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ORIGINAL ARTICLE

Presynaptic autoregulation of norepinephrine release from the sympathetic nerve fibers in the pineal gland of the domestic pig. Pharmacological characterization of α_2-adrenoceptors mediating this process

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Abstract

BACKGROUND: Norepinephrine is the main neurotransmitter controlling melatonin secretion in the mammalian pineal gland. Presynaptic autoregulation of norepinephrine release from the sympathetic nerve fibers in the pineal gland is poorly known.

METHODS: Uptake and depolarization-evoked release of ^{3}H-norepinephrine were investigated *in vitro* using pieces of the pig pineals. Specific antagonists and agonists of α_2-adrenoceptors were employed for the characterization of α_2-adrenoceptors involved in the autoregulation of depolarization-evoked release of norepinephrine in the pig pineal.

RESULTS: The level of neuronal uptake of norepinephrine in the pig pineal was 3.5±0.9 pmol/h/mg of wet tissue and represented about 77% of the total tissue radioactivity. Potassium ions at concentration of 60 mM significantly evoked tritium release. This effect was abolished in the absence of extracellular Ca^{2+} and was diminished in the presence of Cd^{2+}. Antagonists of α_2-adrenoceptors increased depolarization-evoked tritium release. The order of its potency (based on pED_{30}) was rauwolscine > phentolamine > BRL 44408 > WB 4101> RS 79948 = yohimbine >> prazosin >> imiloxan. α_2-agonists decreased K^+-evoked release of tritium with the order of potency: UK14,304 > norepinephrine = guanfacine > oxymetazoline.

CONCLUSION: The processes of neuronal uptake and depolarization-evoked release of norepinephrine from the sympathetic nerve endings in the pig pineal gland have been demonstrated. The studies with the use of adrenergic antagonists and agonists indicate that the sympathetic nerve fibers in the pig pineal gland possess functional presynaptic α_2-adrenoceptors, which are involved in norepinephrine release inhibition. Due to the pharmacological properties these receptors closely resemble the subtype α_{2A}.

Introduction

Norepinephrine released from the sympathetic nerve fibers is considered as the main neurotransmitter regulating melatonin synthesis and therefore responsible for the nocturnal increase in the secretion of this pineal hormone.

Majority of our knowledge about the adrenergic regulation of the mammalian pinealocyte activity derives from the studies performed in the rat. Norepinephrine acts on the melatonin synthesis pathway in the rat pinealocytes via two postsynaptic adrenoceptors: α_1 and β_1 [1, 2]. The stimulation of β_1-adrenergic receptors leads to the increase in cyclic AMP production, activation of transcription and translation of arylalkylamine N-acetyltransferase (enzyme limiting melatonin synthesis and secretion) and the inhibition of the proteosomal proteolysis of this enzyme [3, 4, 5]. The activation of α_1-adrenoceptors, which by itself does not alter the activity of adenylate cyclase and arylalkylamine N-acetyltransferase, potentiates β_1-adrenergic stimulation of cAMP and melatonin production [1, 2]. The presence of postsynaptic α_2-adrenoceptors functionally linked to membrane guanylate cyclase has been also demonstrated in the rat pineal gland [6, 7].

Investigations performed in different mammalian species provided evidence for species heterogeneity in the adrenergic regulation of melatonin secretion in mammals [8, 9, 10, 11, 12]. The interspecies differences concern both the adrenergic receptors involved in the regulation of the pinealocyte activity at postsynaptic level [8, 9] as well as the intracellular mechanisms, which control the rate of melatonin synthesis [10, 11, 12]. In the pineal gland of the domestic pig, which is the subject of the present study, norepinephrine controls melatonin secretion via β_1-adrenoceptors without participation of α_1 and α_2-adrenoceptors [own unpublished results]. The norepinephrine-stimulated increase in the melatonin synthesis in the pig pinealocytes is independent from the activation of transcription and translation, which processes are the central points of the adrenergic cascade regulating melatonin secretion from the rat pineal. Species-specific features were also reported regarding the ultrastructure of the pig pinealocytes [13, 14] as well as the diurnal pattern of changes in plasma melatonin [15, 16].

A well-known attribute of the sympathetic neurotransmission is local autoregulation of norepinephrine release at presynaptic level via α_2-adrenergic receptors. This process has been also demonstrated in the rat pineal gland in the *in vitro* studies [17, 18] as well as in the experiments performed *in vivo* [19].

The distribution and the immunohistochemical characteristic of the sympathetic nerve fibers in the pig pineal gland have been investigated [20], but functional studies of these fibers are lacking. The present study was carried out to investigate the neuronal uptake of norepinephrine as well as the presynaptic modulation of norepinephrine release via α_2-adrenoceptors in the pig pineal gland. Several specific antagonists and agonists of α_2-adrenoceptors were used for the characterization of receptors involved in the autoregulation of norepinephrine release in the pig pineal and their classification as one of four subtypes (α_{2A}, α_{2B}, α_{2C}, α_{2D}).

Material and methods

Animals and tissues

Investigations were performed during spring and summer. Female crossbred pigs (3.5 months of age, weighing 35±2 kg) were purchased, 7–14 days before the experiments started, from a commercial piggery (with natural length of day). Animals were kept in the room, in which natural lighting from windows was supplemented between 06:00-20:00 with fluorescent illumination with light intensity of 500 lx at the level of animal heads. Gilts had free access to water and were fed with standard food. The animals were slaughtered at 17:00. The pineal glands were removed no later than 3 minutes after heart stopped beating, cleaned of adherent connective tissue and blood vessels, then immediately used in the experiments.

Chemicals

The drugs were purchased as follows: guanfacine hydrochloride, UK 14 304, ARC 239, BRL 44408, imiloxan hydrochloride, rauwolscine hydrochloride, RS 79948 from Tocris Cookson Ltd (UK); phentolamine hydrochloride, oxymetazoline hydrochloride, prazosin hydrochloride, WB 4101, yohimbine hydrochloride, norepinephrine bitartrate, pargyline hydrochloride, desipramine hydrochloride and ascorbic acid from SIGMA (USA). Levo-[7-^{3}H]-norepinephrine (spec. act. 15 Ci/mmol) was obtained from NEN Life Sciences Products (USA). All other used chemicals were of analytical grade.

Stock solutions (2 mM) of RS 79948, BRL 44408 and UK 14,304 were prepared in DMSO. Prazosin was dispersed in ethanol and then dissolved in bidistilled water. All other agonists and antagonists were dissolved in bidistilled water. Tyrode medium contained: NaCl - 137 mM, KCl 2.68 - mM, $CaCl_2$ - 1.78 mM, $MgCl_2$ - 1.04 mM, NaH_2PO_4 - 0.42 mM, $NaHCO_3$ - 11.9 mM, Na_2EDTA - 0.069 mM, ascorbic acid - 0.061 mM and glucose - 5.55 mM.

Experimental protocols

Study of norepinephrine uptake

The pineals were divided into two parts (right and left). One part of the gland was preincubated in Tyrode medium containing desipramine (10 μM) for 10 minutes and then incubated in Tyrode medium containing ^{3}H-norepinephrine (0.5 μM) and desipramine (10 μM) for 60 minutes (desipramine sensitive uptake). The second part of the gland was treated in the same way, but in Tyrode medium without desipramine (total uptake). Incubation was performed in a humidified atmosphere of 80% O_2 and 5% CO_2 at 37.5 °C in Kendro incubator BB 6060 (Germany). After the incubation the pieces were flushed five times with Tyrode medium, weighed, digested with Tissue Solubiliser (ICN USA) and the content of tritium was measured using a liquid scintillation method.

In another experiment tissue pieces were incubated (37.5°C, 80% O_2, 5% CO_2) in Tyrode medium containing ^{3}H-norepinephrine (0.5 μM) together with cold norepinephrine 500 μM (unspecific uptake) or ^{3}H-norepi-

nephrine (0.5 μM) alone (total uptake) for 1 h and then processed as described above.

Study of high K^+-evoked norepinephine release and its autoregulation by α_2-adrenoceptors

The pineals were divided into three pieces, which were incubated during 60 minutes in Tyrode medium containing ^{3}H-norepinephrine (0,5 μM) and pargyline (100 μM) in humidified atmosphere of 80 % O_2 and 5% CO_2 at 37.5°C in Kendro BB 6060 incubator. Then the pieces were flushed with Tyrode medium, placed into a nylon net and transferred to separate perfusion chambers (chamber volume - 0.5 ml, total volume of perfusion set - ca 1.4 ml). Tyrode medium containing desipramine (10 μM) was perfused at a flow rate of 0.4 ml/min. The medium was gased with a mixture of 95% O_2 and 5% CO_2. The medium and chambers were maintained at 37.5°C. Medium fractions were collected every 2 minutes. After perfusion the pieces were weighed and digested with Tissue Solubiliser (ICN USA). The content of ^{3}H in the medium and the tissue pieces were measured using a liquid scintillation method.

Four experimental procedures were performed in order to demonstrate the neuronal origin of tritium released in response to the depolarization. (I) Tissue pieces preloaded with ^{3}H-norepinephrine as described above were perfused with Tyrode medium without Ca^{2+} and with EDTA (1 mM) for 150 minutes and then with Ca^{2+} - containing Tyrode medium. (II) Tissue pieces preloaded with ^{3}H-norepinephrine were perfused with Tyrode medium for 150 minutes and later 1 mM of Cd2+ was added to the medium for next 44 minutes of incubation. (III) The explants preloaded with ^{3}H-norepinephrine were perfused with normal Tyrode medium during the whole experiment. (IV) Tissue pieces treated with desipramine (100 μM) during incubation in ^{3}H-norepinephrine solution were perfused with normal Tyrode medium. The chambers with the above-mentioned tissue pieces were perfused with Tyrode medium containing 60 mM of K^+ (replacing equimolar concentration of Na^+) after 110 minutes and 180 minutes of perfusion for 14 minutes.

For the studies of presynaptic autoregulation of depolarization-induced norepinephrine release via α_2-adrenoceptors the tissue pieces preloaded with ^{3}H-norepinephrine were exposed twice (after 110 minutes of perfusion - S1 and 180 minutes of perfusion – S2) to Tyrode medium containing 60 mM of K^+ for 14 minutes. Twenty minutes before and during the second stimulation α_2-antagonists (ARC 239, BRL 44408, imiloxan, rauwolscine, RS 79948, prazosin, WB 4101, yohimbine) and α_2-agonists (norepinephrine, guanfacine, oxymetazoline, UK 14 304) were added to the medium. One piece of each pineal gland was used as control and was treated with medium containing vehiculum.

The fractional release rate (min^{-1}) was calculated as the amount of radioactivity released into each fraction over the total radioactivity present in the tissue at the start of this fraction collection. The high potassium-evoked tritium overflow was calculated by subtraction of the basal release (estimated as the mean fractional release during 10 minutes before stimulation) from each fractional release value during 30 minutes after beginning of perfusion with the high K^+ Tyrode medium and then by summing the obtained differences. The ratio of S2/S1 was calculated and expressed as percent of the control. The pED_{30} values - negative logarithms of concentrations that caused 30% increase or decrease in the depolarization-induced overflow of tritium were interpolated from the averaged concentration-response curves by nonlinear regression using PRISM 3.0 (GraphPad Software Inc., USA). Additionally, the mean basal tritium release for 10 minutes before the first and the second depolarization were compared for the determination of drugs action on spontaneous release of tritium. The data were analyzed using one-way ANOVA followed by Duncan test.

Results

The mean level (n=5) of the dispramine-sensitive (neuronal) uptake of norepinephrine in the pig pineal was 3.5±0.9 pmol/h/mg of wet tissue and represented 77.3% of the total tissue radioactivity. The mean value (n=5) of the specific norepinephrine uptake obtained by the comparison of the total and unspecific (23.1 % of total tissue radioactivity) incorporations of tritium into the tissue pieces was 3.62±0,3 pmol/h/mg of wet tissue.

The basal efflux of tritium from the perfused pineal tissue decreased during 60 minutes of incubation and then was rather stable up to the end of the experiments. Perfusion with Tyrode medium containing 60 mM of K^+ for 14 minutes resulted in tritium overflow corresponding to 3.8–4.5 % of tissue radioactivity before the depolarization (Fig.1). The mean (±SEM) ratio (n=75) of S2/S1 induced by repeated perfusion with 60 mM of K^+ for 14 minutes after 110 and 180 minutes of incubation was 0.91±0.05. The potassium-evoked overflow of tritium was almost completely abolished in the absence of extracellular Ca^{2+} and was diminished significantly in the presence of Cd^{2+} (Fig. 1). Perfusion with 60mM of potassium did not evoke tritium release from the pieces of the pig pineal gland treated with desipramine during loading with ^{3}H-norepinephrine (Fig. 1).

All used drugs at concentrations tested, except prazosin, did not affect the basal efflux of tritium. Prazosin at concentrations 1 μM enhanced basal efflux of tritium about 150% and at concentration 10 μM about 210%.

The used α-adrenoceptors antagonists, except ARC 239, increased concentration-dependently the depolarization-induced overflow of tritium (Fig. 2, 4). The highest increase (253% of the control) in K^+-evoked tritium release was noted after the treatment with rauwolscine (Fig. 2). ARC 239 did not change the depolarization-induced overflow of tritium. The rank order of potencies of adrenergic antagonists (based on the interpolated pED_{30} values) was: rauwolscine > phentolamine > BRL 44408 >WB 4101> RS 79948 = yohimbine >> prazosin >> imiloxan (Fig. 6).

The agonists of α_2-adrenergic receptors: norepinephrine, UK 14,304, oxymetazoline, guanfacine decreased the depolarization-induced overflow of tritium significantly, with the following order of potencies: UK14,304 > norepinephrine = guanfacine > oxymetazoline (Fig. 3, 5, 7).

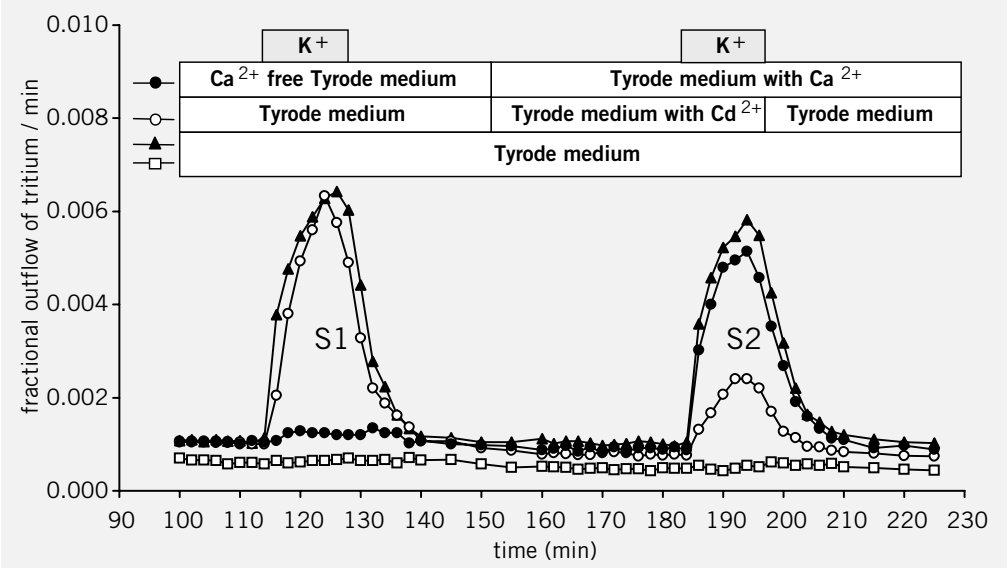


Fig. 1. The effect of exposition to Tyrode medium containing 60 mM of K^+ after 110 (S1) and 180 (S2) minutes of incubation on the mean (n=3) tritium fractional release from the pieces of the pig pineal gland preloaded with ^{3}H-norepinephrine: a) perfused with Tyrode medium without Ca^{2+} and with EDTA (1 mM) for 150 minutes and then with Ca^{2+}-containing Tyrode medium – group I, closed circles; b) perfused with Tyrode medium for 150 minutes, then with Tyrode medium containing 1 mM of Cd^{2+} medium 30 minutes before and during second treatment high potassium Tyrode medium – group II, open circles; c) perfused with Tyrode medium - group III (control), triangles; d) treated with desipramine before and during preloading with ^{3}H-norepinephrine and then perfused with Tyrode medium – group IV, squares. The values S2/S1 differ significantly ($p \leq 0.05$) between the group I (S2/S1 = 32.051), the group II (S2/S1= 0.269) and the group III (S2/S1 = 0.912). In the group IV depolarization did not induced overflow of tritium.

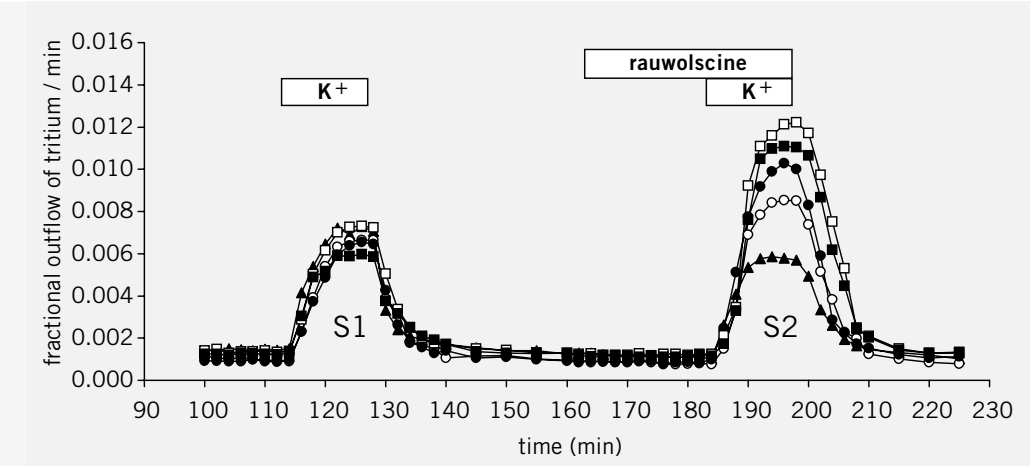


Fig. 2. The effect of perfusion with different concentrations of rauwolscine (open circles 0.01 μM, closed circles 0.1 μM, open squares 1 μM, closed squares 10 μM, triangles – control) 20 minutes before and during the second depolarization (S2) with Tyrode medium containing 60 mM K^+ on the mean (n=3) tritium fractional release from the pieces of the pig pineal gland preloaded with ^{3}H-norepinephrine.

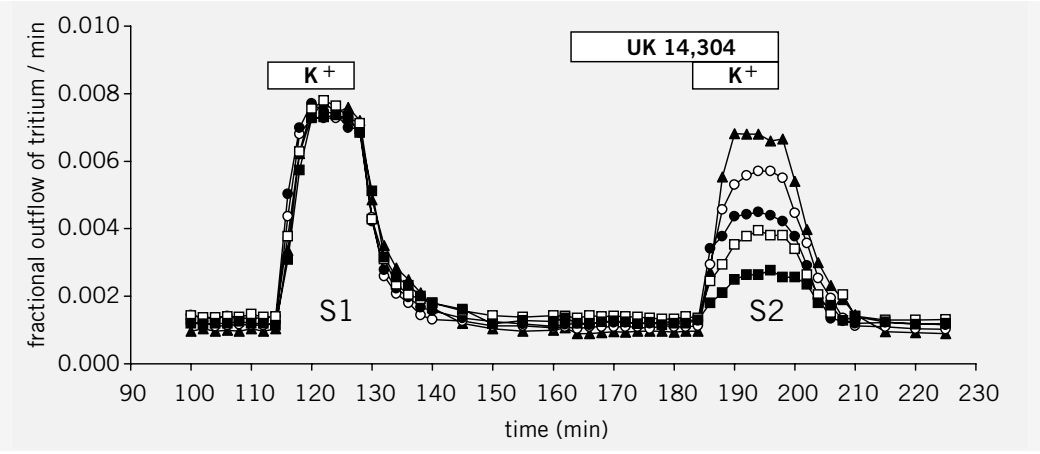
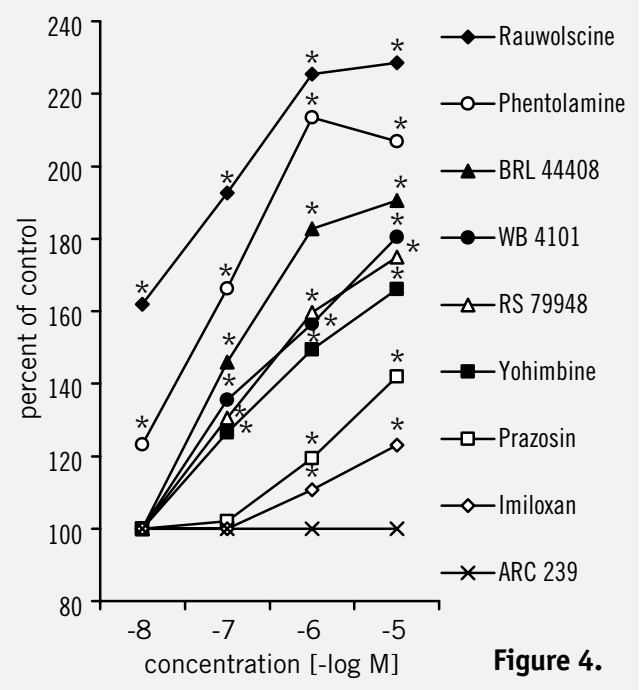


Fig. 3. The effect of perfusion with different concentrations of UK 14,304 (open circles 0.01 μM, closed circles 0.1 μM, open squares 1 μM, closed squares 10 μM, triangles – control) 20 minutes before and during the second depolarization (S2) with Tyrode medium containing 60 mM K^+ on the mean (n=3) tritium fractional release from the pieces of the pig pineal gland preloaded with ^{3}H-norepinephrine.



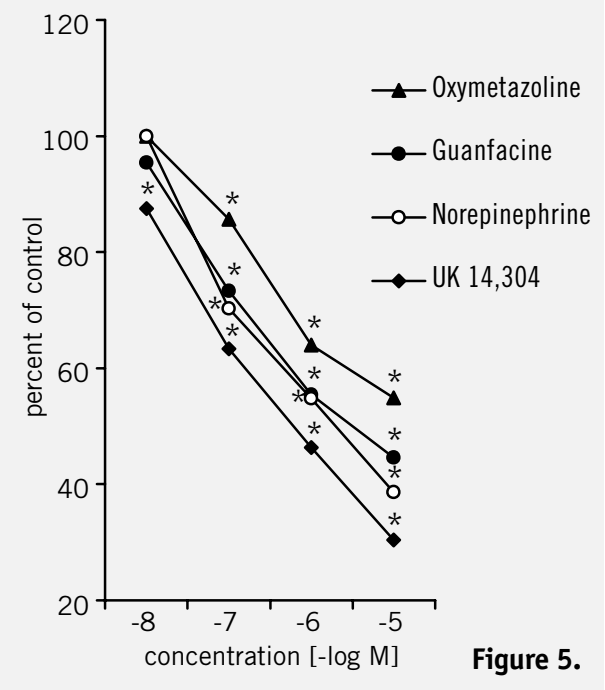
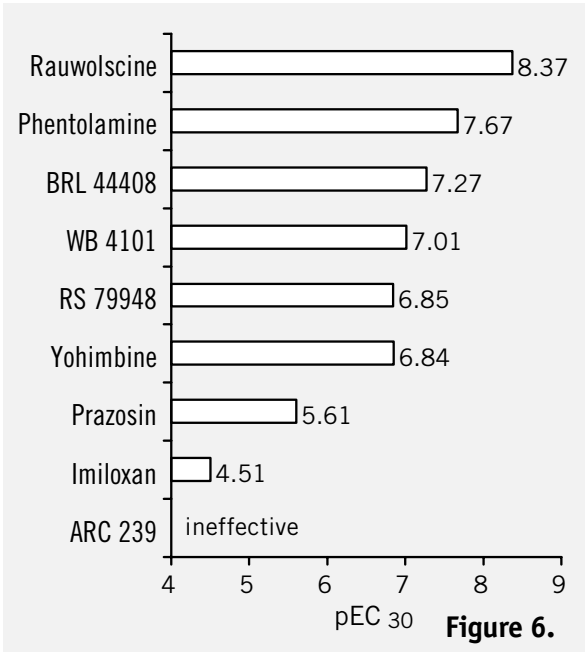


Fig. 4. The effect of α-adrenergic antagonists (expressed as percent of the control S2/S1 value) on depolarization-evoked overflow of tritium from the pieces of the pig pineal gland preloaded with ^{3}H-norepinephrine (means, n=3).
* - values significantly different versus corresponding controls at p≤0.05

Fig. 5. The effect of α-adrenergic agonists (expressed as percent of the control S2/S1 value) on depolarization-evoked overflow of tritium from the pieces of the pig pineal gland preloaded with ^{3}H-norepinephrine (means, n=3). * - values significantly different versus corresponding controls at p≤0.05



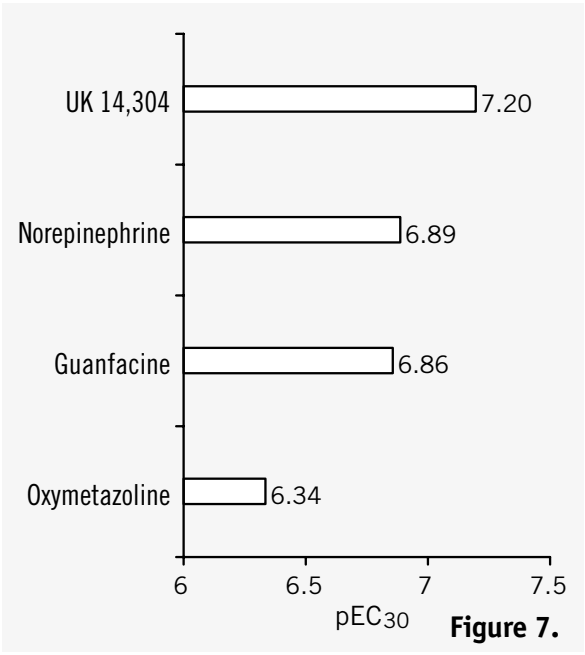


Fig. 6. Values of pEC_{30} of stimulatory action of α-adrenergic antagonists on depolarization-evoked overflow of tritium from the pieces of the pig pineal gland preloaded with ^{3}H-norepinephrine.

Fig. 7. Values of pEC_{30} of inhibitory action of α-adrenergic agonists on depolarization-evoked overflow of tritium from the pieces of the pig pineal gland preloaded with ^{3}H-norepinephrine.

Discussion

The obtained results demonstrate that the pieces of the porcine pineal gland incubated with ^{3}H-norepinephrine specifically accumulate radioactivity via desipramine-blocked mechanism, which suggests that this process represents a neuronal uptake. Depolarization with 60 mM of K^+ evoked overflow of tritium and this effect was significantly decreased in the presence of Cd^{2+} as well as abolished by the removal of Ca^{2+} from the incubation medium. Moreover, K^+-induced tritium overflow was not observed in the tissue pieces pretreated with uptake inhibitor - desipramine before and during loading with ^{3}H-norepinephrine. Several investigations have demonstrated that ^{3}H-norepinephrine represents more than 90 % of tritium released from various tissues (including the rat pineal gland) preloaded with this tritium labeled catecholamine in the presence of pargyline [21, 22]. Summing up, the depolarization-induced release of tritium may be taken as an index of norepinephrine release from the sympathetic nerve fibers in the pig pineal gland.

The neuronal uptake measured in the pig pineal (3.5±0.9 pmol/h/mg of wet tissue) was generally similar to that demonstrated in the pineal gland of the rat [22]. Chuluyan and co-workers [22] have shown that the neuronal uptake of norepinephrine in the rat pineal gland varies in diurnal cycle being higher at 14:00 and at 20:00 than at 24:00 and 04:00. It has been also demonstrated that melatonin decreases the uptake of norepinephrine in the pineal gland removed from the rats killed at 20:00 and does not change this neuronal uptake in the glands taken at the remaining investigated time-points – 14:00, 24:00, 04:00 [22].

In the present study all used adrenergic antagonists and agonists, except prazosin, did not change a spontaneous tritium release from the pieces of the pig pineal. Prazosin at concentration 1 μM and higher increased markedly the spontaneous outflow of tritium. This finding is not surprising, because it has been reported that prazosin enhances the resting outflow of tritium from the tissues preloaded with ^{3}H-norepinephrine, probably due to its reserpine-like properties [23, 24]. The increase in the spontaneous release of tritium had been also described after treatment with ARC 239 [23], however in the present study this effect was not observed.

Stimulatory action of α_2-antagonists and inhibitory action α_2-agonists, observed in the present study of K^+-evoked tritium release, demonstrate that in the pig pineal gland the release of norepinephrine is regulated at the presynaptic level via α_2-adrenoceptors. Autoregulation of norepinephrine release has been previously reported only in the rat pineal gland [17, 18, 19]. In this species the release of norepinephrine from the sympathetic nerve fibers is also modulated by neuropeptide Y via Y_2 receptors [18] as well as by acetylcholine via muscarinic receptors [25]. Moreover, the main pineal hormone – melatonin impaired K^+-evoked release of norepinephrine in the pineals taken from the rats killed during scotophase at 24:00 and 04:00, but remained inactive in pineals excised during photophase at 14:00 and 20:00 [22]. To our knowledge the problem of presynaptic modulation of norepinephrine release in the pineal gland has not been investigated in other mammalian species.

In the present study all used antagonists except ARC 239 (a specific antagonist of α_{2B}-adrenoceptors) increased depolarization-evoked tritium release with the rank order of pED_{30}: rauwolscine > phentolamine > BRL 44408 > WB 4101> RS 79948 = yohimbine >> prazosin >> imiloxan. On the other hand, all used agonists decreased K^+-evoked release of tritium from the pieces of the pig pineal gland. The rank order of pED_{30} was as follows: UK14,304 > norepinephrine = guanfacine > oxymetazoline. An interesting finding of the present study is the action of oxymetazoline as an agonist, because under many experimental conditions the action of oxymetazoline at presynaptic α_2-adrenoceptors as an antagonist has been reported [24]. Similar species or tissue specific differences in stimulatory or inhibitory action were also described in case of another α_2-agonist: clonidine [24]. In our experiments K^+-evoked release of tritium was modified by the drugs considered as specific to α_{2A}-adrenoceptors: BRL 44408, oxymetazoline and guanfacine. On the other hand, antagonists of α_{2B}-adrenoceptors: imiloxan and ARC 239 showed respectively the very low action or the lack of action on depolarization-evoked tritium release. Moreover, rather low potency of prazosin suggests against the involvement of α_{2C}-adrenoceptors in presynaptic regulation of norepinephrine release in the pig pineal. Summing up, α_2-adrenoceptors regulating depolarization-induced norepinephrine release in the pig pineal gland close resemble in pharmacological properties the subtype α_{2A}. Further studies under autoinhibition-free conditions with the use of combination of specific agonists and antagonists should be performed for final confirmation of the classification of these receptors as subtype α_{2A}.

Up till now, functional pharmacological properties of presynaptic α_2-adrenoceptors have not been investigated in the pineal gland in details and their subtypes were not determined. Autoreceptors of type α_2 involved in regulation of norepinephrine release from the sympathetic nerve endings have been classified, depending on species and tissue investigated, as subtypes α_{2A} [23, 24], α_{2B} [26], α_{2C} [27] and α_{2D} [28]. However, in the majority of species autoreceptors regulating norepinephrine release from the sympathetic nerve fibers represent mainly subtype α_{2A} or its ortolog α_{2D} [23, 24, 28].

In conclusion, the present study demonstrated for the first time the processes of the neuronal uptake and the depolarization-induced release of norepinephrine from the sympathetic nerve endings in the pig pineal gland. The experiments with the use of adrenergic antagonists and agonist indicate that the sympathetic nerve fibers in the pig pineal gland possess functional presynaptic α_2-adrenoceptors, which are involved in norepinephrine release inhibition. Based on the pharmacological properties these receptors closely resemble the subtype α_{2A}.

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