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β-Adrenoceptor stimulation enhances transmitter output from the rat phrenic nerve

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1 Neurally-evoked output of newly synthesized [^{3}H]-acetylcholine from the rat phrenic nerve was measured in the absence of cholinesterase inhibitors.

2 Noradrenaline and isoprenaline enhanced neurally-evoked transmitter output markedly. Moreover, immediately after the application of noradrenaline the basal tritium efflux increased significantly.

3 Pretreatment with propranolol (0.1 μmol l^{-1}) or atenolol (0.3 μmol l^{-1}) completely prevented the stimulatory effect of noradrenaline and isoprenaline on evoked transmitter output.

4 The facilitatory effect of isoprenaline declined, when the exposure time was increased. This observation supports the assumption that β-adrenoceptors can be desensitized or inactivated during continued exposure to agonists.

5 It was shown for the first time that stimulation of β-adrenoceptors enhances transmitter output from the motor nerve. It is proposed that these β-adrenoceptors are of the β_1-subtype and are localized on the endings of motor nerves. Circulating catecholamines may facilitate neuromuscular transmission by stimulation of presynaptic β-adrenoceptors.

Introduction

It is generally accepted that sympathomimetic amines affect neuromuscular transmission at motor endplates by a local site of action. Stimulation of β-adrenoceptors which are located on the muscle fibre (postsynaptic site) modifies contractility (Bowman & Raper, 1966; Marsden & Meadows, 1970; Bowman, 1981). Moreover, it has been postulated that α-adrenoceptors are located on the nerve endings, and stimulation of these latter receptors mediates an increased transmitter release (Malta *et al.*, 1979; Bowman, 1981). So far, the present concept of presynaptic α-adrenoceptors and postsynaptic β-adrenoceptors is based on the results of functional experiments, i.e. release of acetylcholine was measured indirectly by the registration of the electrical or mechanical end-organ responses.

We have recently developed a new method of measuring the release of [^{3}H]-acetylcholine (ACh) from the rat phrenic nerve without the inhibition of cholinesterase (Wessler & Kilbinger, 1986; Wessler & Steinlein, 1987). Using this method evidence was provided that the motor nerve is endowed with autoreceptors which are involved in the local control of the stimulated transmitter release (Wessler *et al.*, 1986; 1987a; Somogyi *et al.*, 1987). The aim of the present study was to investigate whether adrenoceptors are also present on motor nerves and whether these receptors can modulate transmitter release. The effects of noradrenaline and isoprenaline on the output of [^{3}H]-ACh from the rat phrenic nerve were examined.

Methods

Output experiments

The experimental protocol has been described in detail previously (Wessler & Kilbinger, 1986; Wessler *et al.*, 1986). In short, small muscle strips (about 20 mg) containing the endplate region together with the phrenic nerve were prepared from the rat left hemidiaphragm (Sprague–Dawley rats). The endplate preparations were superfused in 2 ml organ baths with a physiological salt solution (composition in mmol l^{-1}: NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, glucose 11.2, choline 0.001, ascorbic acid 0.057)

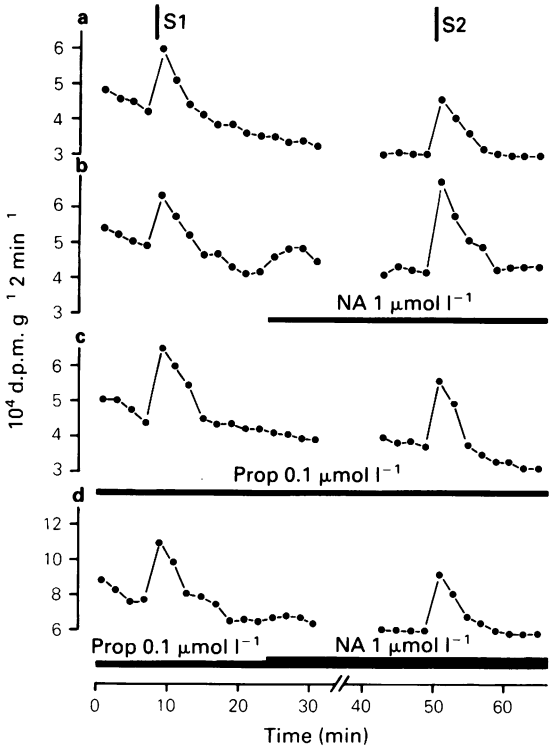

Figure 1 Effects of noradrenaline (NA) and propranolol (Prop) on basal tritium efflux and evoked tritium output. After the labelling and washout period, tritium output was elicited by two electrical nerve stimulations (200 pulses, 10 Hz) at the indicated times (S1, S2). The horizontal bars indicate the presence of noradrenaline and/or propranolol. The antagonist was added 25 min before S1. Given are means, s.e. means were 3–20% of the means. (a) Control experiments ($n = 8$); (b) effect of noradrenaline ($n = 6$); (c) experiments with propranolol alone ($n = 4$); (d) competition experiments with propranolol and noradrenaline ($n = 4$).

bubbled with 95% O_2 and 5% CO_2 (pH of the salt solution: 7.2). Neuronal transmitter stores were labelled by incubation (40 min) with [^{3}H]-choline (10 μCi) and the application of electrical nerve stimulation (square wave pulses of 0.2 ms duration, 8 mA, 1 Hz). After a subsequent washout period (60 min) and the addition of 10 μmol l^{-1} hemicholinium-3 (to prevent re-uptake of choline) tritium efflux was measured in 2 min fractions (Figure 1). Tritium output was evoked by electrical nerve stimulation (200 pulses, 10 Hz) which started 8 min (S1) and 48 min (S2) after the end of the washout period. In some experiments the train length was reduced to 50 pulses applied at 5 Hz. Noradrenaline or isoprenaline were added before (8 min–24 min) the second

Table 1 Neurally-evoked tritium output in control experiments

Drug	*S1* (d.p.m. g^{-1})	*S2/S1*	n
Control	35000 ± 4800	1.00 ± 0.08	8
Propranolol (0.1 μmol l^{-1})	46900 ± 11200	0.84 ± 0.05	4
Atenolol (0.3 μmol l^{-1})	33700 ± 7700	1.16 ± 0.04	4

After the labelling and washout period tritium efflux was measured in 2 min samples. Tritium output was elicited by two (S1, S2) periods of electrical nerve stimulation (10 Hz, 200 pulses). Propranolol or atenolol were added to the incubation medium 25 min before S1. Given are the tritium output evoked by S1 (d.p.m. g^{-1}) and the S2/S1 ratios (means ± s.e. mean of the number of experiments indicated).

stimulation period (S2) and their effects are expressed by the ratios S2/S1. The β-adrenoceptor antagonists propranolol or atenolol were present from 25 min before S1 onwards (see Figure 1).

Electrical nerve stimulation causes an increase in tritium efflux (Figure 1) which can be attributed entirely to the calcium-dependent output of [^{3}H]-ACh from the nerve terminals (Wessler & Kilbinger, 1986; Wessler & Steinlein, 1987). Output of [^{3}H]-ACh was measured from the increase in tritium efflux above the basal level. The basal tritium efflux during the stimulation period was determined by calculating a regression line. Two values obtained immediately before nerve stimulation and two values reaching the pre-stimulation level after the stimulation period (18th–22nd min for S1; 58th–62nd min for S2; see Figure 1) were included for the calculation of regression lines.

Materials

Atenolol (4-[2'-hydroxy-3'-(isopropylamino)propoxy]-phenylacetamide; Sigma Chemie GmbH, Deisenhofen, F.R.G.), methyl-[^{3}H]-choline chloride (NEN, Boston, MA; U.S.A.; 80 Ci mmol^{-1}), hemicholinium-3 bromide (EGA Chemie, Steinheim, F.R.G.), isoprenaline hydrochloride (Boehringer Ingelheim, F.R.G.), noradrenaline bitartrate (Höchst, Frankfurt, F.R.G.), propranolol hydrochloride (Sigma Chemie GmbH).

Statistical analysis

The means ± s.e. mean are given. The significance of differences was assessed by Student's t test. When

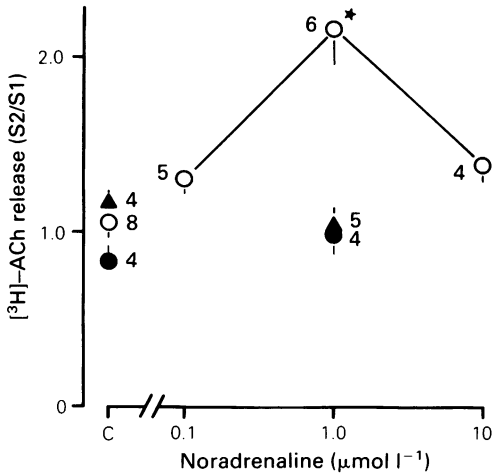

Figure 2 Concentration-response curve for noradrenaline. The experimental protocol is shown in Figure 1. (○) S2/S1 ratios obtained under control conditions (C) or in the presence of noradrenaline. (▲) S2/S1 ratios obtained in the presence of 0.3 μmol l^{-1} atenolol alone (C) or together with 1 μmol l^{-1} noradrenaline. (●) S2/S1 ratios obtained in the presence of 0.1 μmol l^{-1} propranolol alone (C) or together with 1 μmol l^{-1} noradrenaline. Given are the means of the number of experiments indicated; vertical lines show s.e. mean. Significance of difference from control experiments (C): * $P < 0.01$.

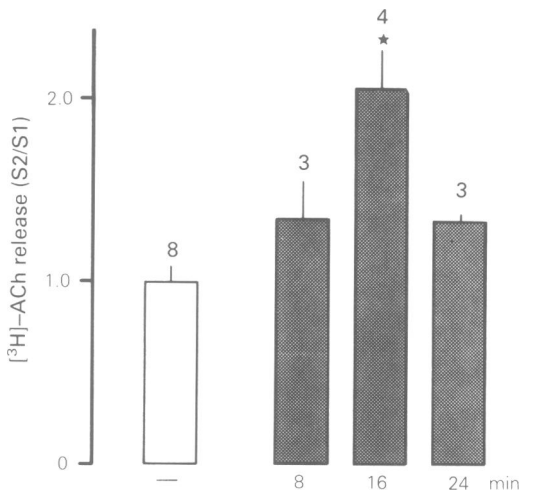

Figure 3 Effect of isoprenaline on neurally-evoked tritium output after different exposure times. The experimental protocol is shown in Figure 1. Open column: S2/S1 ratio obtained under control conditions. Solid columns: S2/S1 ratios obtained in the presence of 0.1 μmol l^{-1} isoprenaline. The exposure time (before S2) is indicated below each column. Each column represents the mean of the number of experiments indicated at the top of the columns; vertical lines show s.e. mean. Significance of difference from control experiments (open column): * $P < 0.01$.

more than one group was compared with one control, significance of differences was evaluated by the Bonferroni test (Wallenstein *et al.*, 1980). P values <0.05 were regarded as significant.

Results

Effect of noradrenaline

Figure 1 illustrates the effect of noradrenaline on neurally-evoked tritium output. Under control conditions S1 caused an output of 35,000 d.p.m. g^{-1} and a S2/S1 ratio of 1.00 was found (Figure 1a, Table 1). Neurally-evoked tritium output increased markedly (115%) in the presence of 1 μmol l^{-1} noradrenaline (Figures 1b and 2). Moreover, immediately after the application of noradrenaline the basal tritium efflux was significantly enhanced by about 30%. The complete concentration-response curve is shown in Figure 2. A biphasic curve was found, the maximal effect occurred at 1 μmol l^{-1} whilst a concentration of 10 μmol l^{-1} was less effective.

Pretreatment with propranolol completely prevented the facilitatory effect of noradrenaline on neurally-evoked tritium output and reduced the effect on the basal tritium efflux (Figure 1d and 2). Likewise, pretreatment with atenolol (0.3 μmol l^{-1}) abolished the enhancing effect of noradrenaline on neurally-evoked tritium output (Figure 2). Tritium output evoked by S1 and the respective S2/S1 ratios obtained in the experiments with the antagonists alone (given from 25 min before S1 onwards) are shown in Table 1.

Effect of isoprenaline

The effect of the β-adrenoceptor agonist isoprenaline was also investigated and the results are illustrated in Figure 3. Like noradrenaline, isoprenaline (0.1 μmol l^{-1}) enhanced (106%) neurally-evoked tritium output (Figure 3). Pretreatment with propranolol of atenolol prevented the enhancing effect of isoprenaline (Table 2). This strongly suggests that the facilitatory effect was mediated by stimulation of β-adrenoceptors. The enhancing effect of isoprenaline strongly depended on the duration of the exposure time. Isoprenaline lost its effect, when the exposure time was increased from 16 min to 24 min (Figure 3).

In further experiments the effect of isoprenaline on tritium output evoked by a short train of pulses (50

Table 2 The effect of isoprenaline on neurally-evoked tritium output in the absence and presence of an antagonist

Drug	*S1* (d.p.m. g^{-1})	*S2/S1*	n
Control	35000 ± 4800	1.00 ± 0.08	8
Isoprenaline	31300 ± 4500	2.06 ± 0.20*	4
Isoprenaline + propranolol	33700 ± 3600	0.81 ± 0.05	3
Isoprenaline + atenolol	38700 ± 2100	0.98 ± 0.04	5

The experimental protocol is described in Table 1. Isoprenaline (0.1 μmol l^{-1}) was given 16 min before S2. In the respective experiments propranolol (0.1 μmol l^{-1}) or atenolol (0.3 μmol l^{-1}) was present in the incubation medium from 25 min before S1 onwards. Given are the tritium output evoked by S1 (d.p.m. g^{-1}) and the S2/S1 ratios (means ± s.e. mean of the number of experiments indicated). Significance of difference from control: * $P < 0.01$.

pulses at 5 Hz) was investigated. Under control conditions S1 released 9900 ± 2000 d.p.m. g^{-1} and a S2/S1 ratio of 1.10 ± 0.11 was found ($n = 5$). Isoprenaline (0.1 μmol l^{-1}) was added 16 min before S2 to the incubation medium. In these latter experiments S1 caused an output of 12,000 ± 1500 d.p.m. g^{-1} ($n = 3$). Also during this short stimulation period isoprenaline enhanced neurally-evoked tritium output (S2/S1 ratio : 2.00 ± 0.18, $n = 3$).

Discussion

It has been shown previously that electrically evoked tritium output is equivalent to a calcium-dependent release of transmitter from the phrenic nerve (Wessler & Kilbinger, 1986; Wessler & Steinlein, 1987).

Noradrenaline and isoprenaline markedly enhanced transmitter output from the rat phrenic nerve. This facilitatory effect disappeared after pretreatment with propranolol or atenolol. It was shown for the first time that stimulation of β-adrenoceptors causes an increase in transmitter output from motor nerves. β-Adrenoceptors are also known to exist on sympathetic neurones where they mediate an increase in transmitter release from these neurones (Starke, 1981). In concurrence with the widely accepted concept of a presynaptic location of modulatory receptors, we propose that the facilitatory β-adrenoceptors which are present within the endplate region of skeletal muscles are also located presynaptically, i.e. on the motor nerve terminals. These presynaptic β-adrenoceptors may be classified as β_1-adrenoceptors, because a low concentration of atenolol (0.3 μmol l^{-1}) completely antagonized the effect of 1 μmol l^{-1} noradrenaline. Therefore, the β-adrenoceptors modulating transmitter output seem to differ in their pharmacological properties from the β-adrenoceptors present on the muscle fibres. The latter receptors are of the β_2 subtype (cf. Bowman, 1981). These different pharmacological properties strengthen the conclusion that two populations of β-adrenoceptors are present, presynaptic β_1-adrenoceptors and postsynaptic β_2-adrenoceptors.

A modulatory role of sympathomimetic amines on neuromuscular transmission has been repeatedly demonstrated (cf. Bowman, 1981). Additionally, it has been shown that cyclic AMP increases the amplitude of endplate potentials (Wilson, 1974). The latter author suggested that cyclic AMP is involved in regulating metabolic activity associated with synthesis, mobilization and storage of acetylcholine. It appears unlikely that the effect of isoprenaline is primarily mediated by an action on synthesis of transmitter, but rather by other mechanisms involved in transmitter release (i.e. increase in release probability, mobilization, recruitment of active release zones) for the following reasons: (1) After the labelling period has been passed [^{3}H]-choline is washed out from the extracellular space and hemicholinium-3 added. Both conditions prevent the synthesis of [^{3}H]-ACh additional to that formed during the labelling period (Wessler & Steinlein, 1987). (2) Uptake of [^{3}H]-choline originating from hydrolysis of phospholipids, which are mainly located extraneuronally (Wessler & Sandman, 1987), is also blocked in the presence of hemicholinium-3. (3) The enhancing effect of isoprenaline was similar either on a train with 200 pulses (10 Hz) or on a train with 50 pulses (5 Hz). A primary action of isoprenaline on synthesis should lead to an increased stimulatory effect during the long train length. Taken together it seems likely that, in the present experiments, the stimulatory effect of β-adrenoceptor activation on transmitter release is located distal from synthesis. This suggestion is further supported by the observation that during repetitive neuronal activity, the first endplate potential is increased in the presence of cyclic AMP (Wilson, 1974).

Sympathomimetic amines can produce an increase in both the frequency of miniature endplate potentials and the amplitude of evoked endplate potentials (Kuba, 1970; Bowman, 1981). Both effects are thought to reflect presynaptic events which are mediated by stimulation of α-adrenoceptors (Malta *et al.*, 1979; Bowman, 1981). However, in the present experiments noradrenaline increased evoked transmitter release by stimulation of β-adrenoceptors. At this stage of the investigation we cannot explain the

discrepancy, probably different experimental conditions (i.e. determination of unlabelled vs labelled ACh, pulse to pulse measure vs trains of 50 or 200 pulses) might have caused the differences. Based on the results of the present experiments we do not exclude the possibility that α-adrenoceptors are also present on the motor nerve. Particularly, the effect of noradrenaline on the basal tritium efflux points to the existence of α-adrenoceptors which is consistent with the above cited studies. Additional experiments with selective agonists on α-adrenoceptors need to be carried out.

The facilitatory effect of isoprenaline declined when the exposure time was increased (Figure 3). Moreover, a concentration of $10\,\mu mol\,l^{-1}$ noradrenaline was less effective than a concentration of $1\,\mu mol\,l^{-1}$, i.e. a biphasic concentration-response curve was found (Figure 2). One possibility to explain these observations is offered by the propensity of β-adrenoceptors to be desensitized or inactivated by a continuous exposure to, or by a high concentration of, agonists (Sibley *et al.*, 1985; Levitzki, 1986). The loss of the β-adrenoceptor-mediated effect developed within several minutes (Figure 3), whereas it has previously been suggested that the presynaptic nicotine receptors of the phrenic nerve desensitize within seconds (Wessler *et al.*, 1987b, c). This difference in the velocity (min vs s) probably reflects different molecular mechanisms underlying the process of desensitization or inactivation. Desensitization of the nicotine receptor is assumed to be linked to a change in the electrical properties of channels (Colquhoun, 1986), naturally a rapid event.

The activation of presynaptic β-adrenoceptors might contribute to the stimulatory action of noradrenaline on neuromuscular transmission, especially under conditions of raised plasma concentrations (phaeochromocytoma, thyrotoxicosis, stress, treatment of Parkinson's disease with L-DOPA). The well known ability of propranolol to reduce hyperactivity of skeletal muscles might be linked with the blockade of both presynaptic and postsynaptic β-adrenoceptors. Taken together, these clinical observations provide evidence that presynaptic β-adrenoceptors might be of physiological significance for neuromuscular transmission.

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References

BOWMAN, W.C. (1981). Effects of adrenergic activators and inhibitors on the skeletal muscles. In *Handbook of Experimental Pharmacology*. Vol 54/II. ed. Szekeres, L., pp. 47–128. Berlin, Heidelberg, New York: Springer Verlag.

BOWMAN, W.C. & RAPER, C. (1966). Effects of sympathomimetic amines on neuromuscular transmission. *Br. J. Pharmacol., Chemother.*, **27**, 313–331.

COLQUHOUN, D. (1986). On the principles of postsynaptic action of neuromuscular blocking agents. In *Handbook of Experimental Pharmacology*. Vol 79. ed. Kharkevich, D.A., pp. 59–113. Berlin, Heidelberg, New York: Springer Verlag.

KUBA, K. (1970). Effects of catecholamines on the neuromuscular junction in the rat diaphragm. *J. Physiol.*, **211**, 551–570.

LEVITZKI, A. (1986). β-Adrenergic receptors and their mode of coupling to adenylate cyclase. *Physiol. Rev.*, **66**, 819–854.

MALTA, E., McPHERSON, G.A. & RAPER, C. (1979). Comparison of pre-junctional α-adrenoceptors at the neuromuscular junction with vascular post-junctional α-receptors in cat skeletal muscle. *Br J. Pharmacol.*, **65**, 249–256.

MARSDEN, C.D. & MEADOWS, J.C. (1970). The effect of adrenaline on the contraction of human muscle. *J. Physiol.*, **207**, 429–448.

SIBLEY, D.R., NAMBI, P. & LEFKOWITZ, R.J. (1985). Molecular mechanisms of hormone receptor desensitization. In *Molecular Mechanisms of Transmembrane Signalling*. Vol. 4. ed. Cohen, P. & Houslay, M.D., pp. 359–374. Amsterdam, New York, Oxford: Elsevier.

SOMOGYI, G.T., VIZI, E.S., CHAUDHRY, I.A., NAGASHIMA, H., DUNCALF, D., FOLDES, F.F. & GOLDINER, P.L. (1987). Modulation of stimulation-evoked release of newly formed acetylcholine from mouse hemidiaphragm preparation. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **336**, 11–15.

STARKE, K. (1981). Presynaptic receptors. *Ann. Rev. Pharmacol. Toxicol.*, **21**, 7–30.

WALLENSTEIN, S., ZUCKER, C.L. & FLEISS, J.I. (1980). Some statistical methods useful in circulation research. *Circ. Res.*, **47**, 1–9.

WESSLER, I. & KILBINGER, H. (1986). Release of [^{3}H]acetylcholine from a modified rat phrenic nerve-hemidiaphragm preparation. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **334**, 357–364.

WESSLER, I., HALANK, M., RASBACH, J. & KILBINGER, H. (1986). Presynaptic nicotine receptors mediating a positive feed-back on transmitter release from the rat phrenic nerve. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **334**, 365–372.

WESSLER, I., KARL, M., MAI, M. & DIENER, A. (1987a). Muscarine receptors on the rat phrenic nerve, evidence for positive and negative muscarinic feedback mechanisms. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **335**, 605–612.

WESSLER, I. & SANDMANN, J. (1987). Uptake and metabolism of [^{3}H]choline from the rat phrenic nerve-hemidiaphragm preparation. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **335**, 231–237.

WESSLER, I., SCHEUER, B. & KILBINGER, H. (1987b). [^{3}H]acetylcholine release from the phrenic nerve is increased or decreased by activation or desensitization of nicotine receptors. *Eur. J. Pharmacol.*, **135**, 85–87.

WESSLER, I., SCHEUER, B. & HILLEN, U. (1987c). Nicotine

receptors of the rat phrenic nerve: Characterized by stimulation, blockade and desensitization. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **335** (Suppl.), R77.

WESSLER, I. & STEINLEIN, O. (1987). Differential release of [^{3}H]acetylcholine from the rat phrenic nerve-hemidiaphragm preparation by electrical nerve stimulation and by high potassium. *Neuroscience*, **22**, 289–299.

WILSON, D.F. (1974). The effects of dibutyryl cyclic adenosine 3',5'-monophosphate, theophylline and aminophylline on neuromuscular transmission in the rat. *J. Pharmacol. Exp. Ther.*, **188**, 447–452.

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