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Characterization of adrenoceptors involved in the electrogenic chloride secretion by cultured rat epididymal epithelium

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1 Short-circuit current (SCC) technique was used to study the adrenoceptors involved in the electrogenic chloride secretion by cultured cauda epididymal epithelium of rats. Stimulation of the epithelium with noradrenaline (primarily β_1-adrenoceptor selective agonist), salbutamol (β_2-adrenoceptor selective agonist) and adrenaline (non-selective β-adrenoceptor agonist) led to a rise in SCC. At a low chart-speed (2 mm min^{-1}), the response profile to these agonists consisted of a peak followed by a sustained response considerably higher than the basal SCC.

2 The EC_{50}s (doses of agonist producing 50% maximum response) of noradrenaline, salbutamol and adrenaline were 300, 115 and 10 nM respectively. Pretreating the tissues with 1 μM atenolol (β_1-selective antagonist) and 10 μM butoxamine (β_2-selective antagonist) shifted the dose-response curves of noradrenaline (shifted EC_{50} = 4000 nM) and salbutamol (shifted EC_{50} = 1050 nM) to the right. Atenolol (1 μM) and butoxamine (10 μM) shifted the dose-response curve of adrenaline to the right with new EC_{50}s of 30 nM and 115 nM, respectively.

3 The rapidly rising phase of the SCC response to noradrenaline and adrenaline observed at low chart-speed consisted of a brief and transient retraction followed by a rebound increase in SCC. At a high chart-speed (1 mm s^{-1}), the retraction and rebound phenomenon manifested as a fast initial spike which could be blocked by phentolamine (non-specific α-adrenoceptor antagonist) in a dose-dependent fashion. Similar initial spikes were observed when the tissues were stimulated with phenylephrine (α_1-selective agonist) but not with isoprenaline (non-selective β-agonist) or forskolin (activator of adenylate cyclase). The response of the initial spike triggered by noradrenaline was dose-dependent and the EC_{50} was 2000 nM.

4 The present study showed that the electrogenic chloride secretion by rat epididymis could be stimulated by α_1-, β_1- and β_2-adrenoceptor agonists. The α_1-mediated response had a faster onset and more transient action than the β-counterpart. It is postulated that epididymal chloride secretion might be regulated by neural (noradrenaline-mediated) and humoral (adrenaline-mediated) controls and that the stimulus-secretion coupling mechanisms might involve both Ca^{2+} (α_1-mediated response) and adenosine 3':5'-cyclic monophosphate (β-mediated response) as intracellular second messengers.

Keywords: Adrenoceptors; rat epididymis; chloride secretion; cell culture

Introduction

Transepithelial chloride secretion in the epididymis has been shown to be stimulated by adrenoceptor agonists (Wong & Chan, 1988). Activation of adrenoceptors on epithelial cells triggers cascades of biochemical reactions leading to increased concentrations of intracellular second messengers like adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Wong & Huang, 1990) and/or Ca^{2+} (Pfeilschifter *et al.*, 1991). These messengers act on different components of the secretory pathway to stimulate chloride secretion (for review see Donowitz & Welsh, 1986). It is believed that signal transduction mechanisms are specific to the adrenoceptors involved (Rasmussen, 1990). For instance, activation of β-adrenoceptors increases the intracellular concentration of cyclic AMP (Levitzki, 1988) whereas activation of α_1-receptors leads to a rise in intracellular Ca^{2+} concentration (Rooney *et al.*, 1989).

It has been demonstrated that the epithelium of the cauda epididymidis is richly innervated by noradrenergic fibres (El-Badawi & Schenk, 1967) which might serve a secretomotor function (Wong *et al.*, 1992). On the other hand, the threshold dose of adrenaline required to stimulate chloride secretion by the epididymis (Wong & Chan, 1988) was close to the circulating adrenaline concentration (Eisenhofer *et al.*, 1985). It is generally believed that target tissues respond to nerve and circulating catecholamine stimulation through α- and β-adrenoceptors respectively (Bevan *et al.*, 1980). The present study aimed to characterize the adrenoceptors involved in chloride secretion by cultured rat epididymal epithelium with a view to understanding the stimulus-secretion coupling mechanisms and the neural and humoral regulation of chloride secretion by the epididymis.

Methods

Tissue culture techniques

The procedures of tissue cultures have been described previously (Cuthbert & Wong, 1986; Wong, 1988a). Male Sprague-Dawley rats weighing 210 to 230 g were used as the source of tissue. The rats were killed by a blow to the head followed by cervical dislocation. The lower abdomen was opened and the caudal part of each epididymis was separated from the rest of the organ. The tissue was finely chopped with scissors and was then digested with 0.25% (w/v) trypsin followed by 0.1% (w/v) collagenase I. The primary cultures were grown on Millipore filters floating on Eagle's Minimum Essential Medium (EMEM) completed with 10% foetal calf serum and other supplements (Wong, 1988a). Cultures were incubated for 4 days at 32°C in 5% CO_2. Thereafter, the monolayers reached confluency and were ready for the measurement of short-circuit current.

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The short-circuit current measurement

The short-circuit current (SCC) measurement has been described previously (Cuthbert & Wong, 1986; Wong, 1988a). Confluent monolayers of rat epididymal cells were clamped vertically between the two halves of the Ussing Chambers. The tissues were short-circuited (transepithelial potential difference clamped at zero) by use of a voltage-clamp amplifier (DVC 1000; World Precision Instruments Inc., Florida, U.S.A.) and the short-circuit current was displayed on a pen recorder (Kipp and Zonen, Delft, The Netherlands). To measure the transepithelial resistance, the transepithelial potential was sometimes clamped intermittently at a value slightly different from zero (0.05–0.3 mV). The resulting current change allowed calculation of the resistance from the Ohmic relationships. The two channels of the amplifier were mostly used simultaneously on parallel monolayers so that studies could be made under control and experimental conditions. At the end of the experiments, the area of the monolayers was measured with an IBM compatible computer equipped with a digitizer (Hipad, Houston Instruments, Austin, Texas, U.S.A.) and the software Autocad. In most experiments, monolayers were incubated in Krebs-Henseleit solution (for composition see below).

Drug addition

Chemical agents used in the present study were added directly to the bathing solution on the basolateral aspect of the monolayers. To study the effects of adrenoceptor antagonists, the tissues were pretreated with the antagonists for 5–10 min before stimulation with the agonists to allow even distribution of the former within the tissues. In all experiments, addition of antagonists alone did not affect the basal short-circuit current. To avoid receptor desensitization (Hausdorff *et al.*, 1990), each monolayer was stimulated with a particular agonist only once (either in the absence or presence of antagonists).

Calculation of pK_B values

The pK_B is a measure of the affinity of a competitive antagonist for its receptors. It is determined from experiments in which the tissue response to various concentrations of a receptor agonist is inhibited by a fixed concentration of the corresponding antagonist. In the present study, the dose-response curves for noradrenaline (primarily β_1-selective agonist) or salbutamol (β_2-selective agonist) were obtained in the absence and presence of atenolol (β_1-antagonist) or butoxamine (β_2-antagonist) respectively. The dose-ratio (DR) was calculated from the EC_{50} in the presence of antagonist divided by that in its absence. The pK_B was calculated from DR according to the equation

$$pK_B = \log (DR-1) - \log B,$$

where B was the concentration of the antagonist expressed in M.

Although in the present study pK_B was determined from one concentration of antagonist only, it provided an estimate of the affinity of the antagonist for the receptors thereby indicating the specificity of its effects.

Solutions and materials

The normal Krebs-Henseleit (K-H) solution had the following composition (mM): NaCl 117, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 11.1. The solution was gassed with 5% CO_2/95% O_2 to give a pH of 7.4. Eagle's Minimum Essential Medium (EMEM), foetal calf serum and non-essential amino acids were purchased from Gibco Laboratories (New York, U.S.A.). Penicillin/streptomycin, Hank's Balanced Salt Solution, sodium pyruvate, 5 α-dihydrotestosterone, trypsin, collagenase I, atenolol, butoxamine, noradrenaline and phenylephrine were from Sigma Chemical Co. (St. Louis, U.S.A.). Adrenaline and salbutamol were purchased from the David Bull Laboratories (Victoria, Australia) and Glaxo (Greenford, England) respectively.

Statistical analysis

Results are expressed as means ± standard error of the mean (s.e.mean). Comparisons between groups of data were made by Student's unpaired *t* test. A *P* value of less than 0.05 was considered statistically significant.

Results

Effects of β-adrenoceptor agonists on SCC

When bathed in normal Krebs-Henseleit solution, the epididymal monolayers exhibited a transepithelial potential difference of 3.3 ± 0.12 mV ($n = 258$ cultures), a basal short-circuit current (SCC) of 6.4 ± 0.21 μA cm^{-2} ($n = 256$ cultures) and a transepithelial resistance of 390.3 ± 15.8 Ω cm^2 ($n = 232$ cultures). Figure 1 shows the effects of β-adrenoceptor agonists on SCC. Addition of noradrenaline (primarily a β_1-selective agonist), salbutamol (β_2-selective agonist) and adrenaline (non-selective β-agonist) to the basolateral bathing solution caused a rapid rise in SCC which attained a maximum within 1 min after stimulation. This was followed by a plateau phase considerably higher than the basal SCC. For quantitative studies, the SCC response to these agonists was defined as the peak increase in SCC after stimulation (see below).

Effects of β-adrenoceptor antagonists

Figure 2 shows the dose-response relationships of noradrenaline in the absence and presence of a β_1-selective antagonist, atenolol. The dose-response curves exhibited a sigmoidal profile. The threshold and EC_{50} of noradrenaline were about 1 and 300 nM respectively and the maximum SCC response was attained at 1 mM. Pretreatment with atenolol (1 μM) shifted the dose-response curve to the right with an EC_{50} of 4000 nM. The dose ratio (DR) was thus 13.3 and the pK_B of atenolol was calculated to be 7.1 (See Methods).

Figure 3 shows the dose-response relationships of salbutamol in the absence and presence of a β_2-selective antagonist, butoxamine. The dose-response curve of salbutamol was sigmoidal and the threshold and EC_{50} were 1.7 and

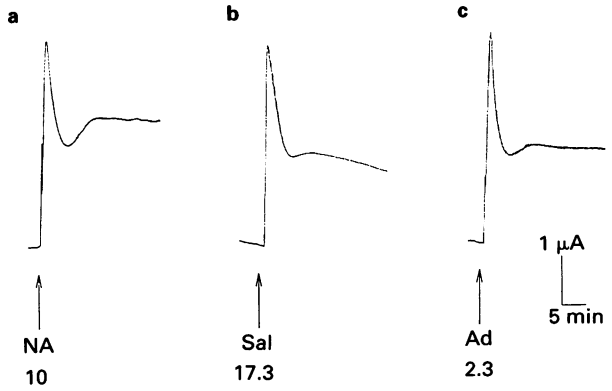

Figure 1 Short-circuit current measurement in three separate monolayers (area 0.4–0.6 cm^2). The tissues were stimulated with (a) noradrenaline (NA, 10 μM), (b) salbutamol (Sal, 17.3 μM) and (c) adrenaline (Ad, 2.3 μM). The agonists were added to the basolateral side of the tissues. The arrows indicate the time at which the drugs were added. Each record is representative of at least six separate experiments.

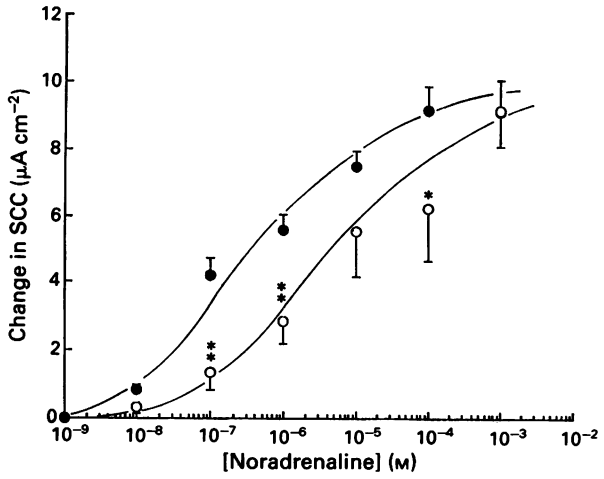

Figure 2 Effects of varying the concentration of noradrenaline on the short-circuit current (SCC) response in the absence (●) and presence (○) of 1 μM atenolol. Each data point is the mean of 4 to 6 separate experiments and each error bar represents one s.e.mean. Asterisks represent the level of significance when the SCC response to a particular dose of noradrenaline in the presence of atenolol was compared with that in its absence (*$P<0.05$; **$P<0.01$).

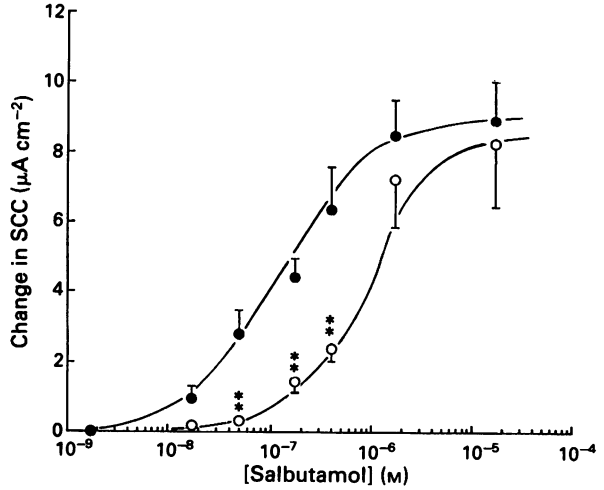

Figure 3 Effects of varying the concentration of salbutamol on the short-circuit current (SCC) response in the absence (●) and presence (○) of 10 μM butoxamine. Each data point is the mean of 4 to 6 separate experiments and each error bar represents one s.e.mean. Asterisks represent the level of significance when the SCC response to a particular dose of salbutamol in the presence of butoxamine was compared with that in its absence (**$P<0.01$).

115 nM respectively. Maximum effect was attained at 17.3 μM. Pretreatment with butoxamine (10 μM) shifted the dose-response curve to the right with an EC_{50} of 1050 nM. The DR was 9.1 and the pK_B of butoxamine was calculated as 5.9.

Figure 4 shows the dose-response relationships of adrenaline in the absence and presence of atenolol or butoxamine. The threshold and EC_{50} of adrenaline were 0.23 and 10 nM respectively and the maximum response was reached at 2.3 μM. Pretreatment with atenolol (1 μM) or butoxamine (10 μM) shifted the dose-response curves to the right with EC_{50}s of 30 and 115 nM respectively. The DR for atenolol was 3 and that for butoxamine was 11.5. The pK_B of atenolol and butoxamine were 6.3 and 6.0 respectively.

Effects of α-adrenoceptor stimulation

During the rapidly rising phase of the SCC response to noradrenaline, a brief and fast retraction followed by a rebound increase in SCC was observed (see Figure 1a). Figure 5a shows the effect of noradrenaline (a non-specific α-agonist in addition to its β_1-selective action) on SCC at expanded time-scale. The retraction and rebound phenomenon manifested as a fast initial spike followed by a delayed and relatively prolonged rise in SCC. The latter corresponded to the peak observed at low chart-speed (see Figure 1a). The initial spike lasted for 8–10 s and was succeeded by the second component before SCC had returned to the basal level. Similar experiments were performed with other adrenoceptor agonists. Figure 5b and c shows the effects of phenylephrine (an α_1-selective agonist) and adrenaline (a non-specific α-agonist in addition to its β-action) respectively on SCC at expanded time-scale. Initial spikes were observed with both agonists. Figure 5d and e show the effects of isoprenaline and forskolin respectively on SCC. Isoprenaline is a pure β-agonist and forskolin is an activator of adenylate cyclase, which forms an integral part of the signal transduction pathway triggered by β-adrenoceptor stimulation. The initial spike was not observed upon stimulation with either of these substances.

Figure 6 shows the effects of noradrenaline (10 μM) on SCC in the absence (a) and the presence of 0.1 μM (b) and 0.2 μM (c) phentolamine, a non-specific α-antagonist. It can be seen that phentolamine at 0.1 μM reduced and at 0.2 μM completely abolished the initial spike triggered by noradrenaline.

Figure 7 shows the dose-response relationships of noradrenaline measured as the maximum SCC reached by the initial spike and the second phase of response. Both curves exhibited a sigmoidal profile. The EC_{50} for the initial spike was 2000 nM while that for the second component was 300 nM. The threshold doses required to elicit the initial spike and the second component were 10 and 1 nM respectively.

Discussion

The present study demonstrated that the electrogenic chloride secretion by cultured rat epididymal epithelium could be stimulated by β_1-, β_2- as well as α_1-adrenoceptor agonists. The response triggered by α_1-stimulation (i.e. the initial spike) had a faster onset and more transient action than the β-counterpart (see Figure 5). The β_1- and β_2-mediated chloride secretion were competitively blocked by atenolol and butoxamine respectively, whereas the α_1-component was inhibited by phentolamine in a dose-dependent fashion.

Short-circuit current was used as a measure of electrogenic chloride secretion across the epididymal epithelium. Although chloride fluxes across the epithelium have not been measured, previous studies with specific chloride channel blockers (Wong, 1988b) as well as bilateral (Wong, 1988a) and unilateral chloride replacement (Leung & Wong, 1992) have shown that the short-circuit current response to secretagogues could be attributed to the opening of apical chloride channels and the subsequent exit of chloride from the cytosol into the apical solution. Like other secretory epithelia, chloride diffuses across the apical membrane along its electrochemical gradient, which is maintained by the active accumulation of chloride inside the cells by the Na^+/K^+ ATPase, the $Na^+/K^+/2Cl^-$ symport and the K^+ channels on the basolateral membrane (Young & Cook, 1987). It has been reported in the epididymal (Wong & Chan, 1988) and tracheal epithelium (Al-Bazzaz & Cheng, 1979) that chloride secretion could be increased upon stimulation of β-adrenoceptors. The present study shows that both β_1- and β_2-receptors are involved in the process. To investigate the β-adrenoceptor subtypes, noradrenaline and salbutamol were used as selective β_1- and β_2-adrenoceptor agonists respectively

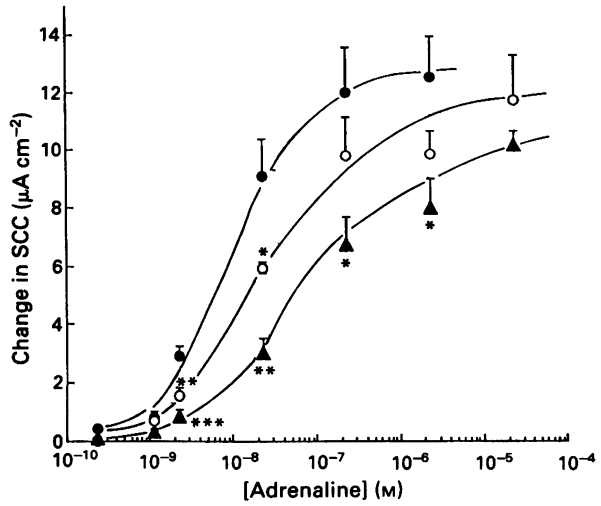

Figure 4 Effects of varying the concentration of adrenaline on the short-circuit current (SCC) response in the absence (●) and presence of 1 μM atenolol (○) or 10 μM butoxamine (▲). Each data point is the mean of 4 to 6 separate experiments and each error bar represents one s.e.mean. Asterisks represent the level of significance when the SCC response to a particular dose of adrenaline in the presence of atenolol or butoxamine was compared with that in their absence (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

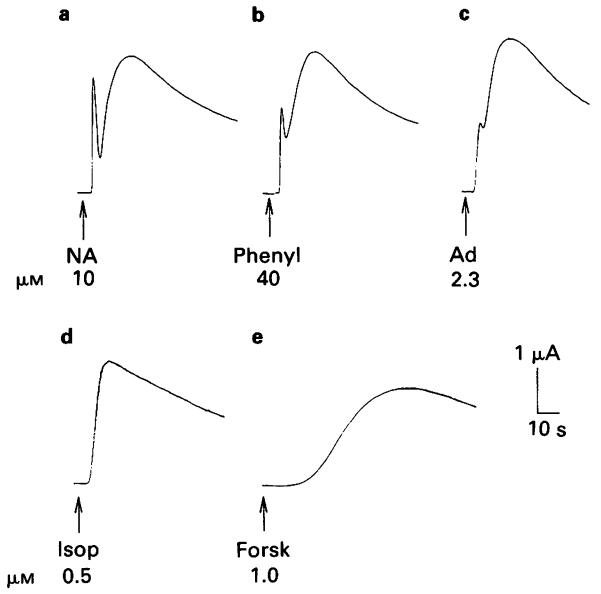

Figure 5 Short-circuit current measurement in five separate monolayers (area 0.4–0.6 cm²) at expanded time-scale. The tissues were stimulated with (a) noradrenaline (NA, 10 μM), (b) phenylephrine (Phenyl, 40 μM), (c) adrenaline (Ad, 2.3 μM) (d) isoprenaline (Isop, 0.5 μM) and (e) forskolin (Forsk, 1.0 μM). The agonists were added to the basolateral side of the tissues. The arrows indicate the time when the drugs were added. Each record is representative of at least six separate experiments.

(for review see Nahorski, 1981). Although a detailed Schild plot analysis has not been performed, the pK_B values of 7.1 and 5.9 determined from a single concentration of atenolol (1 μM) and butoxamine (10 μM) respectively were comparable to those reported in studies on atrial tissues (Leclerc *et al.*, 1981; O'Donnell & Wanstall, 1983). The results suggested that the inhibitory effects of atenolol and butoxamine could be due to the specific inhibition of β_1- and β_2-adrenoceptors

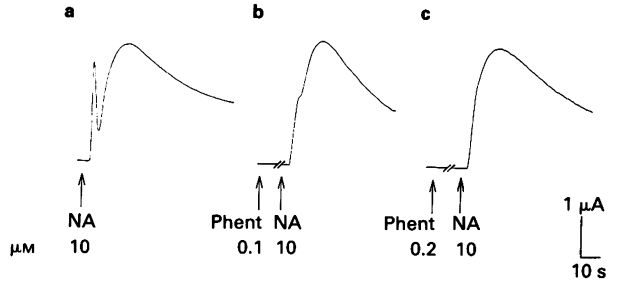

Figure 6 Short-circuit current measurement in three separate monolayers (area 0.4–0.6 cm²) at expanded time-scale. The tissues were stimulated with noradrenaline (NA, 10 μM) in the (a) absence and presence of (b) 0.1 μM or (c) 0.2 μM phentolamine (Phent). The agonists were added to the basolateral side of the tissues. The arrows indicate the time when the drugs were added. Each record is representative of at least six separate experiments.

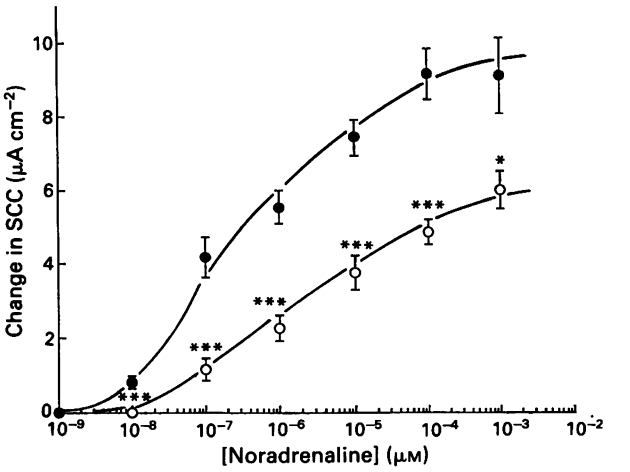

Figure 7 Effects of varying the concentration of noradrenaline on the short-circuit current (SCC) response expressed as the maximum SCC reached by the initial spike (○) and the second peak (●). Each data point is the mean of 4 to 6 separate experiments and each error bar represents one s.e.mean. Asterisks represent the level of significance when the maximum SCC reached by the initial spike at a particular dose of noradrenaline was compared with that reached by the second peak (*$P<0.05$, ***$P<0.001$).

respectively. It was also found that the SCC response to adrenaline could be inhibited by atenolol or butoxamine, suggesting that it could be mediated by both β_1-and β_2-adrenoceptor stimulation.

The present study also provides evidence for the existence of α_1-receptors in the epididymal epithelium. Stimulation of the tissues with an α_1-selective agonist phenylephrine, as well as non-selective α-agonists noradrenaline and adrenaline, gave rise to an initial spike which could be blocked by phentolamine, a non-selective α-antagonist. Recent work in our laboratory has shown that the initial spike could be abolished by agents which perturbed the intracellular Ca^{2+} homeostasis, like thapsigargin and the calcium ionophore A23187 (Wong *et al.*, unpublished). This was not unexpected as α_1-mediated cellular responses are related to changes in intracellular Ca^{2+} concentration (Exton, 1985). The effects of phentolamine on the initial spike could not easily be quantified because as the initial spike was reduced by phentolamine, it was usually masked by the β-mediated second component. It is interesting that in addition to the α_1-mediated initial spike, the SCC response to phenylephrine exhibited a prominent second component which could be reduced by propranolol, a non-selective β-antagonist (Wong

et al., unpublished). This suggested that in the epididymis, phenylephrine might possess a significant β-adrenoceptor effect in addition to its specific α_1-action. The β-effect of phenylephrine on SCC measurement has also been reported in tracheal epithelium (Bainbridge et al., 1989).

The demonstration of both β- and α-mediated chloride secretion by the epididymis may give a clue to the stimulus-secretion coupling mechanisms. Stimulation of β-adrenoceptors in the epididymal (Wong & Huang, 1990) and tracheal epithelium (Smith et al., 1982) has been shown to increase intracellular cyclic AMP concentration, causing a subsequent rise in chloride secretion. On the other hand, the presence of α_1-adrenoceptors in the epididymal epithelium suggests that intracellular Ca^{2+} may also be involved in chloride secretion because α_1-adrenoceptor agonists are prototypes of Ca^{2+} mobilizing agents (Exton, 1985). Measurement of intracellular Ca^{2+} in Fura-2 loaded epididymal cells has demonstrated a transient rise in intracellular Ca^{2+} concentration upon stimulation with noradrenaline (Wong et al., unpublished). Moreover, stimulation of the tissues with the calcium ionophore A23187 has been found to increase Cl^- secretion across the epithelium (Wong, 1988a). However, in the present study the effector processes responsible for the cyclic AMP- and Ca^{2+}-mediated chloride secretion were not investigated. Cyclic AMP has been shown to activate apical chloride channels in the epididymal (Pollard et al., 1991) as well as the colonic (Halm et al., 1988) epithelium. It is believed that cyclic AMP activates protein kinase A and that the subsequent phosphorylation and activation of chloride channels increase chloride secretion (Huang et al., 1992). An increase in intracellular Ca^{2+} concentration has been found to open potassium channels on the basolateral membrane (Petersen & Maruyama, 1984). It has been suggested that the resulting membrane hyperpolarization increases the driving force for chloride exit across the apical membrane and hence chloride secretion (McCann & Welsh, 1990). However, recent studies have also demonstrated the existence of Ca^{2+}-activated chloride conductance in the epididymal (Huang et al., unpublished observations) as well as T-84 colonic tumour cells (Cliff & Frizzell, 1990).

The demonstration of α_1-, β_1- and β_2-adrenoceptor-mediated chloride secretion suggests that the secretory functions of the cauda epididymidis might be under both neural and humoral controls. The cauda epididymidis has been shown to be innervated by intra-epithelial noradrenergic fibres (El-Badawi & Shenk, 1967). Noradrenaline released from nerve terminals may act on α_1- and β_1-adrenoceptors on the epithelium to stimulate chloride secretion. Chloride secretion could also be stimulated by adrenaline (Wong & Chan, 1988) and the threshold dose required to elicit a response was close to the adrenaline concentration in the general circulation (Eisenhofer et al., 1985). Circulating adrenaline may activate various adrenoceptors, including β_2-receptors (Davis et al., 1990), on the epithelium to trigger chloride secretion from the blood to the lumen.

The neurohumoral control of chloride secretion may play a role in the maintenance of a unique and specific microenvironment in the epididymis on which the maturation and storage of spermatozoa depend (Jenkins et al., 1980). It has also been proposed that adrenergic stimulation of chloride secretion in the epididymis may be involved in the emission reflex (Wong & Chan, 1988). Secretion of electrolytes and fluid, coupled with tubular smooth muscle contraction, may facilitate the passage of spermatozoa during ejaculation. Disruption of the control mechanism by, for instance, sectioning the sympathetic nerve supply to the epididymis has been shown to affect adversely sperm transport and motility (Billups et al., 1990a). It was suggested that sympathetic denervation might lead to functional tubular obstruction (Billups et al., 1990b), a reflection of defective fluid transport in the epididymis (Wong, 1990).

The present study demonstrated the co-existence of α_1-, β_1- and β_2-adrenoceptors in a single exocrine tissue. It is believed that mammalian tissues can co-express both β_1- and β_2-adrenoceptors (Nahorski, 1981) and that, under certain circumstances, they may mediate the same physiological response (Carlsson et al., 1972). On the other hand, co-expression of α_1- and β_2-adrenoceptors has been reported in the MDCK cells (Slivka & Insel, 1987). It is interesting that the α_1- and β-mediated chloride secretion in the epididymis follows different temporal profiles. The α_1-response has a faster onset and more transient action while the β-response has a relatively delayed onset but more prolonged effects. A similar temporal sequence of α_1- and β-mediated cellular responses has been found in dissociated kidney epithelial cells (MDCK cells) (Breuer et al., 1988). The present study did not establish whether both responses are mediated by a single epithelial cell, as in MDCK cells (Breuer et al., 1988) or by two separate epithelial cell types with different signal transduction mechanisms.

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