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Pharmacology and thermosensitivity of the dartos muscle isolated from rat scrotum

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1 The dartos is a thin sheet of smooth muscle closely associated with the skin of the scrotum. Although known to play an important role in scrotal thermoregulation, there has been no detailed study into the pharmacology, or thermosensitivity, of the dartos from any species. Here, we investigate these two parameters in the isolated dartos muscle from rat.

2 Field stimulation of the rat dartos caused contractions that were abolished by tetrodotoxin, phentolamine and guanethidine, but unaffected by atropine or L-N^G-nitroarginine. Exogenous noradrenaline also produced contractions blocked by both phentolamine and prazosin. In muscles with raised tone and negated sympathetic function, field stimulation failed to elicit relaxation. The dartos muscle did not contract in response to carbachol, nicotine, histamine, 5-hydroxytryptamine (all up to 100 μM) or substance P (up to 1 μM).

3 Contractile responses to field stimulation and noradrenaline were much greater at 30°C compared with 40°C; indeed, contractions to 1 μM noradrenaline at 30°C were relaxed by around 80% on heating to 40°C. Similar heat-induced relaxations were observed during contractions to both U46619 (100 nM) and high K (70 mM).

4 In contrast, contractile responses to the myosin phosphatase inhibitor calyculin-A (1 μM), either in the presence or absence of external calcium, were resistant to relaxation by heating. In calcium-free medium at 30°C, U46619 continued to produce contractions that were again relaxed by 80% on heating to 40°C. However, in the presence of calyculin-A, this heat-induced relaxation was greatly reduced.

5 Thus, the rat dartos muscle receives a functional sympathetic innervation and contracts to noradrenaline *via* α-adrenoceptors. There is no functional inhibitory innervation. Experiments with calyculin-A suggest that myosin phosphatase is a major contributor to the marked thermosensitivity of the dartos muscle.

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Abbreviations: EGTA, ethyleneglycol-bis(βaminoethyl)-N,N,N′,N′-tetraacetic acid

Introduction

The dartos muscle (*tunica dartos*) is a thin sheet of smooth muscle closely associated with the skin of the scrotum. The smooth muscle bundles are intermingled with fibroelastic tissue, and are arranged as a criss-cross meshwork with blood vessels running through the spaces between the bundles (Shafik, 1973; Cold & Taylor, 1999). Functionally, the dartos plays an important role in scrotal thermoregulation and therefore in the maintenance of the optimal temperature for spermatogenesis (about 2–3°C below body temperature; Tyrrell *et al*., 1942; Shafik, 1973; Spira, 1991). In cold conditions the dartos muscle contracts, possibly *via* a local thermoregulatory network linking temperature receptors on the scrotal skin surface to the smooth muscle cells of the dartos (Maloney & Mitchell, 1996). This contraction causes wrinkling of the scrotal skin and a marked reduction in the surface area of the scrotum, thereby reducing heat exchange (Waites & Moule, 1961; Shafik, 1973); at the same time, the testicles are drawn nearer to the warmth of the abdomen. There is also a vascular effect since, as the dartos contracts, the spaces between the smooth muscle meshwork close down, occluding the blood vessels running within them and again reducing heat loss. The opposite occurs in hot conditions, when the dartos relaxes and heat exchange is increased. Animal studies have shown a clear inverse relationship between the level of contraction of the dartos and the external environmental temperature (Maloney & Mitchell, 1996; El-Darawany, 1999), and have confirmed that the state of contraction of the dartos muscle is one of the major factors controlling scrotal temperature (Maloney & Mitchell, 1996). Furthermore, in man, abnormalities of the dartos may be associated with infertility (Shafik, 1978). Given this central role in testicular thermoregulation and spermatogenesis, it is clearly of importance that the factors (both internal and external) influencing the contractile state of the dartos are fully understood. Previous anatomical investigations have

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suggested that the dartos muscle receives a sympathetic innervation, probably *via* the genital branch of the genito-femoral nerve (Dean & Pegington, 1996). However, to date, there have been no detailed investigations into the functional responses of the dartos during nerve stimulation, or into the temperature sensitivity of these responses. Further, there is little or no information regarding the pharmacology of the muscle. Consequently, in the present study, we describe experiments in which we have recorded tension responses from the isolated dartos muscle of the rat in response to field stimulation and drugs, with specific emphasis on the influence of temperature on the contractile response.

Methods

Male rats (Wistar; 225–350 g) were killed by stunning and exsanguination. The testes were pushed down into the scrotum and the fur removed from the scrotal skin. The scrotum was then opened by making a transverse incision approximately 5 mm below the penis. This incision was continued dorsally in the direction of the anus until the whole of the scrotal skin, including the underlying dartos, could be dissected free from the animal. The anus was included in the dissected material to act as a marker for tissue orientation, but care was taken to exclude it from any tissue subsequently used for experimentation. The scrotum was then pinned out as a flat sheet on a cork board (scrotal skin facing downwards/dartos muscle facing upwards). Using the anus as a reference point, the tissue was bisected along the anus-to-penis midline and then strips of muscle (approximately 10 mm long by 2 mm wide) were cut at right angles to the midline. Using this procedure, at least six muscle strips could be obtained from each animal. However, the mean values reported in the present study include the results from strips obtained from at least three different rats. In preliminary experiments (not shown), tension responses were obtained from strips cut either at right angles to the midline or running parallel to it. No difference was observed between the two methods (presumably as a result of the meshwork arrangement of the dartos smooth muscle) but we decided to use the transverse strips as our standard for all later experiments.

The muscle strips (and associated scrotal skin) were then set up in organ baths (25 ml) containing Krebs bicarbonate buffer (mM: NaCl 118.1, KCl 4.7, $MgCl_2$ 1.0, KH_2PO_4 1.0, glucose 11.1, $NaHCO_3$ 25.0, $CaCl_2$ 2.5) which was gassed continuously with 95%O_2 : 5%CO_2. High K (70 mM) solution was obtained by increasing the amount of KCl and reducing the NaCl appropriately to maintain isotonicity. In the Ca-free medium, $CaCl_2$ was omitted and 1 mM ethyleneglycol-bis(βaminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA) added. The temperature of the inner organ bath was maintained *via* a water jacket supplied by a water heater/circulator; actual temperature in the bath was recorded by a thermometer permanently immersed in the Krebs bathing medium. In some experiments, the temperature of the organ bath was raised from 30°C to 40°C, and then reduced back to 30°C, during an established response to a contractile agent. The time taken to complete the change in either direction was around 8 min. A resting tension of 1 g was placed on the tissue and changes in tension recorded by force-displacement tension transducers (Grass FT03) attached to either Lectromed or Graphtec pen recorders. Again, in preliminary experiments carried out at 35°C, it was found that 1 g resting tension gave optimal tension responses to field stimulation and drugs. Muscles were allowed to equilibrate for 30 min before any experimental procedures were initiated. Electrical field stimulation was applied *via* two parallel electrodes running down either side of the tissue; these were attached to a Grass (S48) stimulator (70V; 1 ms pulse width).

Concentration-response curves to agonists were obtained by cumulative addition of drugs to the bath; responses to each concentration were allowed to reach a plateau before addition of the subsequent dose.

All results are expressed as mean ± s.e.mean and statistical analysis was by Students' *t*-test, with a P value of less than 0.05 taken as representing a significant difference. Q_{10} is defined as the ratio of the change in response associated with a 10°C change in temperature. Thus in the present study, Q_{10} represents the ratio of contraction amplitude at 30°C/contraction amplitude at 40°C.

Drugs used during the course of this study were (obtained from Sigma unless stated otherwise): atropine sulphate; calyculin-A (Tocris); carbachol, guanethidine monosulphate, histamine dihydrochloride, nicotine hydrogen tartrate, L-N^G-nitro-arginine hydrochloride, noradrenaline bitartrate, phentolamine hydrochloride, prazosin hydrochloride, propranolol hydrochloride, 5-hydroxytryptamine hydrochloride, substance P, tetrodotoxin, and U46619. All drugs were dissolved in deionized water except calyculin-A and U46619 which were dissolved in dimethylsulphoxide; the final concentration of dimethylsulphoxide in the bath never exceeded 0.1% (v v^{-1}) and had no discernible effect on the tissue by itself.

Results

Under the *in vitro* conditions used in this study, the resting dartos tissue displayed no spontaneous contractile activity and retained a steady baseline tension in the absence of drugs. Further, in muscles initially set up at 35°C, varying the temperature between 30–40°C had no significant effect on resting tension.

Responses to field stimulation

Field stimulation (1, 2, 5, 10, 20 and 50 Hz; 20 s train every 10 min) of the rat dartos muscle resulted in frequency-dependent contractions that displayed a marked degree of temperature sensitivity. Figure 1 shows the frequency-response curves for field stimulation-evoked contractions from muscles set up and maintained at either 30°C, 35°C or 40°C, and it is clear that the amplitude of the contractions was inversely related to organ bath temperature. At all three temperatures, field stimulation-induced contractions were abolished following incubation for 10 min with 1 μM tetrodotoxin, confirming that they were initiated by nerve activation (data not shown). Further, at all three temperatures, the contractions were greatly reduced in the presence of the non-selective α-adrenoceptor antagonist phentolamine (1 μM; shown for 30°C in Figure 1).

To investigate in more detail the pharmacology of these nerve-evoked contractions, we used a standard sub-maximal frequency of 5 Hz (40 s train) and a standard temperature of

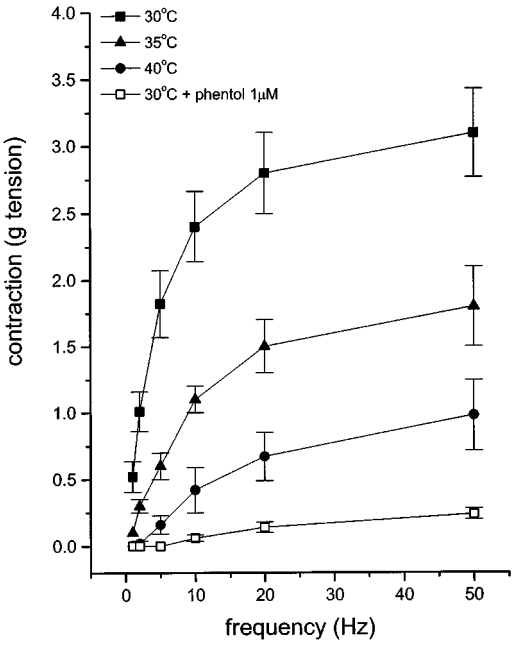

Figure 1 Frequency-response curves for contractions of the rat dartos muscle in response to field stimulation (20 s trains every 10 min). Muscles were set up and maintained at 30°C, 35°C or 40°C. The effect of phentolamine (phentol) on the frequency-response curve at 30°C is also shown. The results are given as mean ± s.e.mean of at least five muscle preparations from at least three different animals.

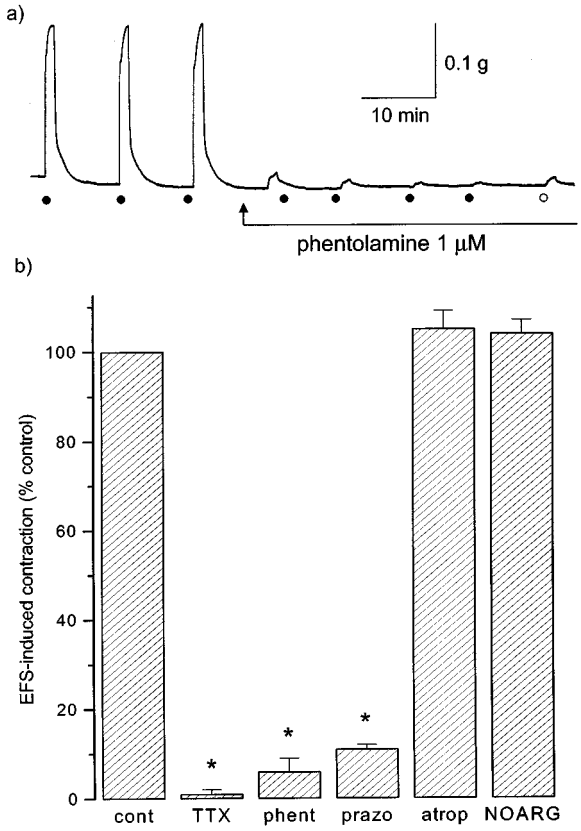

Figure 2 (a) Trace showing contractions of the rat dartos muscle (35°C) in response to field stimulation (40 s trains at 5 Hz at the closed circles) and the effect on these of phentolamine. At the open circle the frequency was increased to 20 Hz. (b) Histogram showing the effects of tetrodotoxin (TTX; 1 μM), phentolamine (phent; 1 μM), prazosin (prazo; 100 nM), atropine (atrop; 100 nM) and L-N^G-nitroarginine (NOARG; 100 μM) on the amplitude of contractions to field stimulation expressed as a percentage of the control (40 s trains at 5 Hz; 35°C). Each point is the mean ± s.e.mean of at least five muscle preparations from at least three different animals. * – $P<0.05$, significantly different from control.

35°C (Figure 2). As mentioned above, contractions to field stimulation were virtually abolished in the presence of either tetrodotoxin or phentolamine. The selective α_1-adrenoceptor antagonist prazosin (100 nM) also greatly reduced the contractions, but they were unaffected by either the muscarinic cholinoceptor antagonist atropine (100 nM) or the nitric oxide synthase inhibitor L-N^G-nitroarginine (100 μM). Negation of field-stimulation-induced contractions by phentolamine suggested that they were mediated by the release of transmitter from sympathetic nerves. This was confirmed in other experiments in which contractions to field stimulation (trains of 5 Hz) were abolished by the adrenergic neurone blocking agent guanethidine (100 μM; $n=4$; data not shown).

Responses to noradrenaline

Exogenous noradrenaline (0.1 – 100 μM) produced sustained, concentration dependent contractions of the dartos muscle (Figure 3a). Like the contractions to field stimulation, responses to noradrenaline showed marked temperature sensitivity. Figure 3b shows the concentration-response curves for noradrenaline-induced contractions from muscles set up and maintained at 30°C, 35°C or 40°C. Again there was a clear inverse relationship between the amplitude of the contractions and organ bath temperature. To examine the pharmacology of these contractions, we used a standard temperature of 35°C and a standard sub-maximal noradrenaline concentration of 3 μM. The response to noradrenaline was completely abolished in the presence of the non-selective α-adrenoceptor antagonist phentolamine (1 μM) and by low concentrations of the α_1-adrenoceptor selective antagonist prazosin (10 nM; data not shown). Conversely, the non-selective β-adrenoceptor antagonist propranolol (1 μM) had no effect on the response to 3 μM noradrenaline.

In all experiments described so far, the muscles were initially set up, and then maintained, at a constant temperature (30°C, 35°C or 40°C). In a further series of experiments, we determined the effect of changing the temperature during an established contraction. Here, the muscles were initially set up at 30°C and then contracted with 1 μM noradrenaline. Once the rise in tone had stabilized, the organ bath temperature was raised from 30°C through to 40°C and then back to 30°C (Figure 4). As the temperature in the organ bath rose, there was a marked relaxation of noradrenaline-induced tone (by around 80 – 90% at 40°C); during the subsequent rise in temperature tone returned to the original level (Figures 4 and 5). This effect was dependent on the magnitude of the temperature change; if the heating phase was stopped at 35°C then the relaxation peaked at around 50% before returning to the original 30°C level during subsequent cooling (Figure 5). Further, in control experiments in which the temperature was raised from 30°C to 40°C and then held there (i.e., without the subsequent

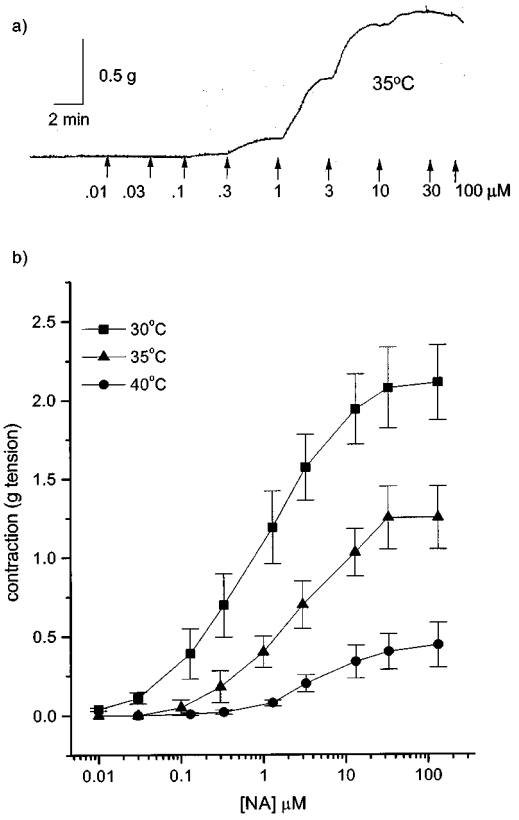

Figure 3 (a) Trace showing contractions of the rat dartos muscle (35°C) in response to cumulative addition of noradrenaline. The concentrations given are final bath concentrations. (b) Concentration-response curves for contractions to noradrenaline in muscles set up and maintained at 30°C, 35°C or 40°C. Each point is mean ± s.e.mean of at least five muscle preparations from at least three different animals.

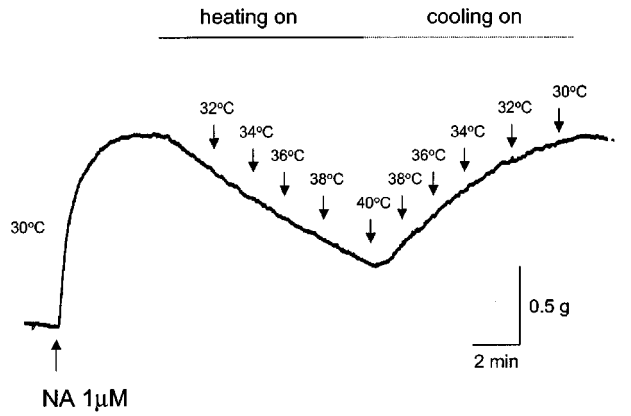

Figure 4 Trace showing contraction of a rat dartos muscle, initially set up at 30°C, to 1 μM noradrenaline (NA) and the effect on this contraction of heating the organ bath to 40°C and then cooling back to 30°C.

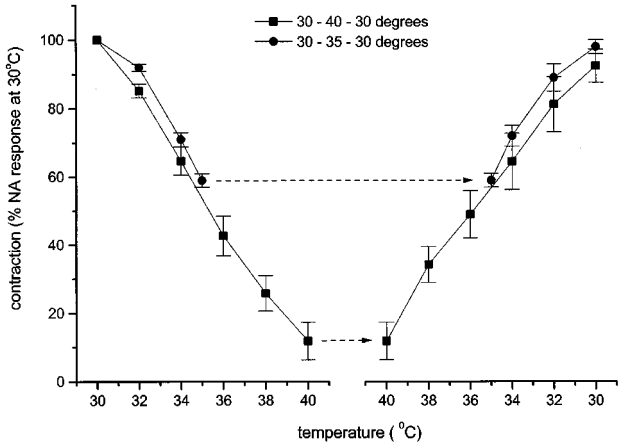

Figure 5 Graph showing the effects of heating to 40°C or 35°C on contractions of the rat dartos muscle, initially set up at 30°C, to 1 μM noradrenaline (NA). The effects of re-cooling back to 30°C are also shown. Responses are calculated as a percentage of the initial contraction at 30°C and are mean ± s.e.mean of at least five muscle preparations from at least three different animals.

cooling phase) the temperature-induced relaxation was maintained (data not shown).

Responses to other contractile agents

To determine whether the observed thermosensitivity of the contractile response of the dartos was specific to noradrenaline, a number of other possible contractile agents were tested on the tissue. At 30°C, the following drugs produced no significant contraction up to a concentration of 100 μM: carbachol, nicotine, histamine and 5-hydroxytryptamine. Substance P was also ineffective, although in this case the highest concentration applied was 1 μM. However, as shown in Figure 6a, the thromboxaneA_2-mimetic U46619 did cause concentration-related contractions. Further, addition of high K to the medium (70 mM) also caused a sustained rise in tone (1.05 ± 0.2 g tension, $n=8$). If dartos muscles at 30°C were contracted by either 100 nM U46619 (1.07 ± 0.1 g tension, $n=13$) or 70 mM K, then subsequent heating to 40°C again resulted in a relaxation of induced tone similar in magnitude to that observed with noradrenaline (Figure 6b).

The role of nerves in the temperature-induced relaxation

To determine whether the thermosensitivity of the dartos muscle might involve activation of a local nerve network, we next tested the effect of tetrodotoxin on the heating-induced relaxation of the response to noradrenaline. In these tissues, set up at 30°C, field stimulation (5 Hz, 40 s train) produced contractions of 1.4 ± 0.4 g tension ($n=5$); following incubation for 15 min in 1 μM tetrodotoxin these field stimulation-induced contractions were abolished. In the continued presence of tetrodotoxin, addition of 1 μM noradrenaline caused a contraction of 1.4 ± 0.3 mg tension ($n=5$). Raising the organ bath temperature to 40°C again caused a relaxation of this noradrenaline-induced tone similar in magnitude to that observed in the absence of TTX (Figure 6b). We also carried out some experiments to determine whether the dartos receives an inhibitory innervation; these were performed at 35°C, intermediate between 30°C and 40°C. In order to observe any relaxations to field stimulation, it was necessary both to raise the tone of the muscle and to negate the effects of the motor transmitter. Motor responses were negated by including 1 μM phentolamine in the bathing medium and by exposing each tissue to 30 μM guanethidine

for 10 min before tone was raised with 1 μM U46619. Under these conditions, field stimulation (20 Hz for 20 s every 2 min) produced no change in muscle tension. The same was true if muscle tone was raised with 3 μM noradrenaline in place of U46619 (phentolamine omitted); again there was no evidence for the release of any relaxant transmitter. These experiments, together with the observations with tetrodotoxin, make it unlikely that the temperature-induced changes in tone involved activation of inhibitory nerves.

The role of myosin phosphatase in the temperature-induced relaxation

The experiments detailed above demonstrated a marked temperature sensitivity of induced contraction in rat dartos, giving a Q_{10} value of around 5 (a 10° increase in temperature reduced the contraction from 100% to 20%). Perusal of the literature revealed that one factor, important in smooth muscle excitation–contraction coupling, which has been shown to have a similar high Q_{10} value (5.2–5.3) is smooth muscle myosin phosphatase (Mitsui *et al*., 1994). We therefore carried out some experiments with calyculin-A, a cell-permeable inhibitor of Type I and Type II phosphatases to determine whether there was any evidence for the involvement of myosin phosphatase in the thermosensitive responses of rat dartos. The calyculin-A concentration employed was 1 μM which has been used in previous studies to inhibit myosin phosphatase activity in other smooth muscle preparations (Ishihara *et al*., 1989; Shiozaki *et al*., 2000; Burdyga & Wray, 2002). In normal calcium-containing medium at 30°C, calyculin-A caused a slow contraction that reached a level of 0.41 ± 0.05 g tension after 15 min (Figure 7a). Unlike the thermosensitivity of the contractile response to the other agents used, the response to calyculin-A was little affected by raising the temperature from 30°C to 40°C (Figures 6b and 7a), with only a 5% relaxation observed at 40°C.

Since calyculin-A can cause calcium independent contraction in smooth muscle (Ishihara *et al*., 1989), we investigated the role of calcium in heat-induced relaxation by observing the effects of calyculin-A and U46619 in calcium-free Krebs solution (30°C; with 1 mM EGTA added). After 30 min incubation in calcium-free medium, contractions to 70 mM K were abolished. However, both calyculin-A (1 μM) and U46619 (1 μM) continued to produced sustained contractions (0.29 ± 0.05 g tension for calyculin-A and 0.51 ± 0.05 g tension for U46619; $n = 5$ in both cases). As in the presence of calcium, raising the temperature from 30°C to 40°C resulted in a marked relaxation of U46619-induced tone ($87 \pm 7\%$ relaxation; $n = 7$) but had little effect on tone induced by calyculin-A ($3 \pm 2\%$ relaxation; $n = 6$). If enhanced myosin phosphatase activity underlies the temperature sensitivity of the dartos then the heat-induced relaxation of the response to U46619 should be attenuated in the presence of calcyculin-A. As shown in Figure 7b, this was indeed the case. In tissues contracted by previous exposure to calcyculin-

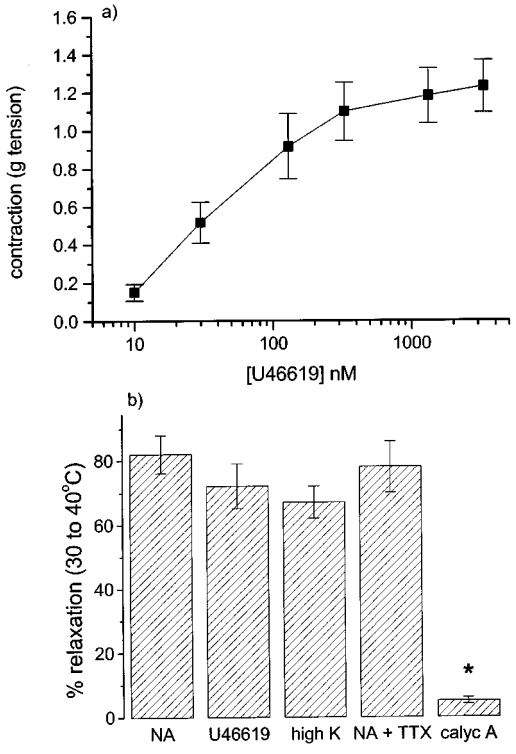

Figure 6 (a) Concentration-response curve for contraction of the rat dartos muscle to U46619 at 30°C. Each point is mean ± s.e.mean of at least five muscle preparations from at least three different animals. (b) Histogram showing the effect of heating to 40°C on the contraction of the rat dartos muscle, initially set up at 30°C, to noradrenaline (NA, 1 μM), U46619 (100 nM), high K (70 mM), NA in the presence of tetrodotoxin (TTX, 1 μM), or calyculin-A (calyc-A; 1 μM). Responses are calculated as the percentage relaxation of the initial contraction at 30°C on heating to 40°C. Each point is mean ± s.e.mean of at least five muscle preparations from at least three different animals. * $P < 0.05$, significantly different from NA.

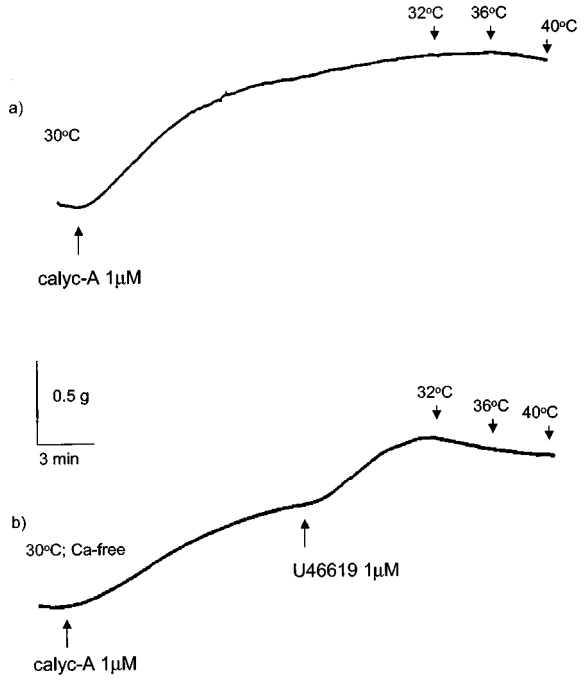

Figure 7 (a) Trace showing the effect of heating to 40°C on contraction of the rat dartos muscle, initially set up at 30°C, to calcyculin-A (calyc-A). (b) Trace showing contraction of the rat dartos muscle (30°C; calcium-free medium) to U46619 in the presence of calyculin-A (calyc-A) and the effect on this contraction of heating to 40°C.

A in calcium-free medium (30°C), addition of U46619 caused a further increase in tone (0.41 ± 0.05 *g* tension; $n=7$). Relaxation of this U46619-induced tone on heating to 40°C was much less ($29 \pm 4\%$; $n=7$) than the effect observed in the absence of calyculin-A ($87 \pm 7\%$ relaxation; $n=7$; $P<0.05$).

Discussion

To our knowledge, this is the first study to report on the pharmacology of the dartos muscle from any species. Further, there have been no published studies into the thermosensitivity of induced contractions in the isolated dartos, or investigations into the underlying mechanisms. This previous lack of information is perhaps surprising given the long-recognized role of the dartos in scrotal thermoregulation (Tyrrell *et al*., 1942; Waites & Moule, 1961; Shafik, 1973; Maloney & Mitchell, 1996). In most mammals, including man, the optimal temperature for spermatogenesis lies 2–3°C below normal body temperature; this has been achieved by externalization of the testes within the scrotum (Waites, 1970). As described in the Introduction, the scrotum is thermoresponsive, contracting in cold environments and relaxing in heat. The dartos muscle is the main effector of these scrotal adaptations. The importance of maintaining the correct scrotal temperature has been emphasized by the many epidemiological studies demonstrating that semen quality in men with oligozoospermia may be enhanced by a variety of treatments aimed at reducing scrotal temperature (see Jung *et al*., 2001). Further, Shafik (1978) reported the case of a 32-year-old patient presenting with prolonged sterility who, on biopsy, was found to have a non-reactive scrotal skin and an absent dartos muscle. Knowledge of the pharmacology of the dartos is therefore of importance in order to predict how drugs might influence the contractile state of the muscle, thereby interfering with its role in scrotal thermoregulation, and influencing spermatogenesis.

Previous anatomical/histological investigations have suggested that the dartos is innervated by the sympathetic branch of the autonomic nervous system (Dean & Pegington, 1996). The results of the present study confirm this and show, in addition, that these sympathetic nerves are motor. Thus, field stimulation resulted in contractions that were abolished in the presence of the α-adrenoceptor antagonist phentolamine and the adrenergic neurone blocking drug guanethidine; further, noradrenaline caused concentration-related contractions that were again blocked by phentolamine. Since the responses to both field stimulation and noradrenaline were also inhibited by low concentrations of prazosin it is likely that it is the α_1-adrenoceptor subtype that is involved (Docherty, 1998). However, a more detailed investigation using a range of selective agonists and antagonist would be required to identify the precise nature of the α-adrenoceptor subtype. An interesting observation is that the maximum response to field stimulation was considerably greater than that to exogenous noradrenaline. One explanation might be that neuromodulators (for example, adenosine triphosphate and/or neuropeptide Y) are also released from the sympathetic nerves and act to potentiate the contractile effects of the co-released noradrenaline. Again, further work would be required to determine the validity of this possibility. Nonetheless, given the potency of the α-adrenoceptor antagonists, it seems clear that the major contractile transmitter is noradrenaline

Over recent years, several smooth muscle tissues within the male genital tract (including the corpus cavernosum, anococcygeus and penile artery) have been shown to receive an inhibitory innervation that utilises nitric oxide as a transmitter (Rand & Li, 1995; Martin, 2000). However, no evidence was obtained in the present study for such a nitrergic innervation, or indeed for any other type of inhibitory innervation, of the rat dartos. When muscle tone was raised with U46619, and sympathetic influence negated by a combination of guanethidine and phentolamine, field stimulation over a range of frequencies failed to elicit relaxation; the same was true when tone was raised with noradrenaline in the presence of guanethidine. The lack of release of nitric oxide during field stimulation was confirmed in experiments observing the effects of L-N^G-nitroarginine (a nitric oxide synthase inhibitor) on the sympathetic contractile responses to field stimulation. Sub-maximal contractions were unaffected, again showing that there was no inhibitory nitric oxide released concomitantly with the motor transmitter, noradrenaline. These results suggest that neuronal control of the rat dartos muscle tone rests solely with the sympathetic nerves.

Perhaps the most striking observation of the present study was the marked thermosensitivity of contractions of the dartos. Responses to field stimulation, noradrenaline, U46619, and high K were all reduced by about 80% on raising the temperature by 10°C, from 30°C to 40°C, giving a Q_{10} value of around 5. It is known that temperature affects several processes involved in smooth muscle excitation-contraction coupling, but in the majority of cases the Q_{10} value is much less than 5 (usually between 1 and 3; Mitsui *et al*., 1994; Burdyga & Wray, 2002). An exception is myosin phosphatase, which has been reported to have a Q_{10} value of 5.3 (Mitsui *et al*., 1994). This led us to investigate the possibility that enhanced myosin phosphatase activity might contribute to the heating-induced relaxation of the dartos. We used calyculin-A, a cell-permeable inhibitor of type I and type II phosphatases, which has used extensively to investigate the role of myosin phosphatase in smooth muscle excitation–contraction coupling (Ishihara *et al*., 1989; Shiozaki *et al*., 2000; Burdyga & Wray, 2002). The contractile state of smooth muscle is determined by the degree of phosphorylation of myosin; calyculin-A results in contraction by inhibiting myosin phosphatase and therefore preventing myosin dephosphorylation (Hartshorne, 1998; Somlyo & Somlyo, 1998). Calyculin-A did indeed cause sustained contraction of the dartos muscle at 30°C; in this case however, heating to 40°C produced very little relaxation. The involvement of calcium in the thermosensitive relaxations of the dartos was investigated by observing the effects of U46619 and calyculin-A in calcium-free medium. At 30°C, both U46619 and calyculin-A continued to produce sustained contractions in calcium-free medium and, as in the presence of calcium, the response to U46619 was relaxed by some 80% on heating to 40°C whereas that to calyculin-A was little affected. However, in the presence of calcyculin-A, the further contraction to U46619 in calcium-free medium was much less sensitive to a rise in temperature. Taken together the results suggest that the presence of calcium is not obligatory for the thermosensitivity of the dartos muscle, but that full myosin

phosphatase activity is an important requirement. This would be in agreement with recent findings using calyculin-A in ureteric smooth muscle from rats and guinea pigs showing that myosin phosphatase is one of the major factors mediating temperature-induced relaxations in these smooth muscles (Burdyga & Wray, 2002).

Smooth muscle myosin phosphatase, a type I phosphatase, is a heterotrimer consisting of a catalytic subunit (37 kD), a regulatory myosin binding subunit (130 kD) that targets the enzyme to its substrate, and a 20 kD subunit of uncertain function (Hartshorne, 1998). Numerous isoforms of the subunits exist, and it is known that enzyme activity can be regulated by cell signalling molecules such as arachidonic acid, protein kinase C, and rho-activated kinase (Hartshorne, 1998; Somlyo & Somlyo, 1998). Given the wide variation in temperature sensitivity in smooth muscle (Burdyga & Wray, 2002), important future work will be to establish the specific subunit isoforms of myosin phosphatase present in the dartos smooth muscle cells. There is also the important question of whether the temperature dependence of the enzyme is an inherent property of one or more of the subunits, or is indirect *via* one of the regulatory signalling molecules.

In conclusion, this study reports for the first time on the pharmacology of the dartos muscle. The rat dartos receives a motor sympathetic innervation and contracts to exogenous noradrenaline, probably *via* α_1-adrenoceptors; there is no functional nitrergic innervation. Consistent with its functional role in scrotal thermoregulation, the contractile response of the dartos displays marked temperature sensitivity. Experiments with calyculin-A implicate myosin phosphatase as a major mediator of this thermosensitivity.

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