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The influence of 2-chloroadenosine on potassium-evoked and neurally-evoked acetylcholine secretion from normal or from latent active zones in the frog

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1 It has been suggested that adenosine receptor agonists do not impair K-dependent acetylcholine (ACh) secretion at motor nerve endings. If true, this result would be discordant with the conventional theories of adenosine action at the neuromuscular junction. It was thus decided to examine the effect of 2-chloroadenosine on quantal ACh release evoked by different K concentrations at frog motor nerve endings.

2 Quantal ACh release evoked by mild increases in the extracellular K concentration (from 2 mM to 6–11 mM) was inhibited by 2-chloroadenosine (10 μM) in a manner similar to the inhibition of neurally-evoked ACh release.

3 ACh secretion evoked by prolonged exposure to 20 mM K Ringer was also inhibited by adenosine derivatives. Under these conditions, alterations in the structure of the secreting active zones have been reported whereby the original release sites now release only a small proportion of the total quantal ACh output.

4 Preparations were bathed for several hours with Ca-free Ringer containing Mg to examine further the importance of intact active zones on inhibition produced by adenosine receptor agonists. This procedure has been reported to produce latent sites of ACh secretion and persistent derangement of the active zones. Shortly after this treatment, neurally-evoked ACh release in normal Ringer solution was found to be inhibited by 2-chloroadenosine (1–5 μM) or adenosine (50 μM).

5 The results suggest that (a) K-evoked ACh release is inhibited by adenosine derivatives even when quantal secretion occurs outside the original active zone and that (b) the cytoskeletal or membrane structures which maintain the structural integrity and lateral regularity of the active zones are not the target sites for inhibition by adenosine derivatives.

Introduction

It has been suggested that adenosine derivatives are negative feedback modulators of transmitter secretion at a number of synaptic loci (Silinsky, 1975; Ribeiro, 1979; Dunwiddie, 1985; Phillis & Wu, 1982; Sebastiao & Ribeiro, 1985). At the skeletal neuromuscular junction, it is generally believed that adenosine receptor activation (Silinsky, 1980) inhibits Ca-dependent acetylcholine (ACh) secretion, either by interfering with Ca entry (Sebastiao & Ribeiro, 1986; but see Silinsky, 1986) or by decreasing the affinity of Ca for a strategic component of the secretory apparatus (Silinsky, 1981; 1984; 1985a, b).

The published evidence most disharmonious with either of the above Ca-dependent mechanisms is the suggestion that adenosine derivatives do not inhibit K-evoked quantal ACh release at motor nerve endings (Buckle & Spence, 1982). If this result is reproducible, then as K-evoked release is dependent both on Ca entry and Ca-evoked exocytosis, the view that adenosine receptor agonists work to impair Ca-dependent processes would be questioned. Such a negative result would be further disconcerting with respect to relating the biochemical effects of adenosine to the electrophysiological studies, as most of the biochemical studies on adenosine and ACh release employed K as the secretagogue (see e.g. Reese & Cooper, 1982; Aas & Fonnum, 1986; Pedata *et al.*, 1986).

High K has been reported to change the ultrastructural appearance of frog nerve endings. Specifi-

cally, it has been found that a large number of latent release sites appear after exposure to 20 mM K whereby the original active zone of ACh secretion now contributes only minimally to the overall quantal output (Ceccarelli *et al.* 1979b; Ceccarelli & Hurlbut, 1980). It has thus been suggested that the absence of an effect of adenosine derivatives on K-evoked release, if true, could be due to such an alteration of the active zone structure (see Silinsky 1985b, for discussion).

The purpose of the work described in this paper was to determine whether adenosine receptor activation inhibits quantal ACh release evoked by different K concentrations with a corollary interest of observing the effects of conditions reported to change the structure of the active zone on the inhibitory effects of adenosine derivatives.

Methods

Electrophysiological and statistical procedures

Cutaneous pectoris nerve-muscle preparations of frog (*Rana pipiens*) were dissected and superfused with flowing Ringer solution. Supramaximal stimulation pulses were delivered to the nerve supply through a suction electrode. Intracellular recordings were made at endplate regions with glass microelectrodes filled with 3 M KCl and with resistances from 8–25 MΩ. Signals from the microelectrodes were fed into a conventional high input impedance preamplifier, the output of which was delivered into an oscilloscope, a computer, a pen recorder (Brush-Gould), and an FM tape recorder. Miniature endplate potential (m.e.p.p.) frequencies were determined from pen records and m.e.p.p. amplitudes from pen records after first being recorded on magnetic tape. Experiments made on synchronous, neurally-evoked release (Figure 3), i.e., release reflected as the endplate potential (e.p.p.), were controlled and analysed by use of an LSI 11/73 computer and 125 kHz 14 bit A/D-12 bit D/A converter on line with hard copy of the digitalized traces being made on an XY plotter.

Statistical procedures were identical to those used previously (see Silinsky, 1984, pp. 244–245). For neurally-evoked ACh release, appropriate averaging techniques were employed to reduce variability so that statistically significant differences might be detected (Silinsky, 1987). For K-evoked release, statistically-significant differences were generally observed at $P \ll 0.01$. Mean values are presented ± standard error of the mean (s.e. mean) in the Figure legends.

Electrophysiological assessment of morphological changes

Nerve terminals in the frog cutaneous pectoris muscle have undergone extensive morphological scrutiny with respect to the ultrastructural properties of secreting synaptic vesicles and active zones (Heuser, 1977; Pumplin & Reese; Ceccarelli *et al.*, 1979a,b; Haimann *et al.*, 1980; Ceccarelli & Hurlbut, 1980). Active zones are the parts of the nerve terminal membrane associated with the secretory apparatus (for review see Ceccarelli & Hurlbut, 1980). They occur at regular intervals (approximately one micron) along the length of the plasmalemma and appear morphologically as gently bulging fibrillar structures flanked by paired rows of large intramembrane particles. Of particular interest is that ultrastructural changes in the secreting active zone in frog cutaneous pectoris nerve-muscle preparations are almost exclusively studied in association with concomitant electrophysiological changes. The distinct electrophysiological alterations that accompany morphological changes provoked by 20 mM K and by Ca-free solutions were used to assess for structural changes in the secreting active zone as follows. For the high K studies, 20 mM K causes a burst of miniature endplate potentials (m.e.p.ps) followed by a decay of m.e.p.p. frequency to relatively stable levels over a period of 1–2 h (e.g. Figure 2). This stable period, which is also associated with the frequent appearance of a small mode m.e.p.ps, delineates the time over which most of the ACh release occurs between the original active zones (Ceccarelli *et al.*, 1979b; Haimann *et al.*, 1980; Ceccarelli & Hurlbut, 1980). For the studies in which the active zone is disorganized by Ca-free Ringer containing Mg, a reduction in m.e.p.p. amplitude in normal Ringer solution after treatment for 2–3 h with Ca-free solutions is related to the morphological disruption of the active zones. This disruptive effect is not reversible, even after 1–2 h in normal Ringer (Haimann *et al.*, 1980).

Composition of solutions and chemicals

Normal frog Ringer solution contained (mM): NaCl 115, KCl 2, $CaCl_2$ 1.8, $NaHCO_3$ (pH 7.2–7.4) and was used in all experiments to test the effects of adenosine derivatives. High K Ringer was of similar composition but contained the indicated amount of KCl. Ca-free Ringer was free of added $CaCl_2$ and contained 1 mM ethyleneglycol-*bis*-(β-aminoethyl ether)-*N*,*N*′-tetra-acetic acid (EGTA) and 3–5 mM Mg. All drugs were obtained from the Sigma Chemical Company.

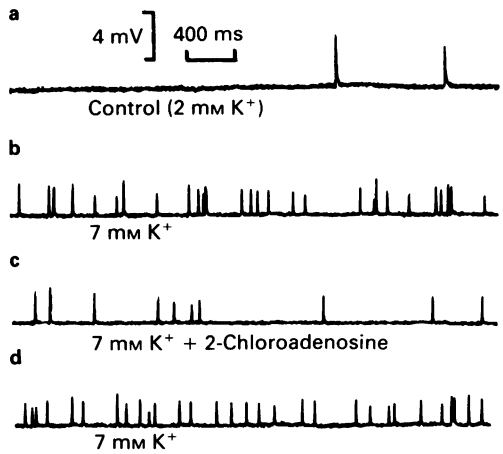

Figure 1 Inhibitory effects of 2-chloroadenosine (10 μM) on release evoked by 7 mM K. (a) Control m.e.p.p. frequency (per s) = 0.43 ± 0.13 (mean ± s.e. mean, n = 28 for all); (b) m.e.p.p. frequency after 20 min exposure to 7 mM K Ringer = 7.39 ± 0.47 (the amplitudes of the m.e.p.ps were decreased by the K-induced depolarization). (c) After 9 min in 7 mM K Ringer + 2-chloroadenosine, m.e.p.p. frequency = 3.61 ± 0.38. (d) Recontrol in 7 mM K Ringer; m.e.p.p. frequency = 8.25 ± 0.52. See text for details of other experiments.

Results

Effects of 2-chloroadenosine on ACh release evoked by 6–11 mM K

The specific adenosine receptor agonist, 2-chloroadenosine, was used in this study as conditions may be chosen whereby this agent does not affect spontaneous quantal release yet produces potent effects on neurally-evoked release (Buckle & Spence, 1982; Silinsky, 1984). Results with this agonist will thus not be confounded by assumptions as to how the changes in resting ACh release observed with other adenosine receptor agonists (Ginsborg & Hirst, 1972; Buckle & Spence, 1982; Silinsky, 1980; 1984) relate to changes in K-dependent quantal secretion.

Figure 1 shows that ACh release evoked by brief mild elevations in the extracellular K concentration is inhibited by 2-chloroadenosine. In this experiment, the control m.e.p.p. frequency of 0.43 s^{-1} (Figure 1a) is elevated 17 fold (b) after 20 min in 7 mM K Ringer. Nine minutes of exposure to 10 μM 2-chloroadenosine produced approximately 50% inhibition of m.e.p.p. frequency (c), an effect that was readily reversible (d). In another experiment (not illustrated), the inhibitory effects of 2-chloroadenosine were studied on ACh release evoked both by 6 mM and 11 mM K in the same fibre. In this cell, 6 mM K Ringer increased resting m.e.p.p. frequency 10 fold. After 13 min in 10 μM 2-chloroadenosine (6 mM K), the m.e.p.p. frequency was reversibly depressed to 53% of the level observed in 6 mM K Ringer. In the same fibre, elevating the extracellular K to 11 mM caused m.e.p.p. frequency to rise approximately 10 fold over that observed in 6 mM K Ringer. After 20 min in 2-chloroadenosine (11 mM K) the m.e.p.p. frequency reversibly declined to 58% of the level observed in 11 mM K. Similar results were observed in two other experiments.

It thus appears that 2-chloroadenosine produces the characteristic inhibitory effect observed for neurally-evoked ACh release (Silinsky, 1984) on release evoked by modest elevations in the extracellular K concentration. Under these conditions, quantal ACh release would be expected to occur exclusively at the active zone (Ceccarelli & Hurlbut, 1980).

The effects of 2-chloroadenosine on ACh release evoked by prolonged exposure to 20 mM K

When frog motor nerve endings are exposed to 20 mM K solutions, the control m.e.p.p. frequency (Figure 2a) is first elevated to immeasurable levels (b) and then, as depletion of ACh quanta ensues (Ceccarelli *et al.*, 1979b), decays over time course 0.5–2 h to lower frequencies which remain relatively stable (c). Figure 2(c–f) illustrates that 2-chloroadenosine is an effective inhibitor of ACh release during the steady-state period of ACh release evoked by 20 mM K. In Figure 2d, the m.e.p.p. frequency was depressed by 2-chloroadenosine to 7% of the stable control value (c), an effect that was reversible (not shown). It should be noted that the fibre studied in Figure 2 was particularly sensitive to the inhibitory effects of 2-chloroadenosine. Specifically, in two other cells of the same preparation, 2-chloroadenosine reversibly decreased m.e.p.p. frequency to 11% and 28% of control. Several other preparations treated in this manner revealed more normal levels of inhibition (approximately 50%) after exposure to adenosine derivatives (see Figure 2e and f).

In the period during and after Figure 2c, correlative morphological studies have shown that exocytosis occurs along the entire surface of the nerve ending with the active zone providing only a small proportion of the total number of releasing regions (see Ceccarelli & Hurlbut, 1980). These present results thus demonstrate that 2-chloroadenosine is a potent inhibitor of quantal ACh release, even when the preponderance of quantal events do not emanate from the original active zone.

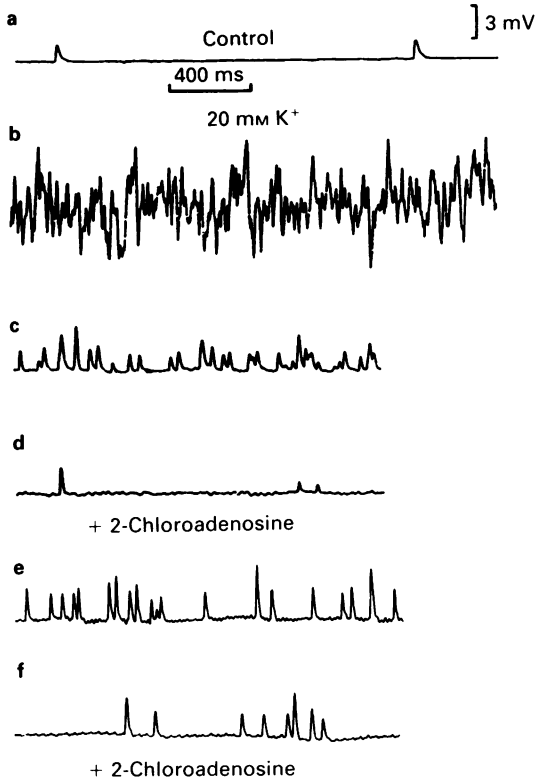

Figure 2 Inhibitory effects of 2-chloroadenosine (10 μM) on release evoked by prolonged exposure to 20 mM K Ringer. (a) Shows control m.e.p.p. frequency (s^{-1}) in normal Ringer = 1.32 ± 0.20 (n = 22). (b) m.e.p.p. frequency 8 min after bathing the preparation in 20 mM K Ringer is elevated to immeasureable levels. (c) Shows results from another experiment; after 3 h in 20 mM K, the m.e.p.p. frequency, after an early period of intense discharge such as shown in (b) has subsided to 64.00 ± 2.35 (n = 4). (d) 20 mM K Ringer + 2-chloroadenosine 8 min after (c). Note profound inhibition of m.e.p.p. frequency (4.75 ± 0.72, n = 8). See text for further details of other fibres in this same preparation. Traces (e) and (f) illustrate effects of 2-chloroadenosine on another preparation treated with 20 mM K Ringer. (e) Control m.e.p.p. frequency after 3.5 h in 20 mM K = 23.55 ± 0.9 (n = 18). (f) After 17 min in 20 mM K Ringer + 2-chloroadenosine, m.e.p.p. frequency = 12.61 ± 0.82 (n = 18). Control m.e.p.p. frequency 40 min after return to nucleoside-free 20 mM K Ringer was 19.6 ± 0.79 (n = 15, not shown).

Effects of adenosine derivatives on ACh release after prolonged treatment with Ca free solutions containing Mg

Based upon the above results, it was decided to investigate further the influence of conditions found to disrupt the orderly appearance of the secreting active zones on the inhibitory effects of adenosine and 2-chloroadenosine. Bathing frog motor nerve endings in Ca-free Ringer containing Mg and EGTA for brief periods has been found to produce latent sites of ACh secretion (Pumplin & Reese,

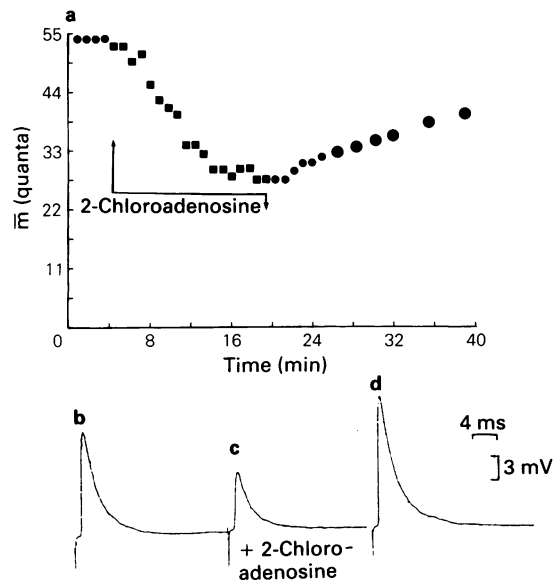

Figure 3 Inhibitory effects of 2-chloroadenosine after pretreating preparations for 3.5 h in Ca free-Ringer containing 1 mM EGTA and 5 mM Mg (Mg-EGTA Ringer). (a) Shows the time course of the inhibitory effects of 2-chloroadenosine in an experiment in which the mean number of ACh quanta released by a nerve impulse (m) was estimated. The first circle (●, which represents control values prior to the application of 2-chloroadenosine) shows transmitter release approximately 1 h after return to normal Ca Ringer containing tubocurarine from Ca-free Ringer; (■) effects of 2-chloroadenosine (2 μM). Small symbols were made from averages of 8 e.p.ps (0.2 Hz); larger symbols in the recovery period reflect the average of 16–32 e.p.ps. Normal Ca Ringer contained tubocurarine (Tc) 6 $mg\,l^{-1}$. The m̄ was calculated by taking the ratio of the e.p.p. amplitude in Tc to the uncurarized m.e.p.p. amplitude and multiplying by a correction factor (=25) representing the concentration of Tc and the affinity constant of Tc for ACh receptors (see Silinsky, 1984, eqn. 1).

Records (b)–(d) are e.p.ps from another experiment. Each record is the averaged response to 8 stimuli delivered at 0.2 Hz in normal calcium Ringer containing Tc 4 $mg\,l^{-1}$. (b) Control in normal Ringer after Ca-free Ringer treatment. (c) Shows the inhibitory effect of 5 μM 2-chloroadenosine as measured 1 h after removal of Mg-EGTA Ringer. (d) Second control made 11 min after (c) and 10 min after (a). Note the rebound increase in ACh release in (d) after exposure to 2-chloroadenosine (see Silinsky, 1980).

1977). If the exposure to Ca-free Ringer was continued for several hours, then derangement of the regular appearance of the active zone has been observed (Ceccarelli & Hurlbut, 1980). Superfusing the preparation with normal Ca solutions after Mg-EGTA treatment restores synchronous evoked ACh release reflected as the e.p.p. (e.g. Figure 3) yet the active zone remains disorganized for at least several hours (Haimann *et al.*, 1980). Figure 3 shows that adenosine derivatives still exert their inhibitory effects in normal Ringer under such disruptive conditions. Figure 3a illustrates the time course of an experiment in which the mean number of ACh quanta released by a nerve impulse ($\bar{m}$) was determined (see legend). In this experiment, 2-chloroadenosine (2 μM) began to inhibit ACh release 1 h and 14 min after 3.5 h of pretreatment of the fibre with 5 mM Mg-EGTA Ringer. Figure 3(b–d) shows electrophysiological records of averaged e.p.ps from another similar experiment (b, post Mg-EGTA control; c, after 10 min in 5 μM 2-chloroadenosine; d, recontrol). Similar results in which adenosine produced an approximately 50% inhibition of ACh release after Mg-EGTA treatment were seen in four other experiments with 2-chloroadenosine (1–2 μM) and in one with adenosine (50 μM).

Discussion

The results are reassuring in that they demonstrate that adenosine receptor agonists exert similar inhibitory effects on K-evoked quantal ACh release detected electrophysiologically and K-dependent ACh secretion assayed biochemically (see Reese & Cooper, 1982, Figure 1; Pedata *et al.*, 1986, Table 1; Aas & Fonnum, 1986). As 2-chloroadenosine does not alter the resting m.e.p.p. frequency under the conditions of these experiments (Buckle & Spence, 1982; Silinsky, 1984), it is impossible to attribute the inhibitory effects of this agonist on K-evoked m.e.p.ps to changes in resting m.e.p.p. frequency, as has been done previously with adenosine (Buckle & Spence, 1982). The results of this study thus demonstrate that Ca-dependent ACh release evoked by increases in extracellular K concentrations is depressed by adenosine receptor agonists.

The results also suggest that alterations in the normal structural appearance of the secreting active zone do not impair the inhibitory action of adenosine receptor agonists. This is in contrast to biochemical perturbations (e.g. an adenylate cyclase inhibitor; Silinsky, 1984) and other physical alterations (e.g. low temperature; Silinsky & Hirsh, 1988) which do prevent the inhibitory effects of adenosine derivatives. The experiments with Ca-free Ringer solution suggest that disruption of the active zone also cannot explain the paucity of inhibitory effects of adenosine on Mg-dependent increases in neurally-evoked m.e.p.p. frequencies during treatment with Ca-free solutions (Ginsborg & Hirst, 1972; see Silinsky, 1985b). An alternative working hypothesis to explain the results of Ginsborg & Hirst (1972) may be formulated as follows. Ca, Sr and Mg have all been shown to allow the movement of synaptic vesicles in frog cutaneous pectoris preparations from 50 A away to apposition with the nerve terminal membrane; subsequent movements associated with fusion and exocytosis are promoted by Ca, Sr but not by Mg (Heuser, 1977). The inability of Mg-dependent release to be inhibited by adenosine derivatives may be due to this ion's inability to bind to Ca binding proteins associated with the exocytosis process (Silinsky, 1985c), proteins that may be the ultimate target sites for the action of adenosine (Silinsky, 1981; 1984). Studies with Mg containing liposomes tend to bear out this prediction (Kharasch *et al.*, 1981).

In summary, these results demonstrate that (a) adenosine derivatives are potent inhibitors of K-dependent quantal ACh release at motor nerve terminals and (b) conditions reported to change the active zone morphologically do not influence the inhibitory effects of adenosine derivatives. In this latter regard, it is noteworthy that the large intramembranous particles, which may represent putative Ca channels and associated Ca binding proteins (Ceccarelli & Hurlbut, 1980), remain in association with exocytotic profiles of ACh secretion under these conditions of altered active zone structure (Ceccarelli *et al.*, 1979a). It thus appears the target Ca binding proteins can be influenced by adenosine and its agonists despite the structural stresses encountered by the active zone.

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