0	7.1 ± 1.9	———	(0.4 ± 0.2)**◆◆

EC_{50}, and E_{max} (mean ± s.e.) were calculated from each tissue by using the Michaelis-Menten equation. When responses (the data in parentheses) could not be fitted by the equation, the E_{max} values were taken from the response at the highest agonist concentration used. $T_{1/2}$ also obtained from the response to at the highest ATP concentration used.

** and *; significantly different form the data of the epididymal portion at $P < 0.01$ and $P < 0.05$, respectively.

◆◆ amd ◆; significantly different form the data of the middle portion at $P < 0.01$ and $P < 0.05$, respectively.

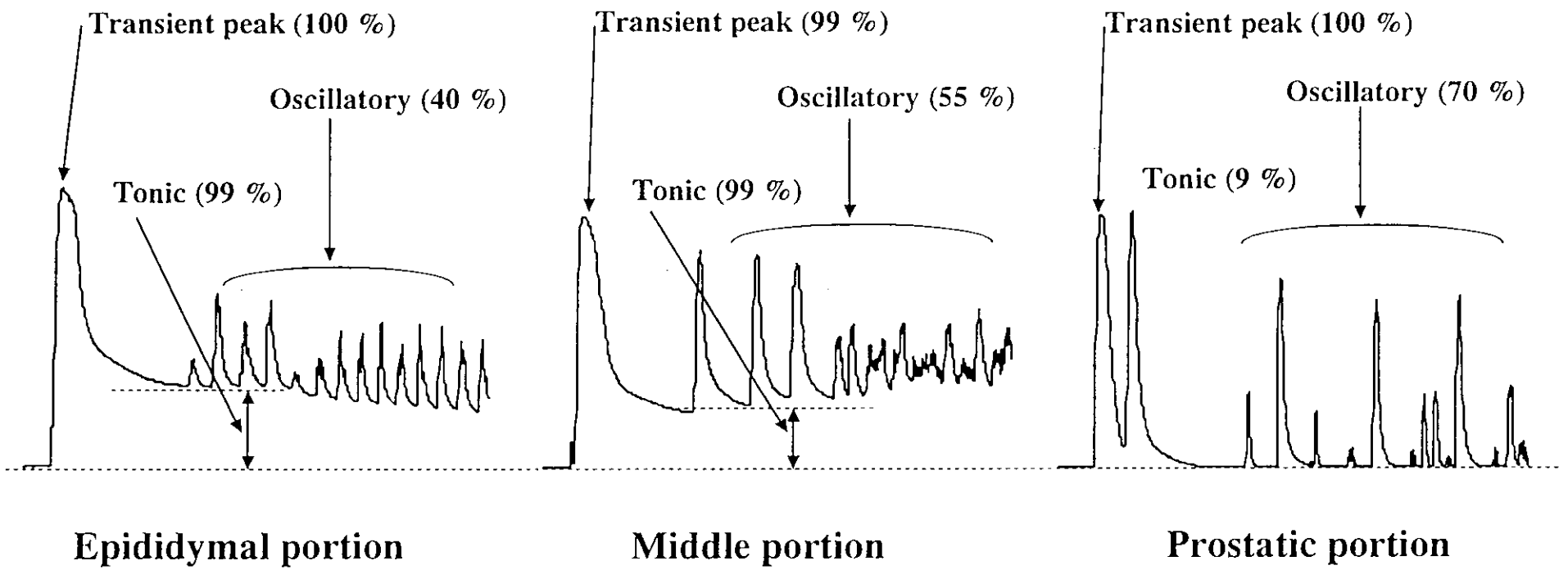


Fig. 3-3 Frequency of observations in three kinds of 100 μM NA-induced contraction. (n = 100).

contraction level was measured as the base line of the repetitive transient contractions. These NA responses were blocked by prazosin, an α_1-antagonist (10 μM), but not by suramin, a P_{2X} antagonist (100 μM).

Contractile responses to adenosine triphosphate (ATP)

The contractile responses to ATP (100 μM) of each portion, from epididymis to prostata are shown in Fig. 3-4a. The time to initial transient peaks were 14.0 ± 1.5, 11.8 ± 2.1 and 11.7 ± 2.3 sec, respectively. In contrast to the tonic contraction evoked by NA, there was no long lasting plateau (tonic responses). The time to recovery was dependent on the ATP concentration and, at each concentration of ATP, increased from the prostatic to epididymal portion. Half decay time ($T_{1/2}$) at the highest concentration of ATP, there were significantly differences between epididymal and middle or prostatic portions. The responses to ATP (100 μM) were sensitive to the P_{2X}-purinergic receptor antagonist, suramin.

Contractile responses to acetylecholine (ACh)

Since ACh may evoke the release of neurotransmitters via neural nicotinic receptors, prazosin (10 μM) and suramin (100 μM) were employed to block the α_1 and P_{2X} receptors on the muscle membrane, in order to investigate the action of smooth muscle muscarinic acetylcholine receptors alone. Fig. 3-5a shows the results of contractile response to ACh. The responses were biphasic in the epididymal and middle portions. In contrast, the response was almost monophasic in the prostatic portion. The EC_{50} and E_{max} of the peak transient contraction in each portion were not significantly different from each other, and the time to peak was 20.0 ±

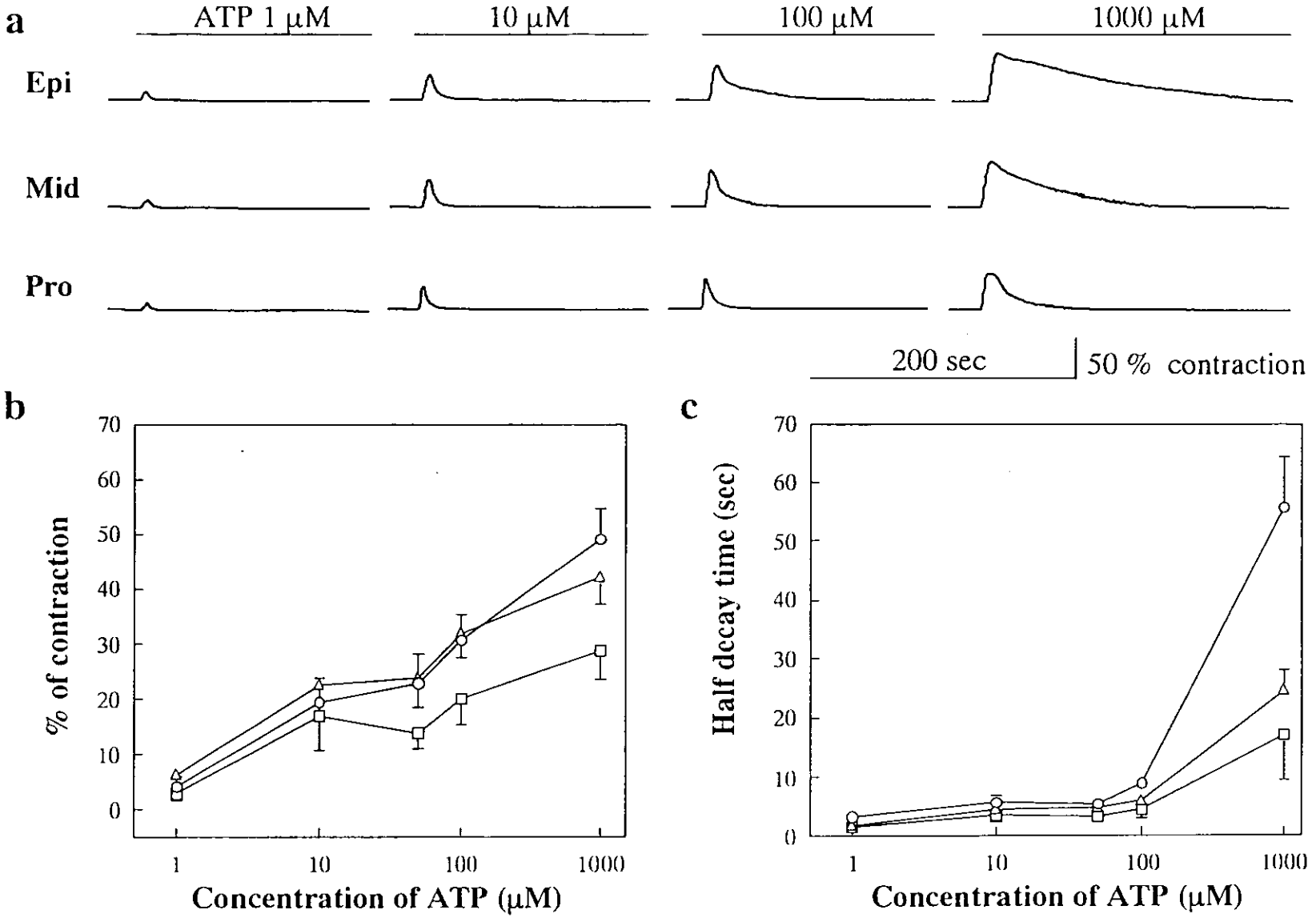


Fig. 3-4 Contractile responses to ATP in the epididymal (Epi, ○), middle (Mid, Δ) and prostatic portions (Pro, □). Typical responses at several given concentrations of ATP (a). ATP concentration-response curves, for peak (b) and half time decay (T1/2) after first maximum peak (c). These responses could not be fitted by the Michaelis-Menten equation. Points represent mean ± s.e. (n = 5).

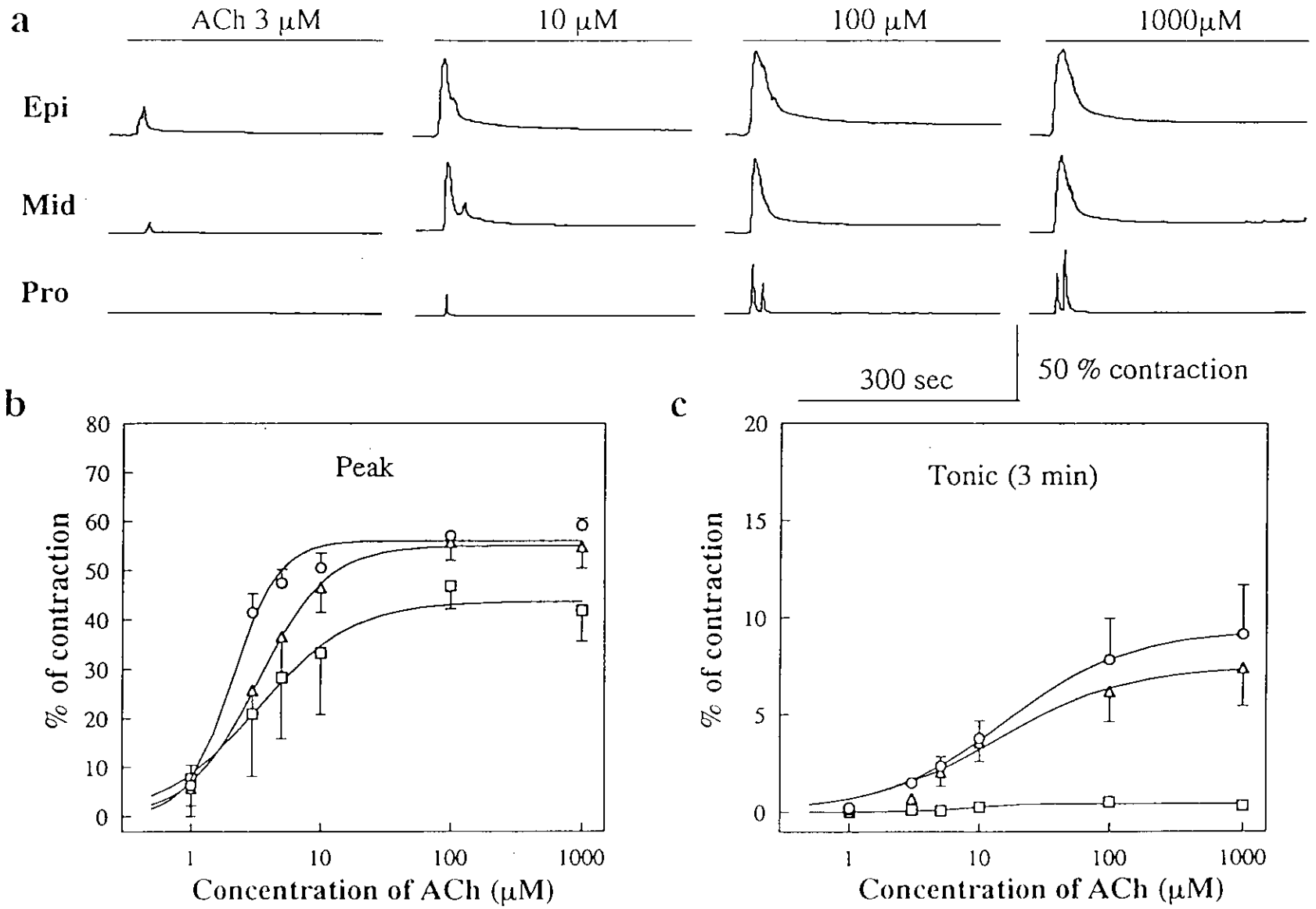


Fig. 3-5 Contractile responses to ACh in the epididymal (Epi, O), middle (Mid, Δ) and prostatic portions (Pro, □). Typical contractile responses at several given concentrations of ACh (a). Concentration-response curves of ACh, for peak (b) and 3 min after first maximum peak (c). Points represent mean ± s.e. (n = 5).

3.5 sec, 19.3 ± 3.1 sec and 19.2 ± 2.8 sec, from epididymal to prostatic portion at 100 μM. The Emax of the tonic contraction was not significantly different between epididymal and middle, but there was significant differences between the prostatic and the other two portions (Fig. 3-5c and Table 1). The ratios of tonic to transient peak were 16.5% and 13.1% at the Emax, in the epididymal and middle portion, respectively. The Emax, however, increased to 6.7% and 8.9% from the prostatic to the middle and epididymal portions, respectively. Atropine, a muscarinic ACh receptor antagonist (1 μM), completely blocked the response to ACh.

Intracellular Ca^{2+} responses to NA, ATP and ACh

Because the previous data of contraction showed that the middle portion exhibited responses intermediate between the epididymal and prostatic portions to all agonists, I focused on the $[Ca^{2+}]_i$ responses in the epididymal and prostatic portions alone. Typical $[Ca^{2+}]_i$ responses to NA, ATP and ACh (each 100 μM) are shown in Fig. 3-6 (cf. color plate figure, p 83). In response to NA, $[Ca^{2+}]_i$ immediately increased to a peak (of 200 to 300 nM) and subsequently showed the oscillation in both preparation. The oscillation ceased after around 200 sec in the epididymis but maintained during stimulation in the prostatic portion. After stimulation with ATP or ACh, as shown in Fig. 3-6, similar oscillatory $[Ca^{2+}]_i$ responses were observed in both preparations. However, the ATP- or ACh-induced oscillation never maintained during stimulation but ceased within 100 to 200 sec even in the prostatic portion. The plateau levels, which attained after decaying the oscillatory responses, were significant higher in epididymal portion than those in the prostatic portion.

Contractile responses to excess external K^+ stimulation

To examine the effect of increases in intracellular Ca^{2+} concentration without induction of receptor-mediated signal transduction pathways, the responses to membrane depolarization evoked by excess external K^+ (over 25 mM) were investigated. Fig. 3-7 shows the typical contractile responses to several concentrations of external K^+ in the external solution. At lower concentrations, repetitive transient contractions occurred in all portions. With increasing the K^+ concentration, tonic contraction appeared after transients and became larger. The time to initial transient peaks in response to 54 mM K^+ were 21.6 ± 1.8, 21.7 ± 2.0 and 25.1 ± 2.5 sec from the epididymal to prostatic portion, and the magnitudes were 42.6 ± 3.9, 40.9 ± 4.2 and 24.7 ± 2.1%. Tonic contractions observed at 3 min after stimulation were 49.2 ± 2.4, 43.2 ± 2.0 and 19.2 ± 2.2%. There were regional differences in the threshold K^+ concentration eliciting contraction, being lower in the epididymal portion than in the prostatic portion. It may be worth noting that tonic contractions of the prostatic portion, which were hardly detected by stimulation with neurotransmitters, could clearly be observed on stimulation with 54 mM K^+. The contractions evoked by external K^+ were inhibited neither by prazosin (10 μM), by suramin (100 μM), nor by atropine (1 μM). It is concluded that the activity of endogenous nerves was not involved in the contractile responses not only to neurotransmitters but also to excess external K^+ in the vas deferens.

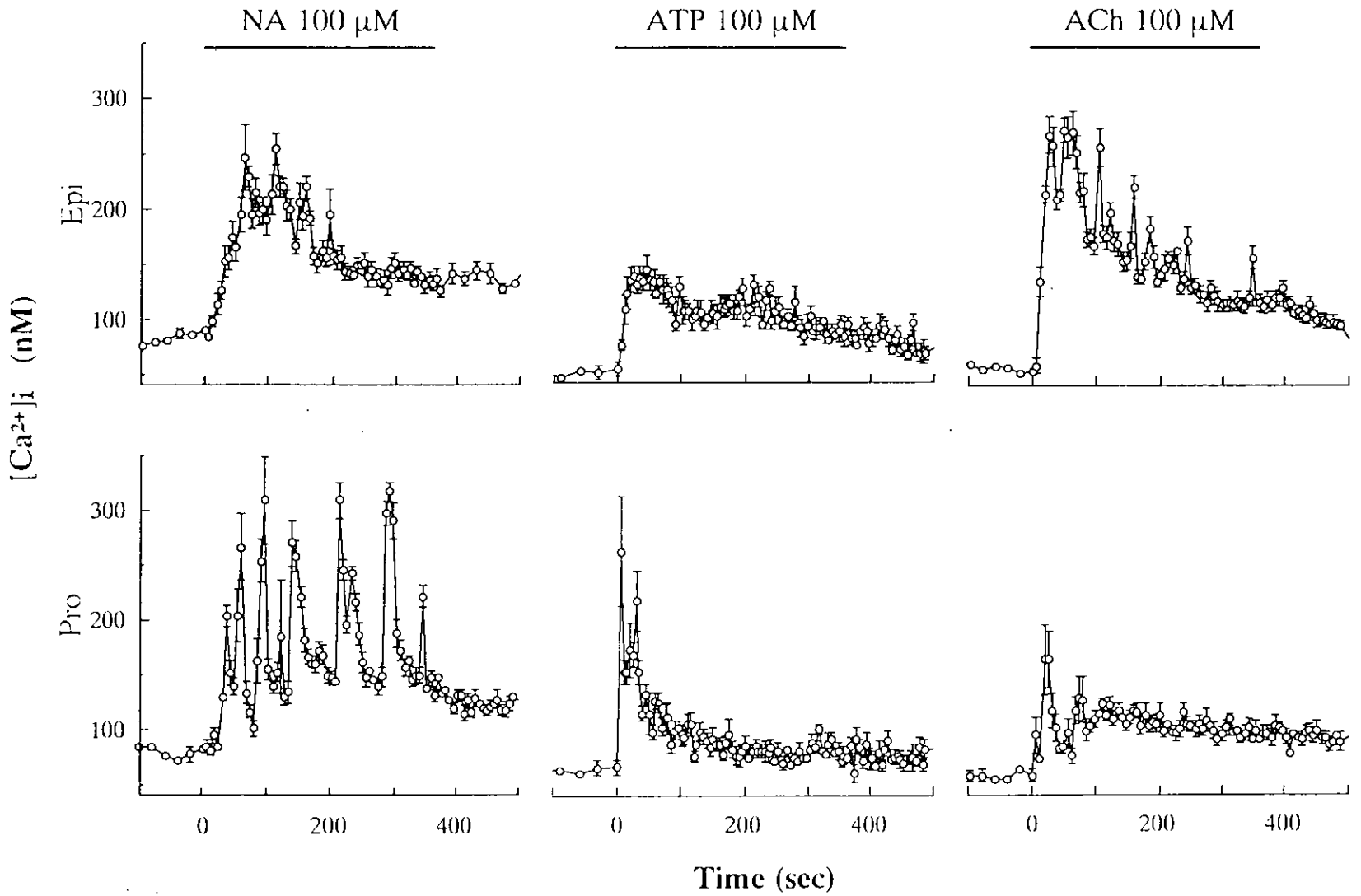


Fig. 3-6 Intracellular Ca^{2+} responses to NA, ATP and ACh. Typical $[Ca^{2+}]_i$ responses in epididymal (Epi) and prostatic (Pro) portions to NA, ATP and ACh obtained from 4 - 6 preparations measured by fura-2 fluorescence. Horizontal bars indicate the time of exposure to agonists: The data represent the average of 7 points on the muscle strip, and vertical bars are the s.e. values.

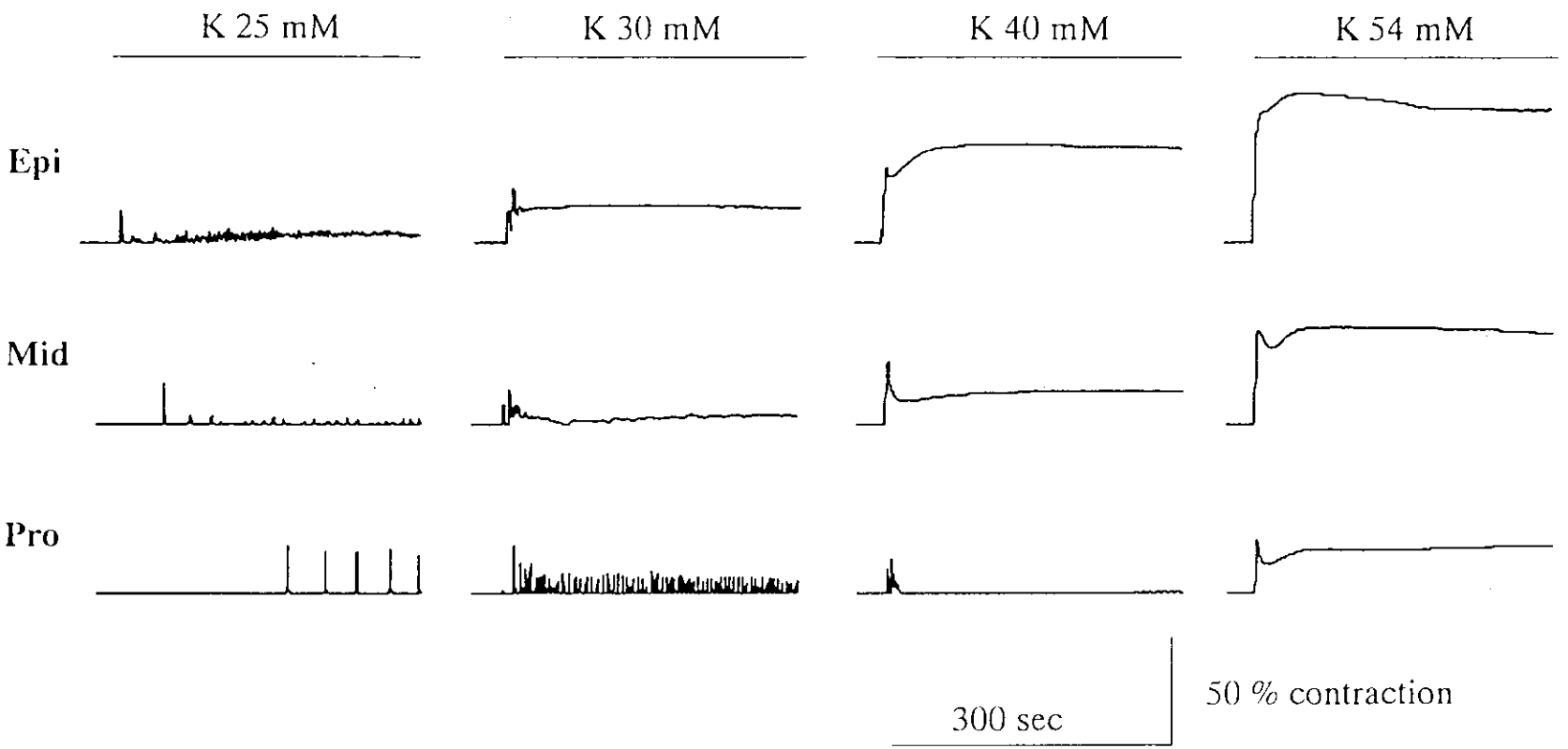


Fig. 3-7 Contractile responses to excess external K^+ stimulation. The typical responses in the epididymal (Epi), middle (Mid) and prostatic portions (Pro). The data are representative of 10 experiments.

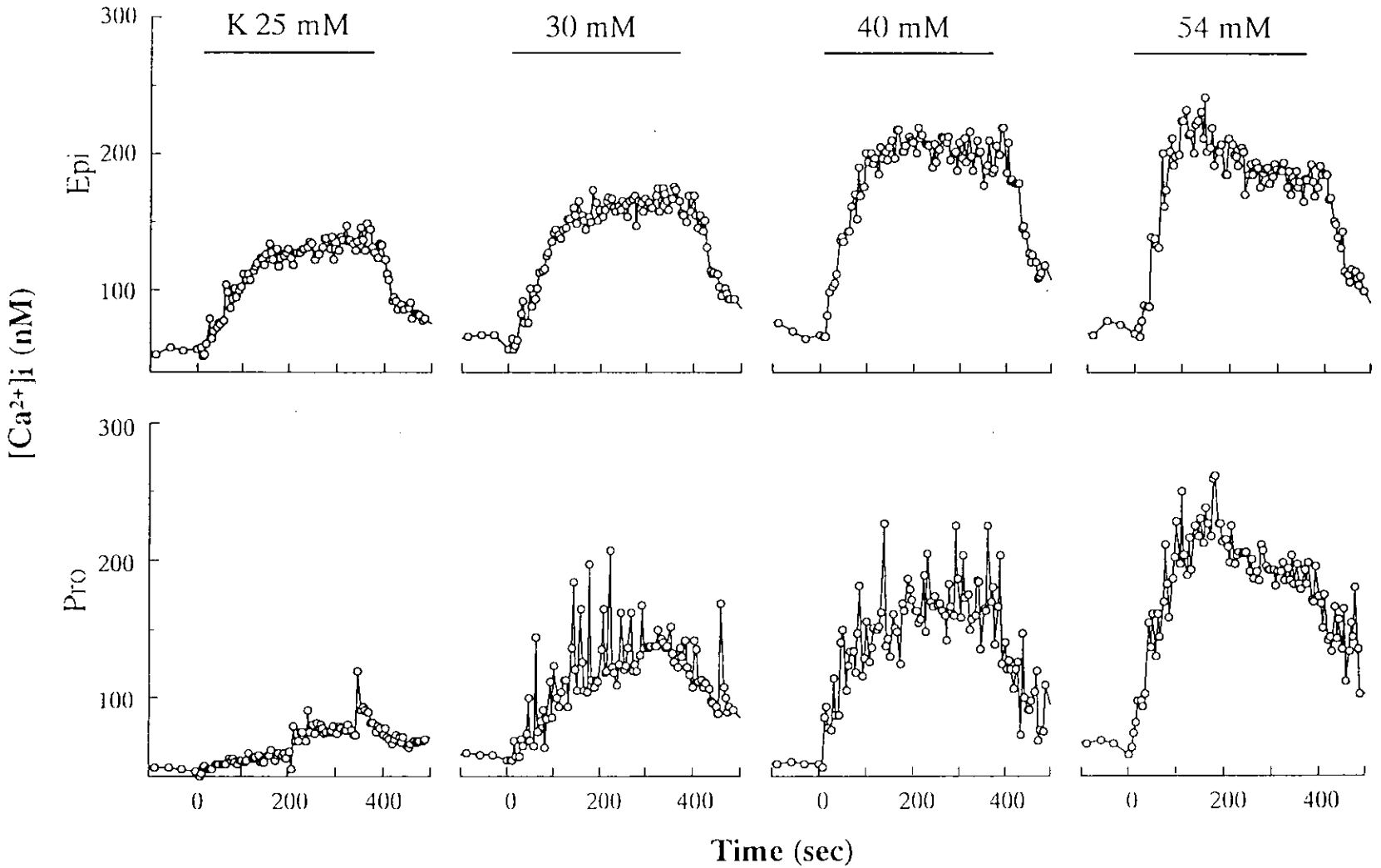


Fig. 3-8 Intracellular Ca^{2+} responses to excess external K^+ stimulation. The $[Ca^{2+}]_i$ responses in epididymal (Epi) and prostatic (Pro) portions were measured by fura-2. The data represent the average of 7 points on the muscle strip out of 3 preparations. $[Ca^{2+}]_i$ of these plateau levels were (in nM) 98·9 ± 7·3, 130·8 ± 13·5, 182·7 ± 6·3 and 192.4 ± 4.4, from 25 mM K^+ to 54 mM, in the epididymal portion.

Intracellular Ca^{2+} responses to excess external K^+ stimulation

The $[Ca^{2+}]_i$ responses to several concentrations of external K^+ are shown in Fig. 3-8. In the epididymal portion, $[Ca^{2+}]_i$ increased to a sustained plateau level even at lower concentrations of external K^+ (over 25 mM). This plateau level was dependent on the K^+ concentration, and seemed to saturate in the K^+ concentrations range between 40 and 54 mM. In the prostatic, high frequency oscillations were often observed at lower K^+ concentration and the plateau phase developed with increasing K^+ concentration. On 25 mM K^+, some preparations showed almost no response in either contraction or $[Ca^{2+}]_i$, suggesting regional variation in the threshold K^+ concentration to induce the contractile response.

Discussion

In this Chapter, it was shown that each of the three portions of guinea pig vas deferens displayed different contractile responses to noradrenaline (NA), ATP and acetylcholine (ACh). Each neurotransmitter elicited contraction in all portions of the vas deferens which was characteristic of that neurotransmitter in terms of the relative magnitude of the tonic phase, time to half-maximal decay (T1/2), the existence of oscillatory contraction and the concentration-response curve. This suggests that there are differences between neurotransmitters in the mechanism of elicitation of contraction. However, there were also clear regional differences in the contractions induced by each neurotransmitter. Transient contractions induced by NA and ACh were observed in all portions, whereas the sustained or tonic phase after the initial transient contraction was observed only in the epididymal and middle portions (Figs. 3-2 and 3-5). The Emax of the NA- and ACh-induced transient contractions were not significantly

different among the portions. The EC_{50} for the transient responses to NA prominently changed depending on the portion. The ATP-induced contraction was transient in all portion, but the duration of a slow decay phase was dependent on the portion (Fig. 3-4). At each concentration of ATP, the decay time of the responses to ATP increased from prostatic to epididymal portion. These results suggest that the regional differences in the contractile responses of the vas deferens to neurotransmitters involve differences in the intrinsic contractile properties of the smooth muscle. This conclusion is supported by experiments with excess external K^+ which artificially induced Ca^{2+} influx into the muscles and consequently elicited contraction. Regional differences in contractions elicited by external excess K^+ were also observed (Fig. 3-7). Principally, the threshold external K^+ concentrations eliciting contraction in the epididymal and middle portions were lower than that in the prostatic portion. Oscillatory contractions, which were observed at lower external K^+ concentrations, were much more prominent in the prostatic portion than in epididymal and middle portions. This might imply differences in the Ca^{2+}-sensitivity of the contractile apparatus and/or in the conductance properties of the smooth muscle cell membrane. Regional differences in the Ca^{2+} influx-dependent contraction may also be involved in the variation in responses to neurotransmitters.

It is generally accepted that elevation of $[Ca^{2+}]_i$ is the principal signal eliciting contraction in smooth muscles (Somlyo & Somlyo, 1994). Therefore I investigated the $[Ca^{2+}]_i$ responses to the neurotransmitters and external excess K^+. The $[Ca^{2+}]_i$ measurements indicated that the $[Ca^{2+}]_i$ responses to all stimulants exhibited regional differences, suggesting that the regional differences in contractile properties involved differences in the mechanisms of intracellular Ca^{2+} elevation and homeostasis. However, sustained elevation of $[Ca^{2+}]_i$ was not necessarily reflected in tonic

contraction. For example, there was almost no tonic contraction at 40 mM external K^+ in the prostatic, in spite of the high level of $[Ca^{2+}]_i$ that was maintained throughout exposure to this concentration of K^+ in experiments with fura-2. This might have been due to modulation of the Ca^{2+}-sensitivity of the contraction or the existence of a threshold level of $[Ca^{2+}]_i$ for eliciting the tonic contraction, as has been reported in smooth muscles of the small intestine and mesotubarium of the guinea pig (Himpens & Somlyo, 1988; Himpens et al., 1990; Somlyo & Somlyo, 1994).

In summary, differences in the Ca^{2+}-sensitivity of the contractile apparatus and the Ca^{2+} homeostatic mechanisms may be involved in the regional differences in contractile responses to neurotransmitters. Further investigations are necessary to elucidate the relationship between contraction and mechanisms of regulation of $[Ca^{2+}]_i$. The physiological relevance of the regional contractile differences are unknown, however, it is possible that the gradient in magnitude of tonic contractions from epididymis to prostata may contribute to the transport of spermatozoa by preventing back flow of the luminal contents.

Chapter 4

Effects of cyclic adenosine 3' 5'-monophosphate (cAMP) on the noradrenaline-induced contractions

Introduction

Caffeine has several effects on smooth muscle cell function; 1) Ca^{2+} release from caffeine-sensitive intracellular Ca^{2+} store (Endo et al., 1990), 2) inhibition of the phosphodiesterase activity (Butcher & Sutherland, 1962) and 3) blocking of the A_1 receptor (Parsons et al., 1988). It is generally accepted that the caffeine-sensitive store is the Ca^{2+}-induced Ca^{2+} release sarcoplasmic reticulum (CICR-SR), and not the inositol triphosphate (IP_3)-sensitive store (I ICR-SR). This may explain why caffeine is able to elicit muscle contraction even in the Ca^{2+}-free solution in several smooth muscles (Watanabe et al., 1992). This effect of caffeine is often used to determine the contribution of Ca^{2+} release from intracellular Ca^{2+} stores during contraction.

On the other hand, caffeine has another effect in inhibiting phosphodiesterase, causing increase in the intracellular cyclic nucleotide (cAMP and cGMP) concentrations (Butcher and Sutherland, 1962). Recently, cyclic nucleotides have been found to act as relaxing agents in pre-contracted smooth muscle. The mechanisms of effects of cyclic nucleotide on muscle relaxation are unclear. These effects of cyclic nucleotides have been reported to be via Ca^{2+} uptake into the SR (Leijten & van Breemen, 1984), decrease of $[Ca^{2+}]_i$ and altering the Ca^{2+} sensitivity of contractile elements (Abe & Karaki, 1992), inhibition of IP_3-dependent Ca^{2+} release (Murthy et al., 1993) and direct phosphorylation of myosin light chain kinase via cAMP-binding protein kinase (PKA,

Adelstein et al., 1978). Moreover, the effects of cAMP may vary depending on stimulation type; NA-induced stimulation is more sensitive to cAMP than high-K^+ stimulation (Willenbucher et al., 1992). Caffeine inhibits the noradrenaline-induced Ca release and influx induced by high K^+ (Ahn et al., 1988).

The experiments described in this Chapter were designed to investigate the effect of caffeine on the NA-induced contraction. Further, $[Ca^{2+}]_i$ was also measured to investigate the effects of caffeine and other drugs on intracellular $[Ca^{2+}]_i$.

Methods

Measurement of contraction and data analysis

Guinea pig vas deferens were isolated and trisected by the methods described in Chapter 2, and expression of contractile response to drug was the same as in Chapter 3. Muscle tension reduction by relaxing agents was indicated as % reduction, and was calculated from the % contractions just prior to and 3 min after application of relaxing agents. The data were presented as mean ± s.e. Statistical significance was evaluated by Student's two tailed paired *t*-test.

Drugs

Forskolin and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). H-89 was obtained from Seikagaku Co. (Tokyo, Japan).

Results

Effects of caffeine, 3-isobutyl-1-methylxanthine (IBMX), and forskolin on tonic contraction induced by noradrenaline (NA)

Typical effects of caffeine on the NA-induced tonic contraction are shown in Fig. 4-1a. Caffeine was applied to the bath 3 min after NA application. Tonic contractions were dramatically reduced by caffeine (20 mM) in the epididymal and middle portions. Caffeine-induced relaxation did not occur in the prostatic portion. Figure 4-1b shows the concentration-response curve of caffeine. Similar concentration-response curves were obtained from epididymal and middle portions. In the prostatic portion, however, the effect of caffeine was not clear, because there was almost no tonic contraction. Some preparations of vas deferens showed repetitive transient contractions (see Figs. 4-2, 4-3 and 4-4). These repetitive transient contractions were also inhibited by caffeine (data not shown). In addition, caffeine alone had no effect on the muscle responses up to 20 mM (data not shown).

To determine whether the inhibition of phosphodiesterase activity by caffeine mediates the relaxant effects on the NA-induced muscle contraction, another methylxthanthine, IBMX, which is similar to caffeine known to be more potent in inhibition of phosphodiesterase, was used. Tonic contractions were reduced by application of IBMX in a manner similar to the caffeine-induced relaxation (Fig. 4-2a). IBMX also inhibited repetitive transient contractions. These IBMX-induced relaxations were concentration-dependent (Fig. 4-2b).

The relaxation induced by caffeine or IBMX may have been due to an increase in the intracellular cAMP concentration by the inhibition of cAMP hydrolysis by phosphodiesterese. This would imply some basal

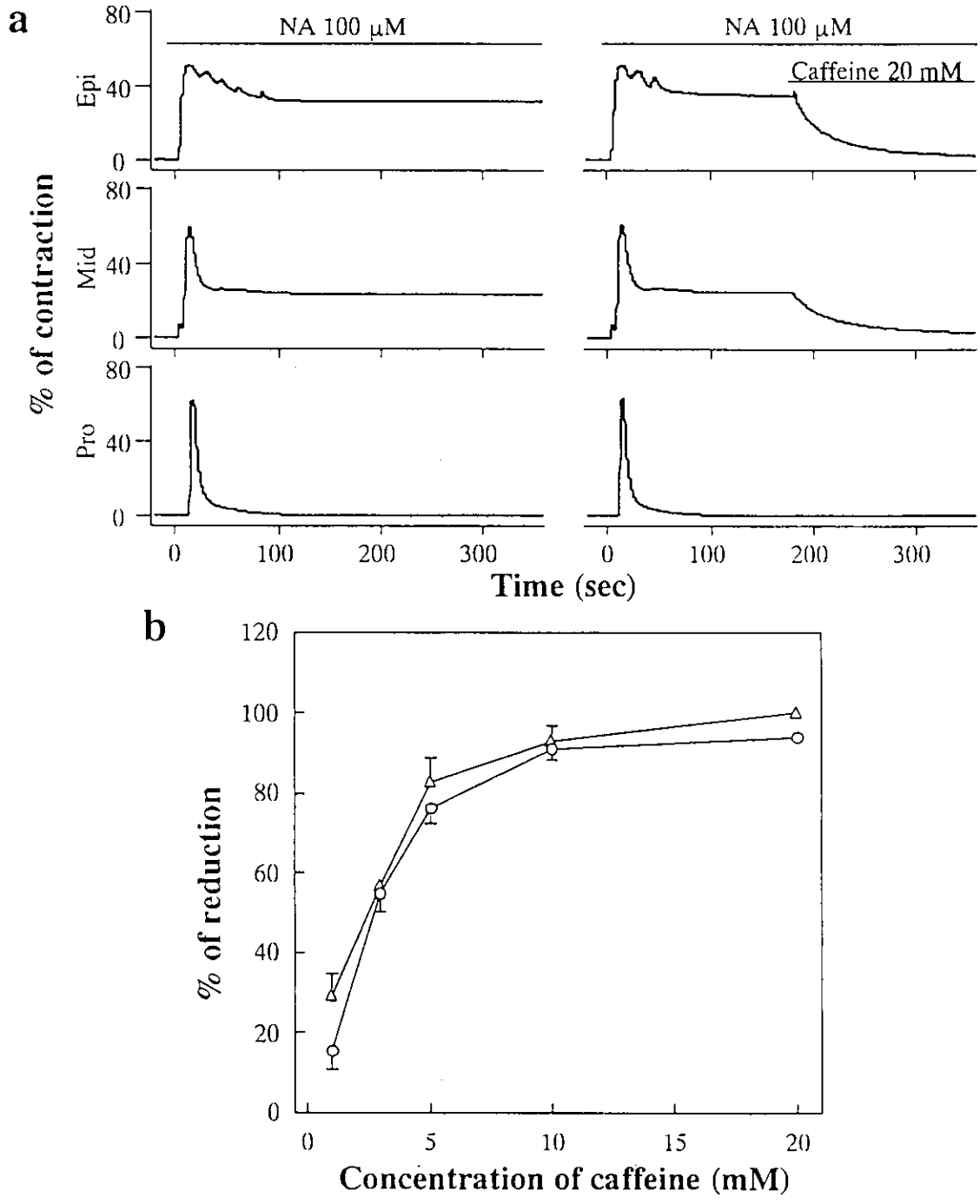


Fig. 4-1 Effects of caffeine on tonic contraction induced by NA. a, Representative responses to NA (100 μM, left) and NA plus caffeine (20 mM, right) in the epididymal (Epi), middle (Mid) and prostatic (Pro) portions. Caffeine was applied to the bath 3 min after NA-application. b, Concentration-response curves of caffeine. The ordinate indicates the percentage of reduction from tonic contraction level just before caffeine application to the contraction level 3 min after caffeine application. Circles and triangles represent the mean response in epididymal and middle portions, respectively (n = 4-5, n = 1 for 20 mM). Vertical bars represent the s.e.

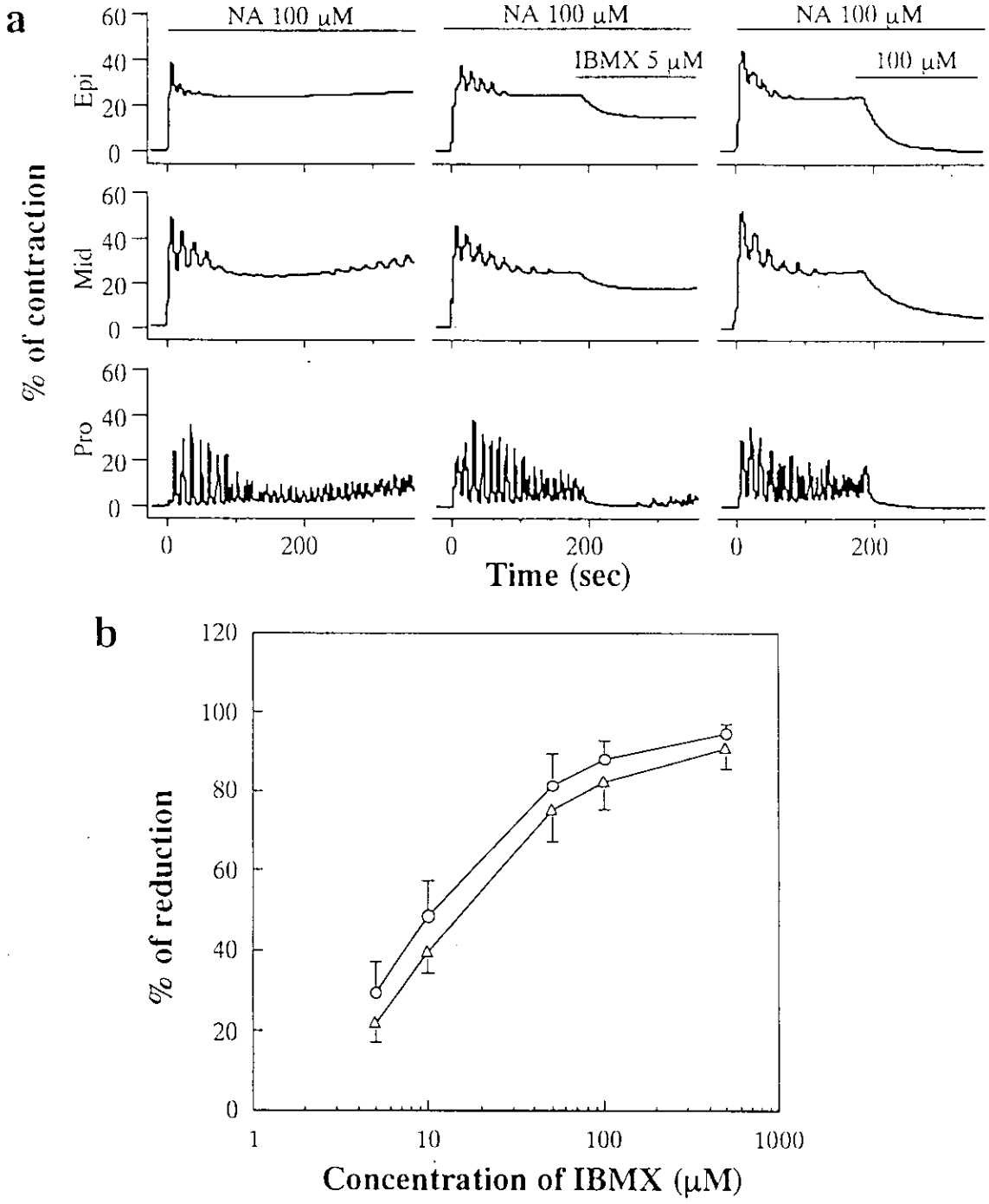


Fig. 4-2 Effects of IBMX on tonic contraction induced by NA. a, Representative responses to NA (100 μM, left) and NA plus IBMX (5 and 100 μM). b, Concentration-response curves of IBMX. Symbols and vertical bars are the same as those in Fig. 4-1.

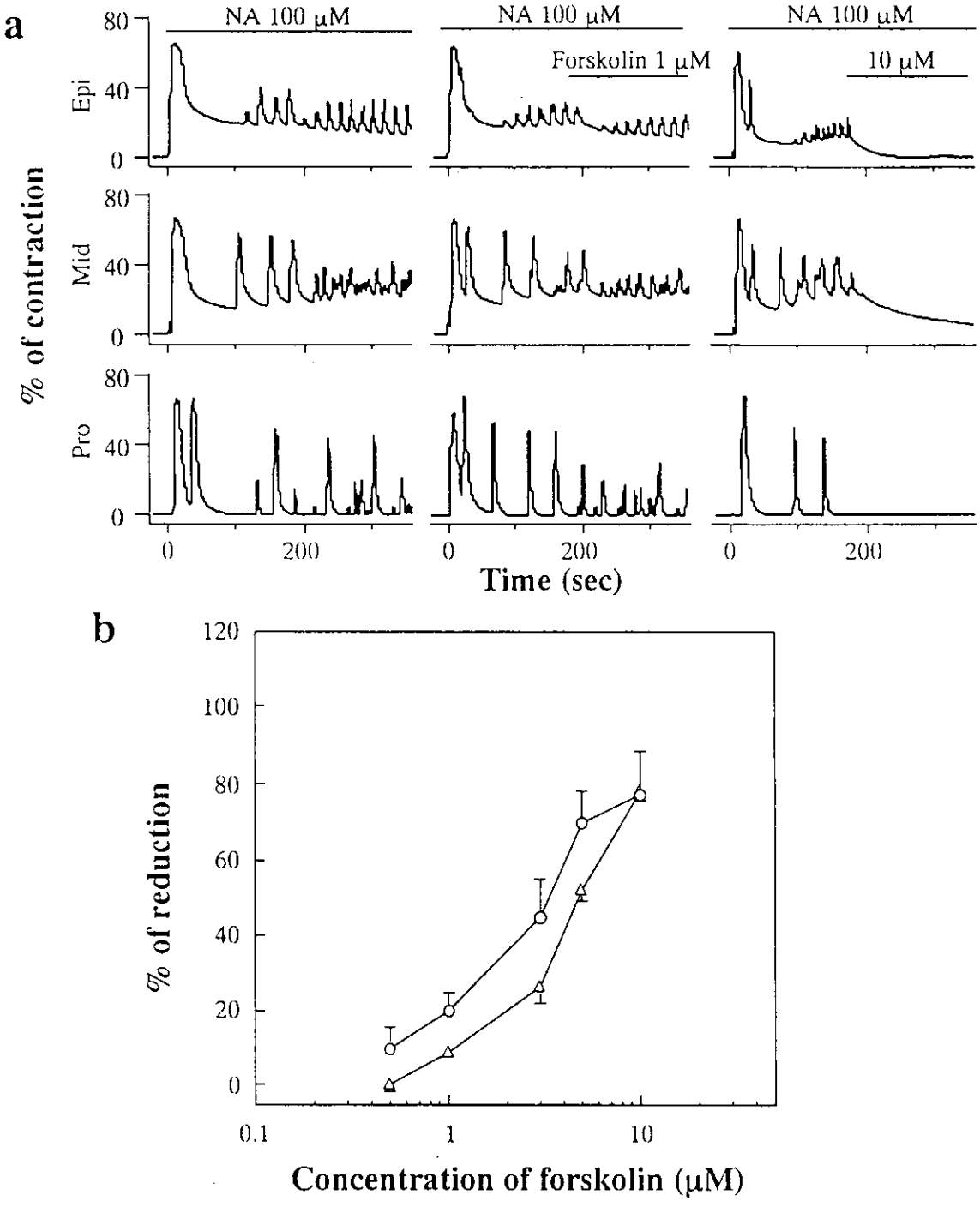


Fig. 4-3 Effects of forskolin on tonic contraction induced by NA. a, Representative responses to NA (100 μM, left) and NA plus forskolin (1 and 10 μM). b, Concentration-response curves of IBMX. Symbols and vertical bars are the same as those in Fig. 4-1.

activity of adenylate cyclase which synthesizes cAMP from ATP. The adenylate cyclase activity can be directly activated by forskolin. Figure 4-3 shows the effects of forskolin on the contraction induced by NA. Forskolin reduced the NA-induced tonic contraction in a concentration-dependent manner. The repetitive transient contractions were also inhibited by forskolin. Caffeine, IBMX and forskolin were reversible.

Effects of pretreatment with IBMX and forskolin on NA-induced contraction

To determine whether cAMP affects the transient phase or not, IBMX and forskolin were applied prior to stimulation of the muscle with NA. Figure 4-4 shows that IBMX inhibited both the transient and tonic phases of the NA-induced contraction in a concentration-dependent manner. The effects of IBMX were more prominent in the prostatic portion than the epididymal portion. Forskolin also inhibited concentration-dependently both the transient and tonic phases of the NA-induced contraction (Fig 4-5). The epididymal side was less sensitive to forskolin than the prostatatic side. IBMX or forskolin alone failed to evoke any response in the guinea pig vas deferens.

Effects of a protein kinase inhibitor on IBMX- or forskolin-induced relaxation

In many kinds of cells, cAMP works through the activity of cAMP-dependent protein kinase (PKA). To determine whether the PKA activity is involved in the caffeine-, IBMX- or forskolin-induced relaxation, the effect of a relatively specific PKA inhibitor, H-89, was examined. Pretreatment with H-89 (10 μM) had no effect on the IBMX-induced

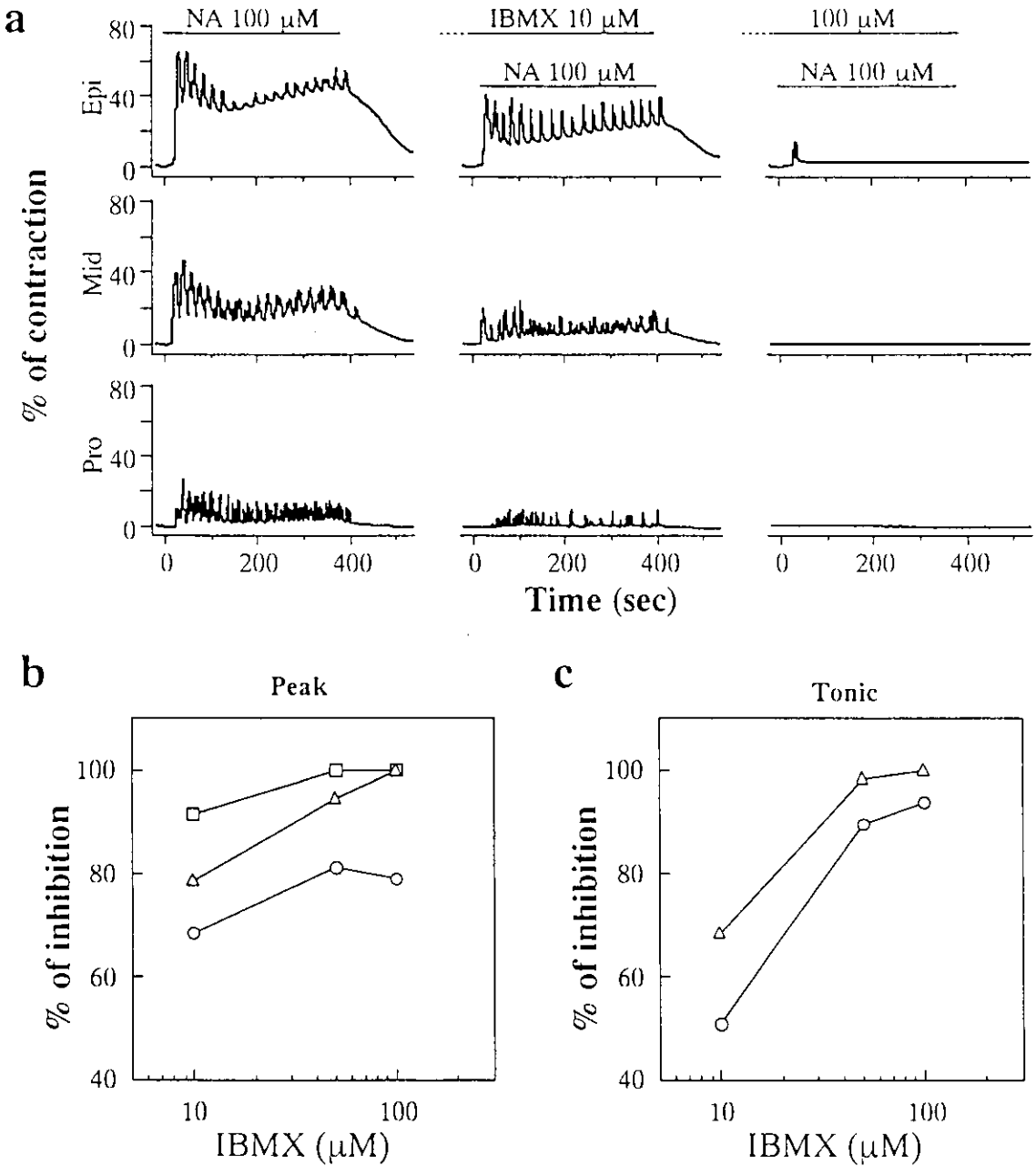


Fig. 4-4 Effects of pretreatment with IBMX on NA-induced contraction. a, Typical responses to NA (100 μM) before and after treatment with IBMX (10 and 100 μM). IBMX was applied to the bath 3 min before NA-application. Concentration-response curves of initial maximum peak contraction (peak, b) and tonic contraction measured at 3 min after NA-application (tonic, c, n = 2). Circles and triangles are the same as those in Fig 4-1. Squares represent the mean response in prostatic portion.

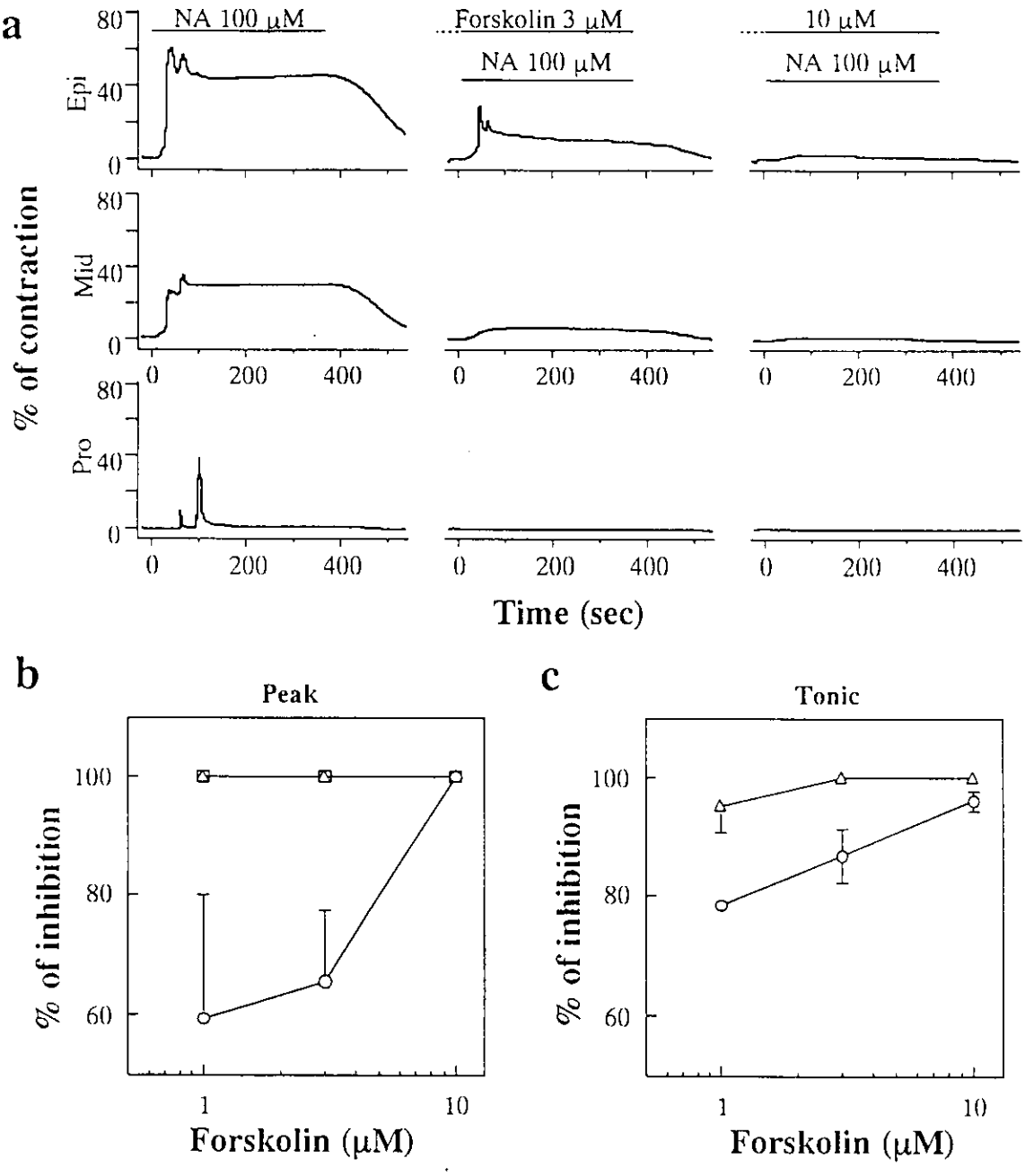


Fig. 4-5 Effects of pretreatment with forskolin on NA-induced contraction. a, Typical responses to NA (100 μM) before and after treatment with forskolin (3 and 10 μM). IBMX was applied to the bath 3 min before NA-application. Concentration-response curves of initial maximum peak contraction (peak, b) and tonic contraction measured at 3 min after NA-application (tonic, c, n = 2). Circles and triangles are the same as those in Fig. 4-1. Symbols are the same as those in Fig. 4-4. Vertical bars represent the s.e.

relaxation or the tonic contraction induced by NA (Fig. 4-6). The forskolin-induced responses were also not affected by H-89 at this concentration (data not shown). Whether the IBMX-induced inhibition of the NA-induced contraction involved PKA activity was then checked by applying H-89 and IBMX to the bath 4 and 3 min prior to NA-application, respectively (Fig. 4-7). IBMX-induced inhibition was also resistant to H-89 (10 μM).

Effects of forskolin on $[Ca^{2+}]_i$ response evoked by NA

Figure 4-8 shows the $[Ca^{2+}]_i$ response to NA and the effect of forskolin on it. As shown in Chapter 3, NA induced a marked increase in $[Ca^{2+}]_i$ superimposed with the oscillations in both epididymal and prostatic portions. Forskolin (10 μM, sufficient to induce relaxation; Fig. 4-3) was applied to the bath 3 min after stimulation with 100 μM NA (right-hand two traces). In both of epididymal and prostatic portions, forskolin caused insignificant inhibition of NA-induced $[Ca^{2+}]_i$ increase. The $[Ca^{2+}]_i$ concentrations of the plateau or basal level of oscillations observed 3 min after forskolin-application were 90 ± 9 and 111 ± 30 nM (n = 5) in the epididymal and prostatic portions, respectively. These values are not significantly different from the $[Ca^{2+}]_i$ measured at 6 min after NA-application in control muscles (97 ± 5 and 95 ± 24 nM, n = 5, in epididymal and prostatic portions, respectively). These data indicate that forskolin induces relaxation of NA-induced contraction without any distinctly coincidental decrease in the $[Ca^{2+}]_i$.

In contrast to the effect of forskolin on the $[Ca^{2+}]_i$ increase evoked by NA, pretreatment with forskolin (10 μM) on the $[Ca^{2+}]_i$ responses to NA (100 μM, Fig. 4-9) caused complete inhibition of NA-induced $[Ca^{2+}]_i$

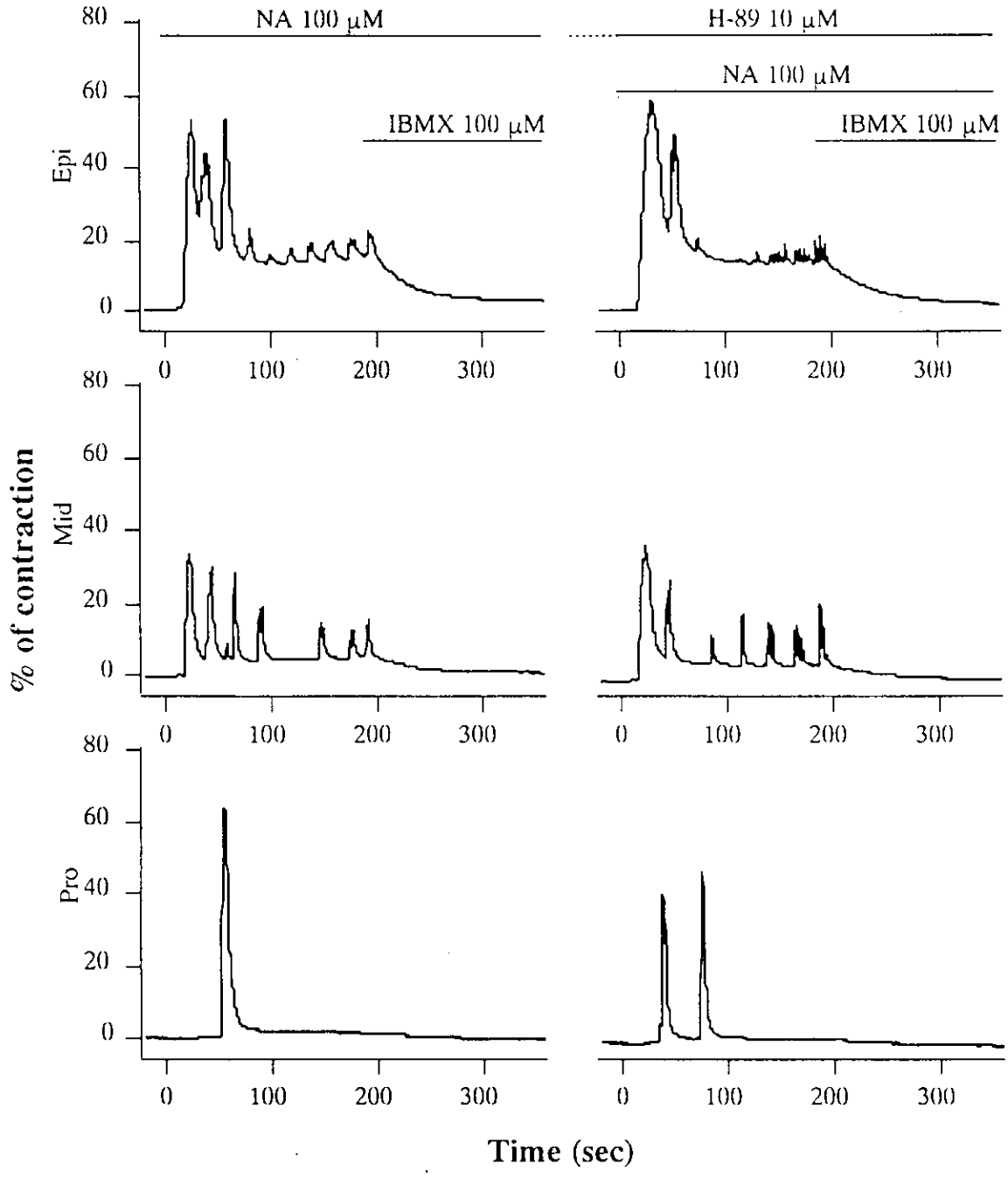


Fig. 4-6 Effects of a relatively PKA-specific inhibitor, H-89, on the cAMP-mediated relaxation. Representative responses to NA (100 μM) and IBMX (100 μM) before and after treatment with H-89 (10 μM). H-89 was applied one min before application of NA (n = 2).

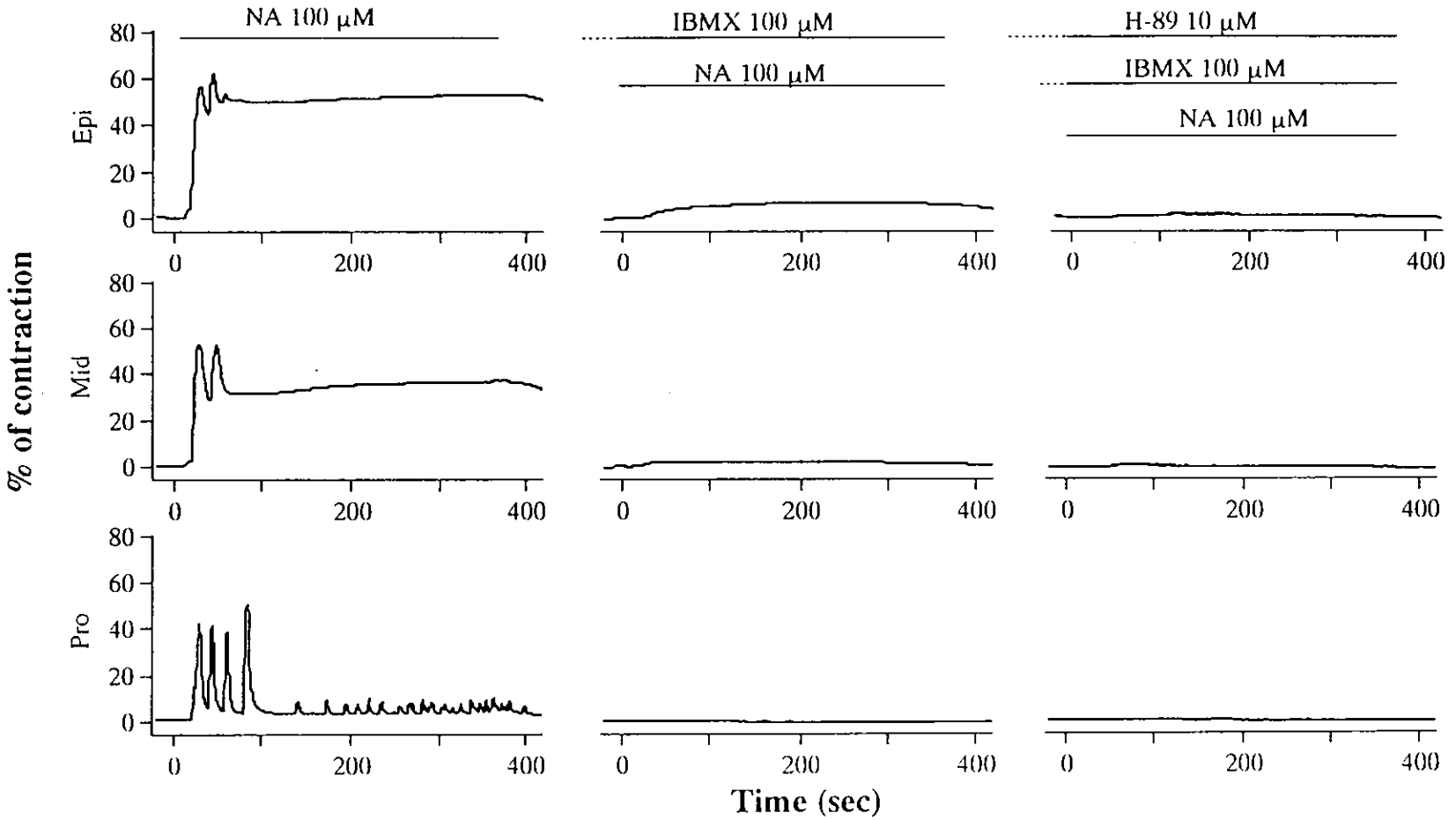


Fig. 4-7 Effects of a relatively PKA-specific inhibitor, H-89, on IBMX-induced responses. Representative responses to NA (100 μM, left) before and after pretreatment with IBMX (100 μM, central) as well as with H-89 (10 μM, right). H-89 and IBMX were applied 4 and 3 min, respectively, before application of NA (n = 2).

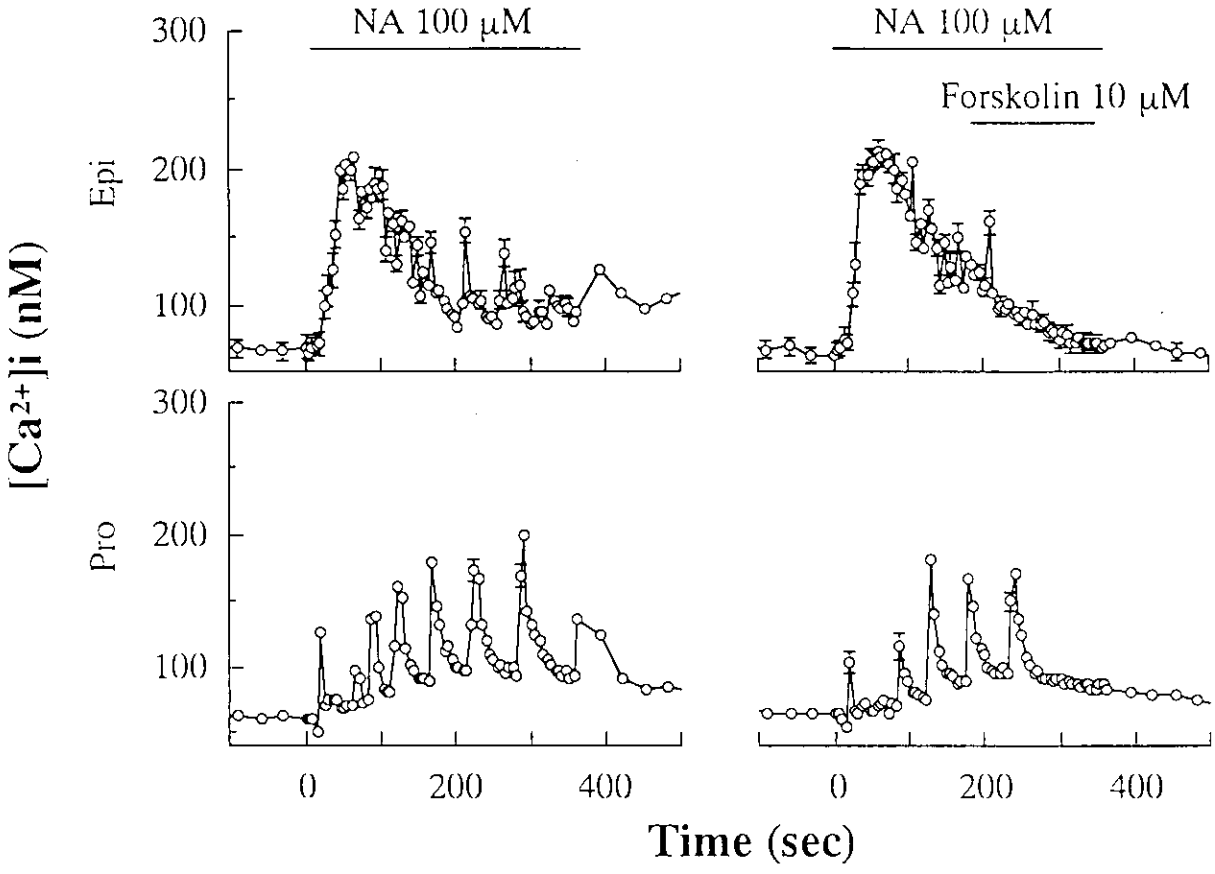


Fig. 4-8 Effect of forskolin on NA-induced $[Ca^{2+}]_i$ responses to NA (100 μM, left) and forskolin (10 μM, right) in epididymal (Epi) and prostatic (Pro) portions. Forskolin was applied to the bath 3 min after NA-application. The data represent the average of 7 points in the measurement area on the one muscle strip and vertical bars show s.e. (n = 4).

response. The inhibitory effect of forskolin was observed in other 3 experiments carried out both in the epididymal and prostatic portions.

Effects of forskolin on $[Ca^{2+}]_i$ responses evoked by excess K^+

As shown in the Chapter 3, external excess K^+ induces contraction and a $[Ca^{2+}]_i$ increase by a mechanism bypassing the receptor activation. The effects of forskolin pretreatment on these responses were examined (Fig. 4-10). In contrast to NA stimulation, the excess K^+ induced a sustained $[Ca^{2+}]_i$ increase without exhibiting oscillations in both epididymal and prostatic portions. Even in forskolin-treated muscles excess K^+ induced a $[Ca^{2+}]_i$ rise to nearly same level as that in control. In contrast to the $[Ca^{2+}]_i$ responses, the contractile responses induced by excess K^+ were different between control and forskolin-pretreated muscles. In the epididymal portion, biphasic contraction in control became almost monophasic by pretreatment with forskolin. In the prostatic portion, contraction in control response was almost completely suppressed by pretreatment with forskolin. The maxima of peak contractions in control and forskolin-pretreated muscles were 60.9 ± 4.1 and 36.1 ± 3.5%, respectively, in the epididymal portion and 48.9 ± 10.8 and 10.7 ± 6.2%, (n = 3) respectively, in the prostatic portion. The tonic contraction (measured at 3 min after K^+ stimulation) were 59.2 ± 7.5% and 6.5 ± 3.2% in epididymal portion and 31.9 ± 9.5% and 0.58 ± 0.3% in prostatic portion of control and forskolin-pretreated muscles, respectively (n = 3).

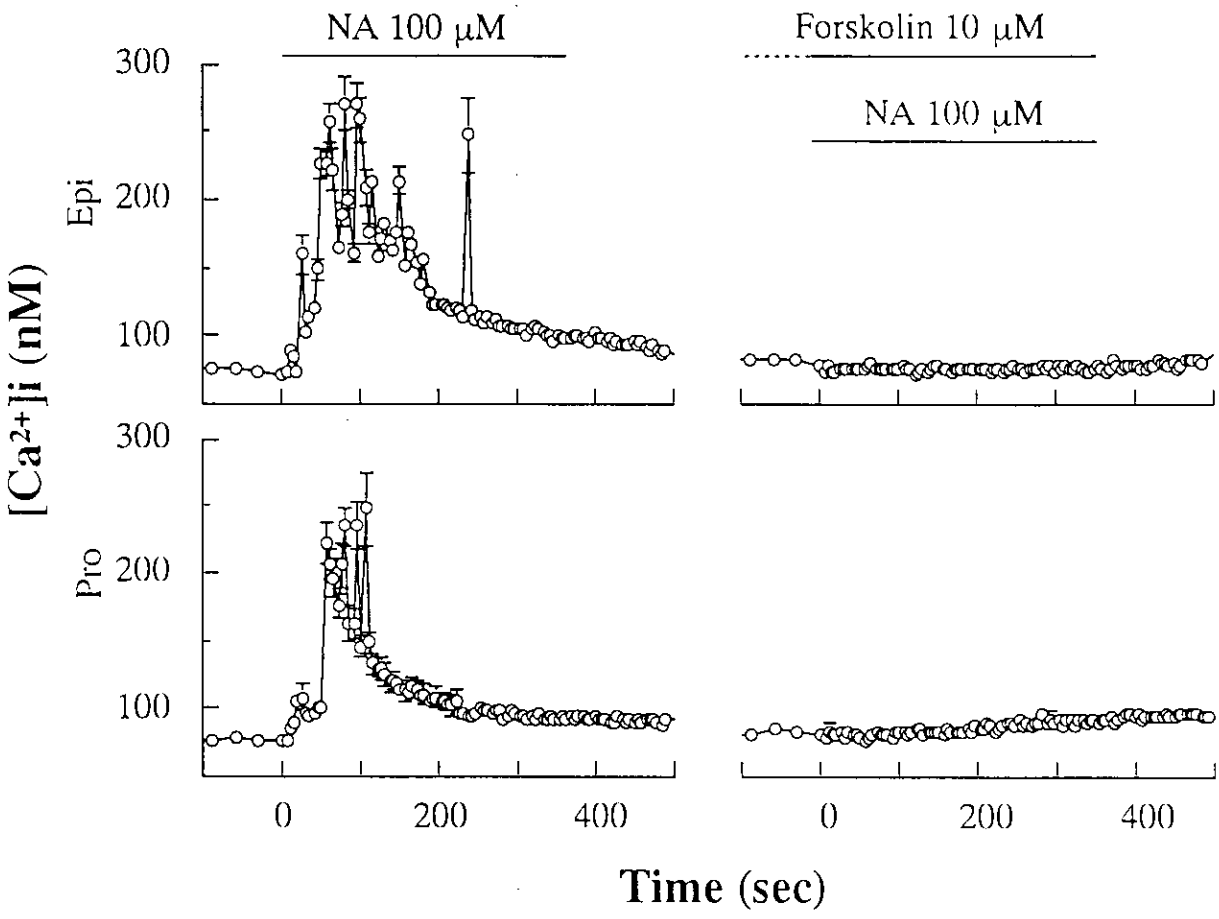


Fig. 4-9 Effects of pretreatment with forskolin on NA-induced $[Ca^{2+}]_i$ responses. $[Ca^{2+}]_i$ responses to NA (100 μM) without (left) and with (right) treatment with forskolin (10 μM). The data represent the average of 7 points in the measurement area on the one muscle strip, and vertical bars are the s.e. (n = 4).

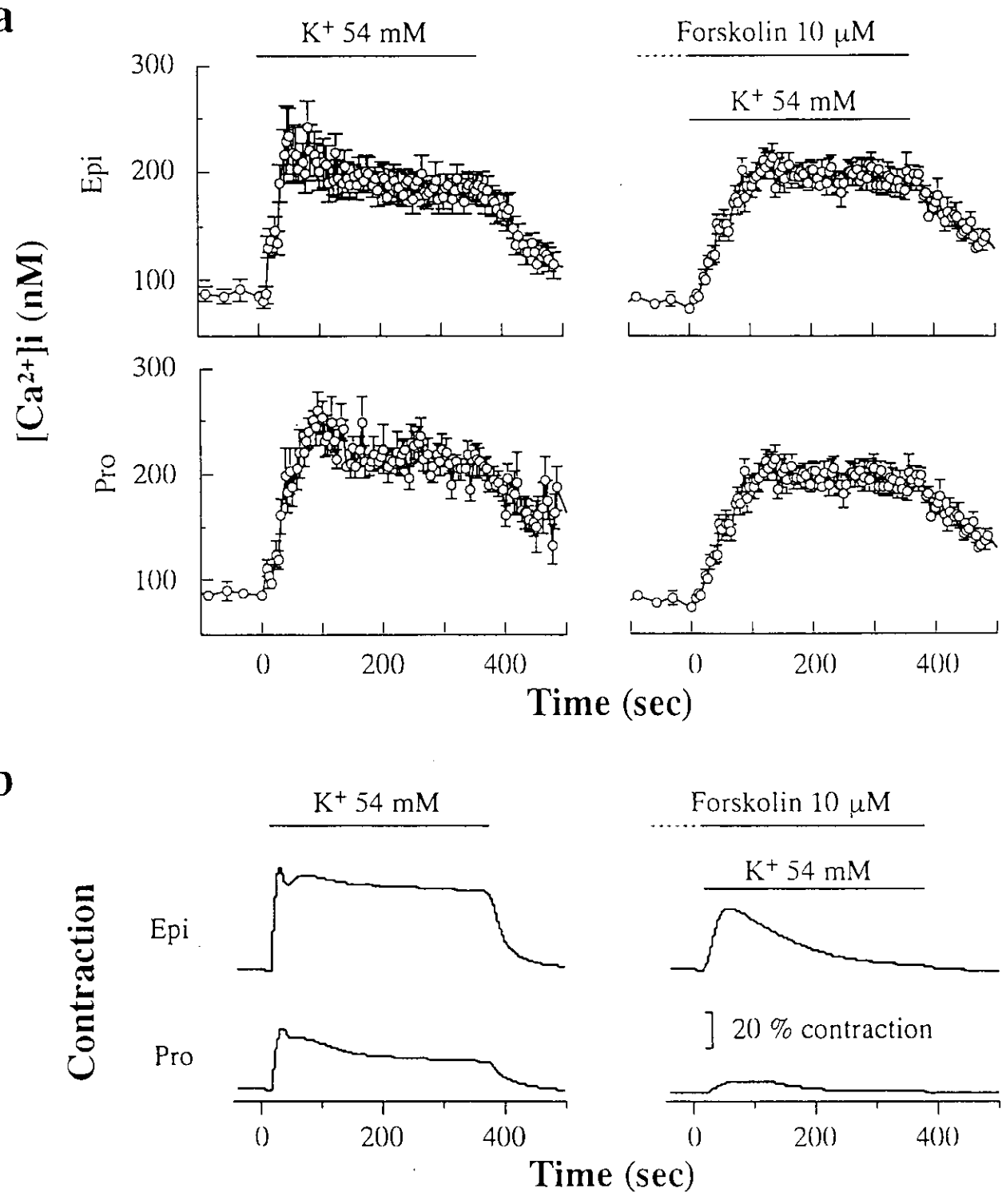


Fig. 4-10 Effects of pretreatment with forskolin on excess external K^+-induced $[Ca^{2+}]_i$ responses. $[Ca^{2+}]_i$ responses (a) and contractile responses (b) to excess external K^+ (54 mM) without (left) and with (right) forskolin pretreatment. The data represent the average of 7 points in the measurement area on the one muscle strip, and vertical bars are the s.e. (n = 2). The data a and b were obtained from different muscles.

Discussion

In this study, it is shown that increased intracellular cAMP induced by methylxanthines or forskolin inhibits the NA-induced muscle contraction in the guinea pig vas deferens. This cAMP-related muscle relaxation seems to be independent of reduction in intracellular Ca^{2+} levels and may involve down-regulation of α_1-adrenergic receptors and modulation of the contractile elements and/or Ca^{2+}-binding proteins. These mechanisms are not necessarily exclusive and may occur in combination. This is shown in Fig. 4-11 schematically.

The intracellular cAMP level is regulated by two well known enzymes; one is a plasma-membrane-bound enzyme adenylate cyclase which synthesizes cAMP from ATP, the other is cAMP phosphodiesterase which rapidly and continuously destroys cAMP to 5'-AMP (Alberts et al., 1994). Since forskolin directly activates adenylate cyclase and methylxthanthines inhibit phosphodiesterase activity, the intracellular cAMP concentration is artificially elevated by these agents. Caffeine, IBMX and forskolin inhibited the NA-induced contraction. These effects were concentration-dependent. Barnette et al. (1992) and Yamagishi et al. (1994) suggested that intracellular methylated xanthines act on the cGMP-specific phosphodiesterase by which the intracellular cGMP concentration is increased. In this experiment, however, the effects of forskolin and methylxanthines (caffeine and IBMX) on NA-induced contraction were very similar to each other. Therefore, it is suggested that the suppressing effects of these agents on contraction predominantly result from elevation of intracellular cAMP level.

The elevation of the intracellular cAMP concentration triggers activation of cAMP-binding protein kinase (PKA). PKA is known to mediate smooth muscle relaxation (Jin et al., 1993). In these experiments,

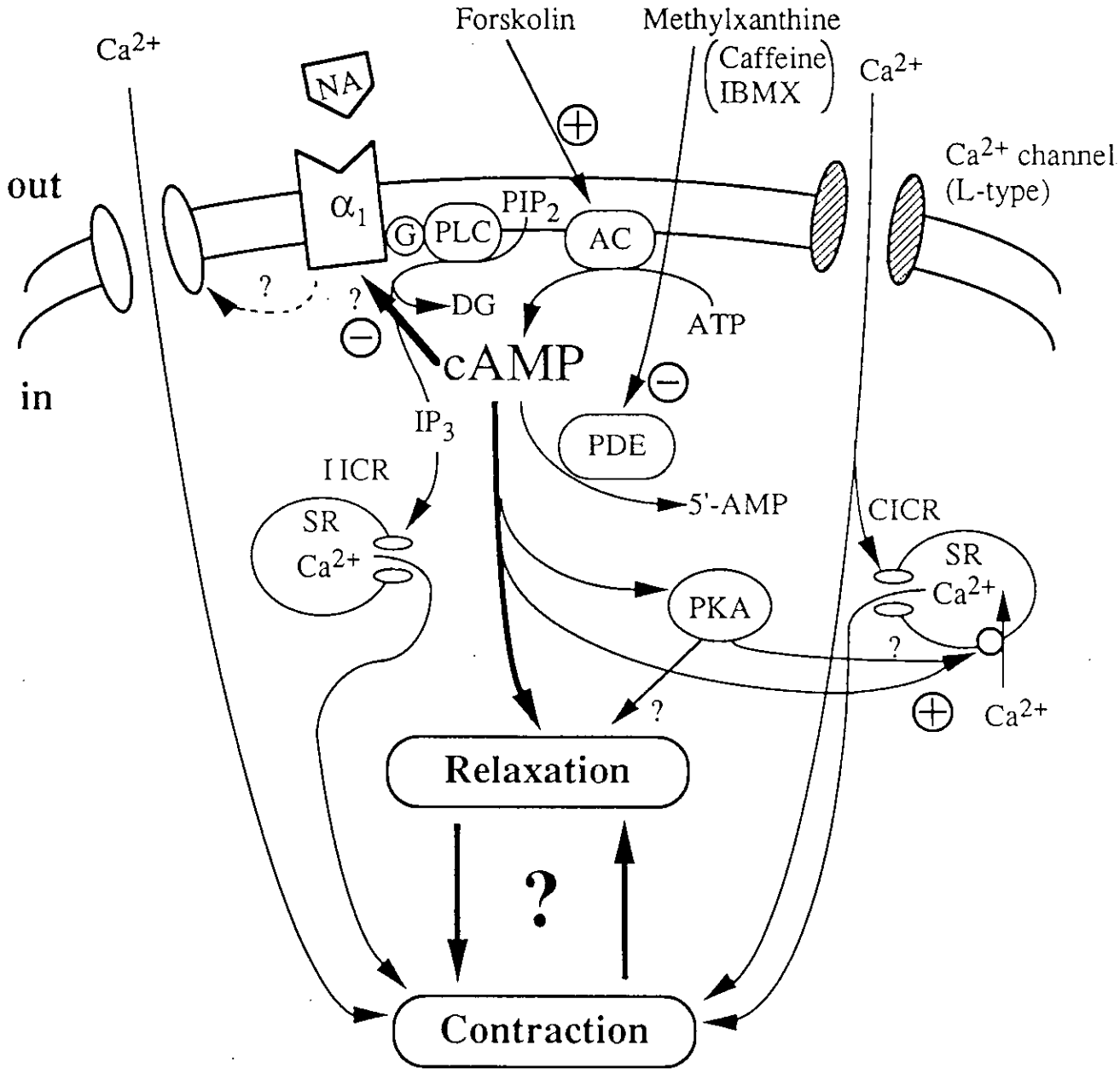


Fig. 4-11 A schematic model for cAMP-mediated relaxation of NA-induced contraction in guinea pig vas deferens. AC, adenylate cyclase; PDE, phosphodiesterase; PKA, protein kinase A; SR, sarcoplasmic reticulum; PIP_2, phosphatidylinositol-4,5-bisphosphate; DG, diacylglycerol; IP_3, inositol-1,4,5-triphosphate; CICR, Ca^{2+}-induced Ca^{2+} release; I ICR, IP_3-induced Ca^{2+} release; G, GTP-binding protein; PLC, phospholipase C.

however, a relatively PKA-specific inhibitor, H-89 (10 μM), failed to affect the IBMX- or forskolin-induced relaxation. Thus, it appears that cAMP directly affects the NA-induced contractile response.

In contrast to the cAMP effect on the contraction induced by NA, cAMP could not largely alter the NA-induced $[Ca^{2+}]_i$ responses. Both the epididymal and prostatic portion, the $[Ca^{2+}]_i$ levels at 6 min after NA-application in the forskolin-treated muscle and control muscle were not different significantly and were significantly larger than the resting $[Ca^{2+}]_i$ level. However, NA-induced contraction was immediately suppressed when caffeine, IBMX and forskolin were applied to the bath, and was almost relaxed to initial length at 3 min after application of these agents. These facts suggest that NA-induced contraction was suppressed by cAMP without coincidental decrease of $[Ca^{2+}]$. These facts suggest that NA-induced contraction was suppressed by cAMP via Ca^{2+}-independent mechanism(s). A number of researchers have reported similar cyclic nucleotide-induced Ca^{2+}-independent relaxation of smooth muscle. In the rat mesenteric artery smooth muscle cells permeabilized by α-toxin the $[Ca^{2+}]_i$ level was constant during cAMP-mediated relaxation (Nishimura & Breemen, 1989). Cyclic AMP also inhibited muscle tension more strongly than $[Ca^{2+}]_i$ in vascular smooth muscle exposed to high K^+ (Abe & Karaki, 1992). Similar results were obtained also from coronary artery (Yamagishi et al., 1994). $[Ca^{2+}]_i$-independent relaxation by cAMP was also reported by Willenbucher et al. (1992) in rabbit colon and Smith et al. (1993) in canine colon. They concluded that cAMP reduced the Ca^{2+} sensitivity of the contractile elements. Taken together, it can be conceded that cAMP-induced $[Ca^{2+}]_i$-independent relaxation mechanism(s) may exist irrespective of tissue and animal species.

Cyclic AMP has also been reported to directly affect the contractile enzyme, myosin light chain kinase (MLCK). Cyclic AMP and PKA

reduced myosin light chain phosphorylation down to 2-fold smaller than the control level in chicken gizzard (Adelstein et al., 1978). The direct action of cAMP on MLCK and on actin-myosin interaction in chicken gizzard was also suggested by Ozaki et al. (1990). It is possible to speculate, therefore, that cAMP directly inhibits MLCK not via PKA in the guinea pig vas deferens.

It was shown that pretreatment with either forskolin or IBMX inhibited both contractile and Ca^{2+} responses to NA. On the other hand, the contractions induced by excess external K^+ were attenuated by pretreatment with forskolin, whereas the Ca^{2+} responses were not significantly affected. These results suggest that cAMP also affects the NA-induced $[Ca^{2+}]_i$ increase presumably due to Ca^{2+} influx via receptor-operating Ca^{2+}-channels. Although further investigations are necessary to elucidate the precise mechanisms of cAMP effects on NA-induced responses, it is likely that inhibition of NA-induced $[Ca^{2+}]_i$ increase by cAMP may involved down-regulation of the α_1-adrenergic receptor.

Chapter 5

Summary and Conclusion

The vas deferens smooth muscles play an essential role in the transport of spermatozoa and seminal emission from the testis to the urethra. However the mechanisms and regulatory factors of their contraction remaind in almost unknown. Thus, the contractile responses to neurotransmitters and excess external K^+ in the guinea pig vas deferens have been studied in this thesis, with reference to cellular mechanisms of muscle innate contraction and relaxation.

In Chapter 2, the basic contractile properties of the guinea pig vas deferens were investigated. Contractile responses to 100 μM of noradrenaline (NA), adenosine triphosphate (ATP) and acetylcholine (ACh) were markedly altered by cooling the bath solution to 25 °C.

The NA-induced contractions were inhibited by prazosin but not by propranolol or yohimbin, indicating that the α_1-adrenoceptor was dominant in the vas deferens. Pirenzepine (M_1 antagonist) was more potent at inhibiting the ACh-induced contractions than antagonists of other muscarinic acetylcholine receptor subtypes, suggesting that the M_1-muscarinic acetylcholine receptor was dominant. In the ATP responses, prior treatment with α,β-methylene ATP blocked ATP-induced contractile responses. Suramin, recently accepted as a P_{2X} purinergic receptor blocker, attenuated contractions induced by a high concentrations of ATP (100 μM) and completely abolished responses to the low concentration (10 μM).

NA-, ATP- and ACh-induced contractions were almost completely inhibited by deprivation of extracellular Ca^{2+}, although nifedipine (L-type Ca^{2+} channel blocker, 10 μM) did not inhibit completely the tonic phase

of the NA- and ACh-induced contractions. The ATP-induced responses were inhibited by nifedipine. These data suggest that the NA-, ATP- and ACh-induced contractions require the influx of Ca^{2+} from the extracellular space, and that a nifedipine-insensitive pathway, presumably receptor-operative Ca^{2+}-channels may contribute to the NA- and ACh-induced influx.

In Chapter 3, the contractile responses to various concentrations of NA, ATP and ACh, and to excess external K^+ in the epididymal, middle and prostatic portions of the guinea pig vas deferens were investigated by measuring the isotonic contraction and monitoring the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) using fura-2 fluorescence. In the epididymal portion, the contraction evoked by each of these agonists was biphasic comprising a transient followed by a tonic phase. In the middle portion, NA and ACh evoked biphasic contractions whereas the ATP-induced contraction was an almost monophasic transient. In contrast, in the prostatic portion, only transient contractions were evoked by ACh and ATP, while the NA-induced contraction was oscillatory. The maximum responses of tonic contraction to each of the neurotransmitters were largest in the epididymal portion, decreased in the middle and were almost absent in the prostatic portion. These regional differences in the contractile properties of the vas deferens were also evident upon stimulation with excess external K^+. Such regional differences in contraction may involve regional differences in the Ca^{2+} homeostasis and/or the sensitivity of the contractile apparatus to intracellular Ca^{2+} ions. The physiological relevance of the muscle innate regional contractile differences are unknown, however, it may contribute to the transport of spermatozoa by preventing back flow of the luminal contents, together with neuronal regulation.

In Chapter 4, the effects of caffeine, 3-isobutyl-1-methylxanthine (IBMX) and forskolin on the NA-induced contraction were investigated. All drugs reduced the tonic contraction induced by NA in a concentration-dependent manner. Methylxanthines (caffeine and IBMX), by inhibiting the phosphodiesterase activity, cause an increase in intracellular cAMP concentration. Forskolin, by directly stimulating adenylate cyclase, also causes an increase in intracellular cAMP concentration. It is thus suggested that the reduction of NA-induced tonic contraction by these drugs may be mediated by cAMP. Pretreatment with these drugs inhibited the NA-induced contraction in a concentration-dependent manner, suggesting that cAMP affected not only the tonic phase but also the initiation of contraction evoked by NA. The effects of cAMP on NA-induced $[Ca^{2+}]_i$ responses were investigated using the same protocol as for the contractile responses. Forskolin (10 μM) did not have significant sustained effects on the NA-induced $[Ca^{2+}]_i$ rise. In only the epididymal portion, a small transient $[Ca^{2+}]_i$ decrease was observed. In contrast, pretreatment with forskolin (10 μM) completely inhibited the $[Ca^{2+}]_i$ increase. With excess external K^+ stimulation, the same concentration of forskolin did not inhibit the $[Ca^{2+}]_i$ increase. However, although sufficient increases of $[Ca^{2+}]_i$ were observed, contractile responses to excess K^+ were attenuated by pretreatment with forskolin. The inhibiting effect of forskolin suggests that relaxation mechanism(s) independent of decrease in $[Ca^{2+}]_i$ exists in the smooth muscle and that cAMP may be involved in this Ca^{2+}-independent relaxation.

In summary, it is concluded as follows:

1) The contractile responses to neurotransmitters were demonstrated to be potentiated by cooling the ambient temperature, whereas such hypothermic potentiation was not observed for excess K^+-induced contraction of guinea pig vas deferens.

2) The α_1-adrenergic, M_1-muscarinic acetylcholine and P_{2X}-purinergic receptors play essential roles in mediating the neurotransmitter-induced contractions of the vas deferens.

3) Extracellular Ca^{2+} is necessary for the neurotransmitter-induced contraction of vas deferens. Nifedipine-insensitive Ca^{2+} influx pathways may be indispensably involved in the NA- and ACh-induced contractions.

4) In the guinea pig vas deferens, there are regional differences in the contractile responses to various neurotransmitters. These differences may involve variation in the mechanisms of Ca^{2+} homeostasis and the sensitivity of contractile apparatus to intracellular Ca^{2+} ions.

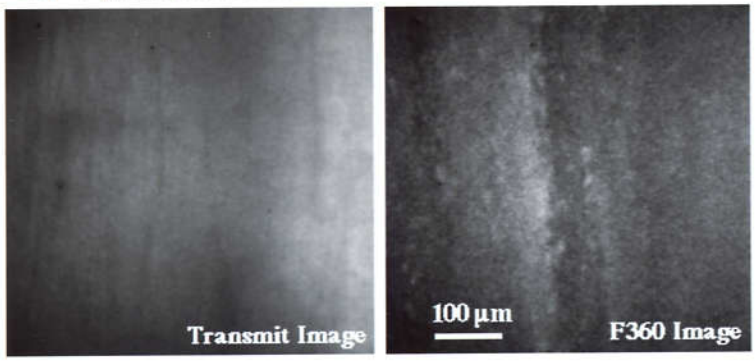
5) Increases of intracellular cAMP level may directly relax NA- and excess external K^+-induced contractions by a mechanism independent of reduction in $[Ca^{2+}]_i$. Increased cAMP may also inhibit the NA-induced Ca^{2+} influx via nifedipine-insensitive Ca^{2+} pathways, thereby inhibiting NA-induced $[Ca^{2+}]_i$ rise, and contraction.

Color plate figure

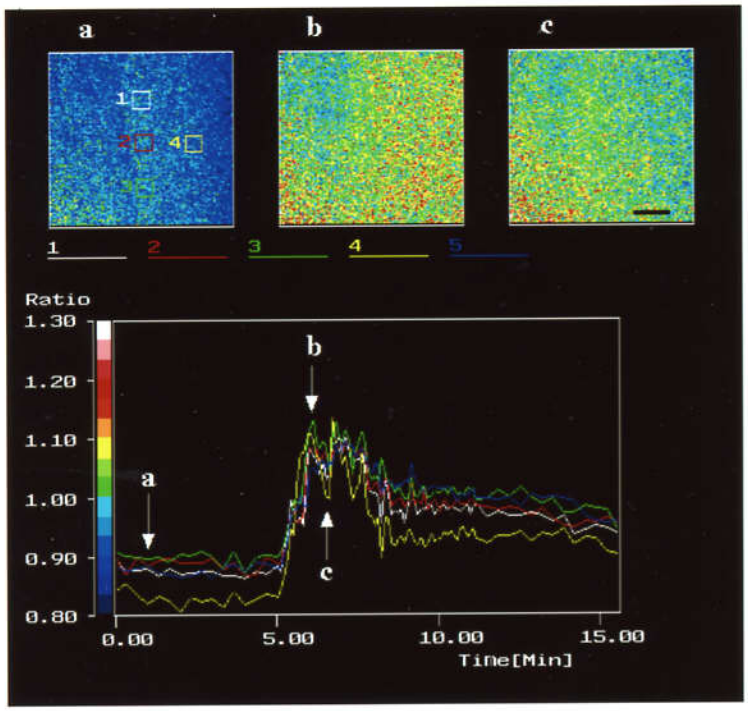
Color plate figure. (next page).

A), A transmit image (left) and an F360 image after loading with fura-2 in the epididymal portion. Scale: 100 μm. B and C), Representative $[Ca^{2+}]_i$ responses to NA (100 μM) in the epididymal portion and prostatic portion, respectively. Superimposed panels, the ratio images at the time indicated by arrows. Scale: 100 μm.

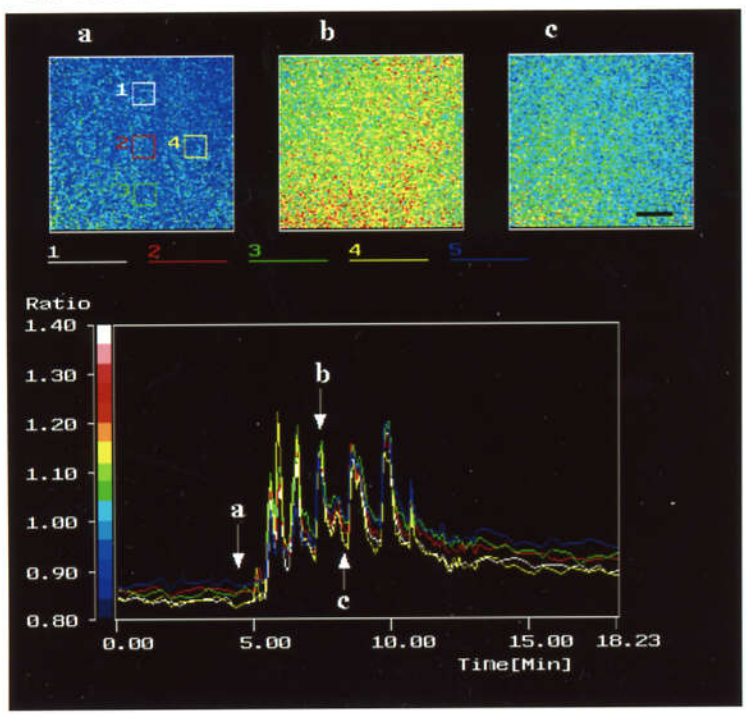
A. Epididymal Portion



B. Epididymal Portion



C. Prostatic Portion



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