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EVIDENCE FOR SPECIFIC ADENOSINE RECEPTORS AT CHOLINERGIC NERVE ENDINGS

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1 An electrophysiological study was made to determine if adenosine and adenine nucleotides affect cholinergic nerve endings to frog skeletal muscle through relatively non-specific nucleotide receptors or through specific adenosine receptors.

2 Non-hydrolysable derivatives of adenosine triphosphate failed to alter the mean number of acetylcholine (ACh) quanta released by a nerve impulse ($\bar{m}$) or the miniature endplate potential frequency (m.e.p.p.$_f$) but N^6-methyladenosine and 2-chloroadenosine, two adenosine analogues with an unsubstituted ribose moiety (R-site agonists), produced marked reductions in $\bar{m}$ and m.e.p.p.$_f$.

3 In contrast, 2′-deoxyadenosine, a derivative with an unsubstituted purine ring (P-site agonist), generally produced increases in $\bar{m}$ and m.e.p.p.$_f$, which further increased after removing the drug. Other P-site agonists such as 5′-deoxyadenosine (in the presence of theophylline) and 9-β-D-arabinofuranosyl adenine also increased $\bar{m}$ and m.e.p.p.$_f$.

4 The results suggest that two types of adenosine receptors may be present at cholinergic nerve endings, one type (R-site) mediating depression and the other type (P-site) producing enhancement of ACh release.

Introduction

The observations that adenine nucleotides are released from cholinergic nerves to skeletal muscle (Silinsky & Hubbard, 1973; Silinsky, 1975) and that adenosine and adenine nucleotides depress the release of acetylcholine (ACh) (Ginsborg & Hirst, 1972; Ribeiro & Walker, 1975; Cook, Hamilton, & Okwuasaba, 1978; 1979) have led to considerable speculation and controversy regarding the structural specificity of adenosine compounds in cholinergic transmission (Stone, 1978; Cook *et al.*, 1978; 1979). At cholinergic nerve terminals in the autonomic nervous system (ANS), the specificity for adenosine derivatives is very low; most modified adenosine compounds including non-hydrolysable adenine nucleotides and even coenzyme A act as effective inhibitors of ACh release (Cook *et al.*, 1978; 1979). In the only studies at skeletal motor nerve endings, adenine nucleotides appear equipotent with the parent nucleoside, adenosine, in inhibiting the release of ACh (Ginsborg & Hirst, 1972; Ribeiro & Walker, 1975). Such non-selectivity is in contrast to the evidence suggesting that many biological effects are mediated by specific receptors for adenosine, receptors that may be linked to adenylate cyclase. For example, two types of receptive sites for adenosine or adenylate cyclase have been postulated (Londos & Wolff, 1977); one in which agonist activity is favoured by an unsubstituted ribose moiety (type R), the other in which selective activation is associated with an unsubstituted purine ring (type P). Whilst in the process of investigating the physiological mechanisms of adenosine's actions, I made the incidental observation that, in contrast to the ANS, non-hydrolysable adenine nucleotides did not inhibit ACh release at the skeletal neuromuscular junction. It was thus decided to test the possibility that the receptors for purine compounds in this system, rather than being non-selective, may be specific adenosine receptors.

Methods

The frog cutaneous pectoris nerve-muscle preparation was used in conjunction with conventional electrophysiological methods for intracellular recording and nerve stimulation. The mean number of ACh quanta released by a nerve impulse ($\bar{m}$) was determined by standard methods (del Castillo & Katz, 1954; Martin, 1955; Stevens, 1976). Generally, the ratio of the mean endplate potential amplitude (e.p.p.) to the mean miniature endplate potential amplitude (m.e.p.p.) was used to calculate $\bar{m}$, but at low $\bar{m}$ the method of

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failures was used as well. Nerve stimulation was delivered at a frequency of 0.5 Hz and 2 to 4 identical ($\pm$0.2 mV) computer averages of e.p.ps in response to 128 stimuli were obtained before adding a drug (e.g. Figure 1b, open circles). In order to increase and thus facilitate measurements of m.e.p.p. frequencies (m.e.p.p.$_f$), the control Ringer solution (0.5 to 0.7 mM $CaCl_2$, 6 to 8 mM $MgCl_2$, 115 mM NaCl, 2 mM KCl, 1 μg/ml neostigmine bromide, buffered to pH ~ 7.1) was of slightly elevated tonicity. Similar results were obtained at normal tonicity and m.e.p.p.$_f$ (see also Ginsborg & Hirst, 1972). All drugs were obtained from the the Sigma Chemical Co. with the exception of the deoxyadenosine derivatives which were purchased from P.L. Biochemicals.

Results

Neither α,β-methylene ATP nor β,γ-methylene ATP, two non-hydrolysable derivatives of adenosine triphosphate (ATP), produced effects on ACh release in concentrations as high as 0.5 to 1 mM. In contrast, a selective R adenosine receptor agonist N^6-methyladenosine (Londos & Wolff, 1977) inhibited ACh release (Figure 1a). In the experiment presented in Figure 1a, the control e.p.p. (upper trace), which reflects an $\bar{m}$ = 12.2 and is associated with a m.e.p.p.$_f$ of 5.4/s was reduced in a concentration-dependent fashion by 12.5 μM N^6-methyladenosine ($\bar{m}$ = 7.0, middle trace); (m.e.p.p.$_f$ = 3.7/s) and 25 μM N^6-methyladenosine ($\bar{m}$ = 5.8, lower trace); (m.e.p.p.$_f$ = 2.2/s). The experiment shown in Figure 1a is representative of seven experiments (see figure legend). The concentration-effect relationship (as well as the rapid onset and recovery from inhibition) for N^6-methyladenosine was similar to adenosine, with maximal levels of inhibition (~50% of control) being produced generally at concentrations near 250 μM. Another R-site agonist, 2-chloroadenosine, produced similar experimental records to those shown in Figure 1a but at 1 to 2 orders of magnitude lower concentration. It is possible that the extreme sensitivity of ACh release to inhibition by 2-chloroadenosine may be due to the insensitivity of this drug to deamination and/or uptake (Clarke, Davoll, Phillips & Brown, 1952).

As opposed to the behaviour of these derivatives modified at the purine ring, the ribose-modified compound 2′-deoxyadenosine (10 to 200 μM) failed to exert an inhibitory effect (and actually enhanced ACh release) in 3 of 4 experiments (see below), with a modest 13% inhibition of $\bar{m}$ and 11% inhibition of m.e.p.p.$_f$ being observed during drug exposure in the remaining experiment. These results differ from the pronounced inhibitory effects seen with 2′-deoxyadenosine on cholinergic neurones in the ANS (Cook *et al.*, 1979) but are consistent with the minimal effects of this agent on R-receptors for adenosine (Londos & Wolff, 1977). The results described thus far (failure of non-hydrolysable nucleotides to inhibit ACh release, agonist selectivity) suggest that there are type R adenosine receptors on cholinergic nerve endings.

Adenosine derivatives can also increase ACh release. In the experiment shown in Figure 1b, brief exposure to 100 μM 2′-deoxyadenosine (between arrows) produced an increase in $\bar{m}$ and m.e.p.p.$_f$ (filled squares) over control levels (open circles) which further increased after the drug was removed (filled circles). This long-lasting oscillatory excitation was characteristics of experiments in 2′-deoxyadenosine (see figure legend).

It seems plausible that the effect of 2′-deoxyadenosine represents the behaviour of a different receptor population, possibly one in which activation is favoured by an unsubstituted purine ring (type P adenosine receptors). Further support for the presence of P-receptors was obtained from experiments with 5′-deoxyadenosine, a potent P-receptor agonist (Londos & Wolff, 1977). It was found that in the presence of a relatively high concentration (1.8 mM) of theophylline (which blocks the inhibitory effects of all nucleosides), 5′-deoxyadenosine (25 to 400 μM) produced reversible increases in $\bar{m}$ (ranging from 15% to 43%) and m.e.p.p.$_f$ (ranging from 13% to 66%) in all four fibres studied. In addition, two experiments with the selective P-receptor agonist 9-β-D-arabinofuranosyl adenine (Londos & Wolff, 1977), revealed long-lasting increases in ACh release as well, adding support for the presence of P-sites. Finally, agents that affect both P- and R-receptors produced a characteristic pattern of an initial inhibition followed by some reflection of prolonged increases in release during and especially after drug treatment. This dual effect was seen in all reversible experiments with 3′- and 5′-deoxyadenosine and, in some experiments, with 2-chloroadenosine and adenosine as well; these four agents activating both P- and R-sites.

Discussion

The results suggest that cholinergic nerve endings to skeletal muscle possess two distinct populations of adenosine receptors and that adenine nucleotides may need to be hydrolysed to adenosine before exerting their immediate effects. If this is so, then an elevation in the extracellular adenosine concentration (resulting from released and hydrolysed adenine nucleotides and associated with elevated levels of ACh release) could act through R receptors (which are probably situated at the external surface of the membrane (Londos, Wolff & Cooper, 1979) as a negative feedback modulator of ACh release (see also Silinsky, 1975). Although these experiments were made at low levels of

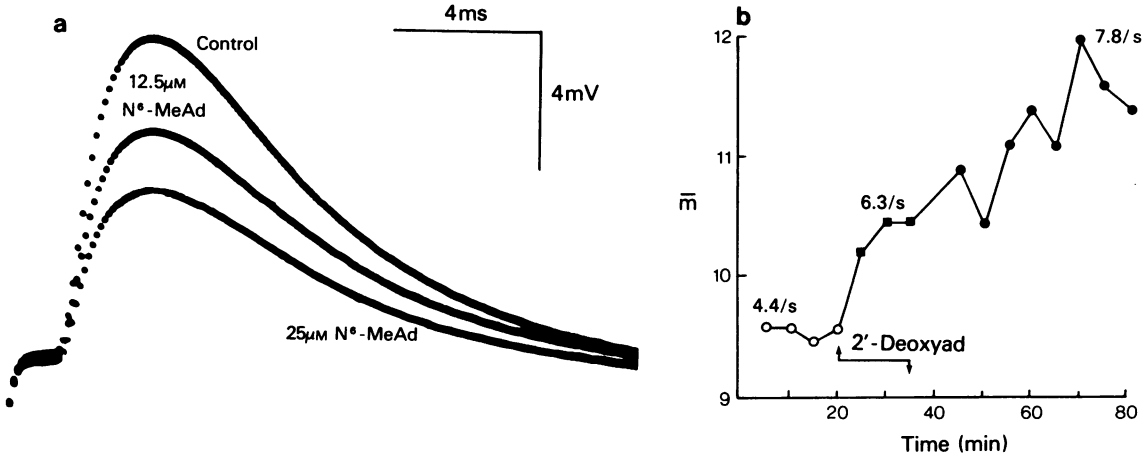

Figure 1 Effects of adenosine derivatives on acetylcholine (ACh) release. (a) Depression of ACh release by N^6-methyladenosine (N^6-MeAd). E.p.ps, directly reflect the mean number number of ACh quanta released by a nerve impulse (m). In this experiment, the control $\bar{m} = 12.2$ (a, upper record) and m.e.p.p.$_f = 5.4 \pm 0.3$/s (mean $\pm$ s.e. mean,) are reduced to $\bar{m} = 7.0$ (a, middle record) and m.e.p.p.$_f = 3.7 \pm 0.3$/s by 12.5 μM N^6-methyladenosine and to $\bar{m} = 5.8$ (a, lower record) and m.e.p.p.$_f = 2.2 \pm 0.2$/s by 25 ϱM N^6-methyladenosine. At these concentrations the effects of N^6-methyladenosine were readily reversible. Similar results were seen in 6 other experiments with a mean reduction in $\bar{m}$ being ~33% in the seven experiments in 25 μM N^6-methyladenosine. Each record in (a) is the computer-averaged e.p.p. in response to 128 stimuli. Vertical calibration: 4 mV; horizontal calibration) 4 ms. (b) Long-lasting enhancement of ACh release by 2'-deoxyadenosine (100 μM): (○) $\bar{m}$ in control Ringer; (■) $\bar{m}$ in presence of 2'-deoxyadenosine (2'-Deoxyad, applied between arrows); (●) $\bar{m}$ upon return to control Ringer after 2'-deoxyadenosine pretreatment. Each symbol represents mean of 128 e.p.ps/m.e.p.p. amplitude. M.e.p.p.$_f$ shown at 5 min (control), 30 min (2'-deoxyadenosine), and 70 min (post-drug control). In the three other experiments with 2'-deoxyadenosine (10 to 200 μM) the post-drug increases in $\bar{m}$ over control ranged from 25% to 86% and in m.e.p.p.$_f$ 23% to 77%. Note that even in the one experiment where 2'-deoxyadenosine depressed ACh release (see text), post-control levels of $\bar{m}$ and m.e.p.p.$_f$ were elevated above the pre-drug level.

evoked ACh release to enable both $\bar{m}$ and m.e.p.p.$_f$ to be determined, it is noteworthy that marked depressant effects on ACh release occur at higher $\bar{m}$ as well. For example, in several experiments in higher $[Ca^{2+}]$, 1 μM 2-chloroadenosine depressed $\bar{m}$ by a value estimated to be greater than 500 quanta per impulse. In addition to this inhibition, adenosine, under certain circumstances (e.g. a high enough concentration at an appropriate locus, possibly intracellularly), might produce a persistent increase in ACh release and thus add an element of plasticity to cholinergic nerve endings. However, as both P- and R-sites have thus far been associated with the enzyme adenylate cyclase (Londos & Wolff, 1977) any firm conclusions must await further experiments in which cyclic nucleotides, adenosine derivatives, and other purine compounds are applied intracellularly (using liposomes as a vehicle). Such experiments are currently in progress.

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