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On the type of receptor involved in the inhibitory action of adenosine at the neuromuscular junction

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1 The effects of adenosine and adenosine analogues (L-N^6-phenylisopropyladenosine (L-PIA), D-N^6-phenylisopropyladenosine (D-PIA), N^6-cyclohexyladenosine (CHA), N^6-methyladenosine, 5'-N-ethylcarboxamide adenosine (NECA) and 2-chloroadenosine) on evoked endplate potentials (e.p.ps) and on twitch tension were investigated in innervated sartorius muscles of the frog.

2 Adenosine and its analogues decreased, in a concentration-dependent manner, the amplitude of both the e.p.ps and the twitch responses evoked by indirect stimulation. The order of potencies in decreasing twitch tension was: L-PIA, CHA, NECA > 2-chloroadenosine > D-PIA > N^6-methyladenosine, adenosine. L-PIA was about ten fold more potent than D-PIA.

3 None of the adenosine analogues tested affected the twitch responses of directly stimulated tubocurarine-paralyzed muscles.

4 In concentrations that did not modify neuromuscular transmission, theophylline and 8-phenyltheophylline (8-PT) but not isobutylmethylxanthine (IBMX), antagonized the inhibitory action of 2-chloroadenosine at the neuromuscular junction. 8-PT behaved as a competitive antagonist and was about forty fold more potent than theophylline.

5 It is concluded that the R-type adenosine receptor at the neuromuscular junction should not be classified in the A_1/A_2 system. The possibility of calcium-linked adenosine receptors having pharmacological profiles distinct from those originally defined as modulating adenylate cyclase is discussed.

Introduction

Adenosine decreases neuromuscular transmission at the neuromuscular junction (Ginsborg & Hirst, 1972; Ribeiro & Walker, 1975; Ribeiro & Dominguez, 1978; Brãnisteanu *et al.*, 1979; Ribeiro *et al.*, 1979a; Ribeiro, 1982) through an R-type adenosine receptor (Silinsky, 1980). However, R-type adenosine receptors appear to encompass two receptor populations, the A_1/R_i site and the A_2/R_a site (van Calker *et al.*, 1979; Londos *et al.*, 1980). The present work was undertaken to characterize further the type of receptor involved in the inhibitory action of adenosine at the neuromuscular junction.

A brief account of some of the results has already appeared (Sebastião & Ribeiro, 1985).

Methods

The experiments were carried out at room temperature (22–25°C) on innervated sartorius muscles of the frog (*Rana ridibunda*).

Endplate potentials recordings

When recording evoked endplate potentials (e.p.ps), the preparations were mounted in a Perspex chamber through which the solutions flowed continuously at a rate of 5 ml min^{-1} via a roller pump; the bath volume was 5 ml, the level being kept constant by suction. The solutions were changed by transferring the inlet tube of the pump from one flask to another. The e.p.ps were recorded in the conventional way (Fatt & Katz, 1951) with intracellular electrodes filled with 3M KCl and of 10 to 20 MΩ resistance; the bath electrode was an Ag-AgCl pellet. The nerve was stimulated supramaximally with rectangular pulses of 20 μs duration applied once every 2 s. Evoked responses of 64 consecutive stimuli were averaged after amplification with a Datalab DL-400 computer. The output of the computer was coupled to a pen-recorder. The usual procedure was to continue to record averages in the same solution until a stable value was obtained, i.e. until two successive averages differed by less than 5%.

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Muscle contraction recordings

When recording twitch responses the preparations were set up in a 25 ml bath through which the solutions flowed continuously with the aid of a roller pump. The perfusing conditions were the same as those used when recording e.p.ps except that the flux rate was 25 ml min^{-1} during the first 1.5 min after changing the solutions. The nerve was stimulated supramaximally with rectangular pulses of 20 μs duration applied once every 5 s. The tension development (T) was recorded isometrically at a resting tension of 0.05 N with a Sanborn transducer and displayed on a Hewlett-Packard recorder. The maximum rate of rise of tension (*dT/dt max*) was recorded simultaneously with T. In some experiments the twitch responses evoked by direct stimulation of the muscle fibres were also recorded; supramaximal pulses of 0.8–1.5 ms duration, 0.2 Hz, were applied by means of two palador wires. In these experiments tubocurarine was added to the bath in a concentration (2×10^{-5} M) sufficient to cause complete blockade of neuromuscular transmission.

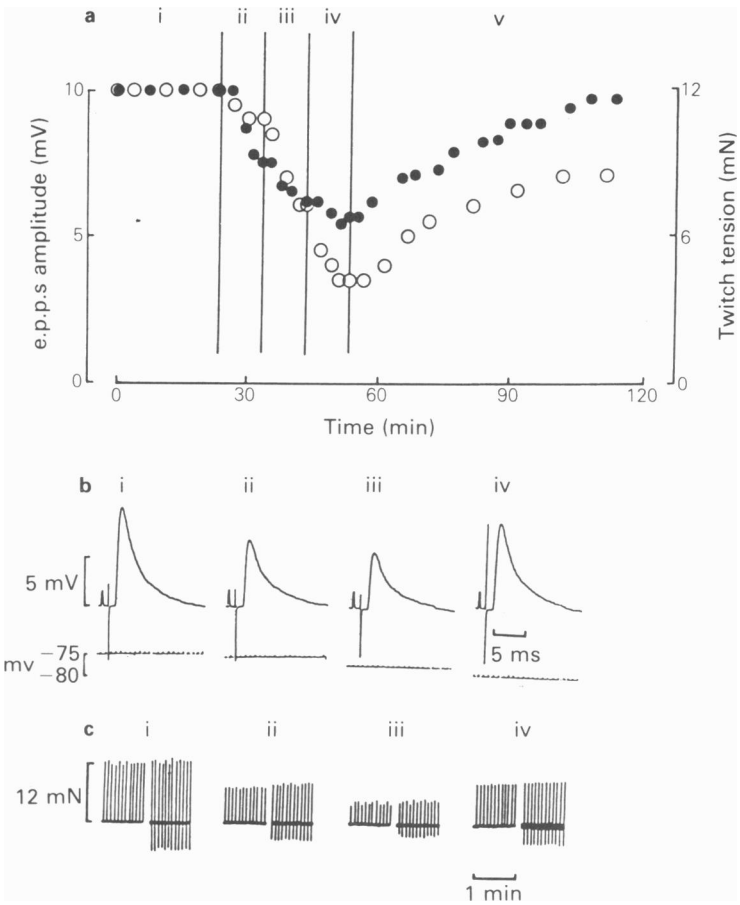

Figure 1 Effects of L-N^6-phenylisopropyladenosine (L-PIA) on the average amplitude of endplate potentials (e.p.ps) and on the twitch responses evoked by indirect stimulation of frog-sartorius muscles. Solutions contained 10 mM Mg^{2+} when recording e.p.ps or 3.0 mM Mg^{2+} when recording twitch responses. (a) Time course of the effects of different concentrations of L-PIA. The ordinates are the computed averages of 64 successive e.p.ps (left) (●) or the twitch tension (right) (○). In (i) and (v), bathing solution; (ii) L-PIA (100 nM); (iii) L-PIA (250 nM); (iv) L-PIA (500 nM). (b) Upper part: pen-recorder traces of averaged e.p.ps corresponding to (i) 20 (ii) 40 (iii) 50 (iv) 110 min for time in (a). Each response is preceded by a 2 mV calibration pulse. Lower part: membrane resting potential. (c) Pen-recorder traces of twitch responses and maximum rate of rise of tension corresponding to (i) 20 (ii) 40 (iii) 50 (iv) 110 min for time in (a) Each panel is composed of twitch responses followed by the maximum rate of rise of tension recorded concomitantly. Twitch amplitude in the normal bathing solution (Mg^{2+} 1.2 mM): 38 mN.

Solutions and drugs

The normal bathing solution (pH = 7.0) contained (mM): NaCl 117, KCl 2.5, NaH_2PO_4 1, Na_2HPO_4 1, $MgCl_2$ 1.2 and $CaCl_2$ 1.8. When recording e.p.ps the magnesium concentration was adjusted to prevent twitches of the muscle in response to nerve stimulation. When recording twitch responses the magnesium concentration was increased (2.5–3.5 mM) in order to reduce the safety margin of neuromuscular transmission.

Drugs used were: adenosine, 2-chloroadenosine, N^6-methyladenosine, theophylline, (+)-tubocurarine hydrochloride (Sigma); N^6-cyclohexyladenosine (CHA), L-N^6-phenylisopropyladenosine (L-PIA), D-N^6-phenylisopropyladenosine (D-PIA), 1,3-isobutylmethylxanthine (IBMX), 8-phenyltheophylline (8-PT) (R.B.I.); 5'-N-ethylcarboxamide adenosine (NECA) was a kind gift from Byk Gulden, Konstanz, F.R.G.. L-PIA and D-PIA were made up as 50 mM stock solutions in dimethylsulphoxide (DMSO) containing 120 mM NaCl; 8-PT was made up in a 10 mM stock solution in methanol 80% v/v containing 0.2 M NaOH. Dilutions of these stock solutions were used.

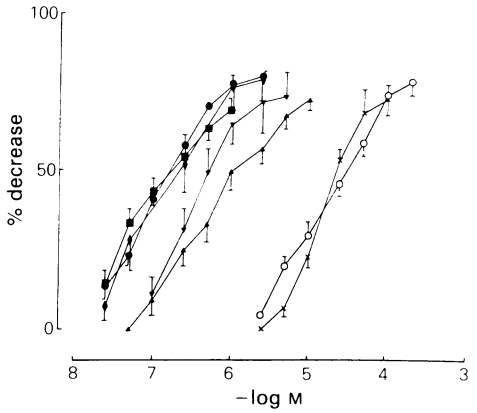

Figure 2 Concentration-response curves for the effects of L-N^6-phenylisopropyladenosine (L-PIA) (■), N^6-cyclohexyladenosine (CHA) (●), 5'-N-ethylcarboxamide adenosine (NECA) (◆), 2-chloroadenosine (▼), D-N^6-phenylisopropyladenosine (D-PIA) (▲), N^6-methyladenosine (×) and adenosine (○) on the twitch responses evoked by indirect stimulation of frog sartorius muscles. The ordinates are percentage decreases of the twitch amplitude recorded in high Mg^{2+} (2.5–3.5 mM) bathing solutions (averaged twitch amplitude in Mg^{2+} bathing solutions was 54 ± 2.5% of the twitch amplitide in the normal (Mg^{2+} 1.2 mM) bathing solution). 0% is the twitch amplitude in the Mg^{2+} bathing solutions and 100% represents a complete inhibition of the twitches. The vertical bars represent ± s.e.mean and are shown when they exceed the symbols. Averaged twitch amplitude in the normal bathing solutions: 24 ± 2.9 mN. Each point is the average of 4 to 5 experiments.

Statistics

The significance of the differences between the means was calculated using Student's *t* test. *P* values of 0.05 or less were considered to represent significant differences.

Results

Adenosine and adenosine analogues

Comparison between the effects of L-N^6-phenylisopropyladenosine on endplate potentials and on twitch amplitude Figure 1 illustrates the effects of L-PIA on the average amplitude of evoked endplate potentials (e.p.ps) and on the twitch responses evoked by indirect stimulation of frog sartorius muscles. In the experiment in which evoked e.p.ps were recorded the magnesium concentration of the solutions was 10 mM and in the experiment in which twitch tension was recorded, magnesium concentration was 3.0 mM. In these conditions, L-PIA decreased both the amplitude of the e.p.ps and the twitch amplitude within the same range of concentrations and in a quantitatively similar way. Since both intracellular and twitch tension recording experiments were performed in similar continuous perfusing conditions the time course for obtaining the steady effects for each concentration of L-PIA was of the same order (Figure 1a). L-PIA affects the amplitude of e.p.ps without modifying their decay phase (Figure 1b) and affects to the same extent both

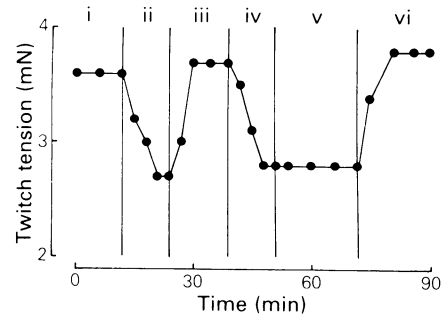

Figure 3 Comparison of the time course of the effects of 5'-N-ethylcarboxamide adenosine (NECA) with N^6-cyclohexyladenosine (CHA) on twitch responses evoked by indirect stimulation of a sartorius muscle: (i), (iii) and (v) bathing solution, (ii) NECA (100 nM), (iv) CHA (100 nM), (vi) 8-phenyltheophylline (8-PT; 2.5 μM). Solutions contained 2.5 mM Mg^{2+}. Note the quick recovery during washout for NECA. The effect of CHA showed no recovery during a 20 min washout period. Addition of 8-PT in a concentration (2.5 μM) devoid of effect on neuromuscular transmission when applied to the same preparation, caused a rapid return of the twitch responses to control levels.

Table 1 ED_{50} values and the relative potencies obtained from the data shown in Figure 2

	L-PIA	CHA	NECA	Cl-Ad	D-PIA	Met-Ad	Adenosine
ED_{50} (nM)	81	105	111	352	777	17250	17714
Relative potency	219	169	160	50	23	1	1

L-PIA: L-N^6-phenylisopropyladenosine; CHA: N^6-cyclohexyladenosine; NECA: 5'-N-ethylcarboxamide adenosine; Cl-Ad: 2-chloroadenosine; D-PIA: D-N^6-phenylisopropyladenosine; Met-ad: N^6-methyladenosine.
Relative potency: ED_{50} of adenosine/ED_{50} of each agonist.
ED_{50} values were determined on the regression lines (method of least squares, correlation coefficients ranged from 0.997 to 0.999) of the linear part of the concentration-response curves shown in Figure 2, and represent the concentration of each agonist that produced 50% of the maximal effect; this was taken as 80% decrease in twitch amplitude, which was about the maximal effect obtained for CHA and NECA.

the amplitude and the maximum rate of rise of the twitches (Figure 1c).

Rank order of potency of the adenosine analogues All the adenosine analogues tested (L-PIA, CHA, NECA, 2-chloroadenosine, D-PIA and N^6-methyladenosine) decreased in a concentration-dependent manner both the maximum rate of rise of tension and the twitch amplitude of partially magnesium-paralyzed frog sartorius preparations. Concentration-response curves for the effects of adenosine and its analogues on the twitch amplitude are shown in Figure 2. The effects of L-PIA and D-PIA could not be attributed to its solvent, DMSO, since the maximum concentration of DMSO (0.02% v/v) present in the PIA solutions applied to the muscles was devoid of effect either on e.p.ps or twitch responses. The full effect of adenosine and its analogues was usually seen in the first 10 min after their application to the preparations. The effects of NECA (Figure 3), 2-chloroadenosine, N^6-methyladenosine and adenosine were usually washed out in 10–20 min. The effects of CHA (Figure 3), L-PIA and D-PIA were little affected by washing; however, addition of 8-phenyltheophylline (8-PT) in concentrations (1–2.5 μM) devoid of effect on neuromuscular transmission, caused a quick return of the twitch responses to control levels (Figure 3) (cf. Dunwiddie *et al.*, 1984).

Table 1 shows the mean values for the concentration of each adenosine agonist that produced half maximal effect (ED_{50}) calculated from the data of Figure 2. These data were obtained in 2.5–3.5 mM Mg^{2+} solutions which decreased the twitch amplitude to 54 ± 2.5% of its value in the normal (Mg^{2+} 1.2 mM) bathing solution. It is evident from Table 1 that the

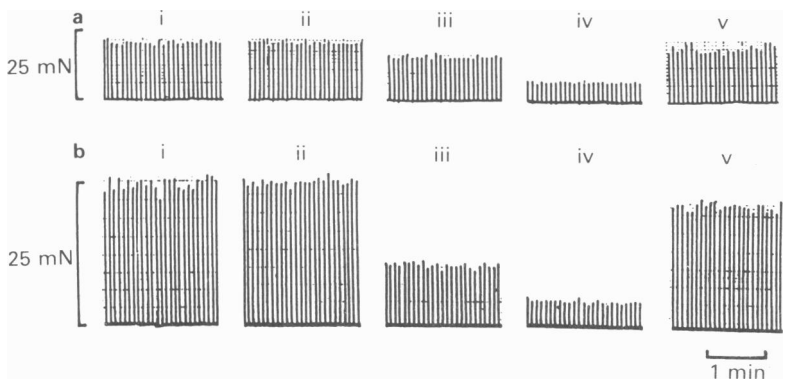

Figure 4 Antagonism by 8-phenyltheophylline (8-PT) and theophylline of 2-chloroadenosine-induced inhibition of twitch responses evoked by indirect stimulation of frog-sartorius muscles. (a) Antagonism by 8-PT: (i) twitch responses in the bathing solution, before applying 8-PT; (ii) 14–15 min after applying 8-PT (2.5 μM); (iii) effect after 19–20 min in 8-PT (2.5 μM) + 2-chloroadenosine (1 μM); (iv) effect after 24–25 min in 2-chloroadenosine (1 μM); (v) 19–20 min after returning to a 2-chloroadenosine-free solution containing 8-PT (2.5 μM). Solutions contained 2.0 mM Mg^{2+} which decreased the twitch response to 57% of its amplitude in the normal (Mg^{2+} 1.2 mM) bathing solution. (b) Antagonism by theophylline: (i) twitch responses in the bathing solution before applying theophylline; (ii) 14–15 min after applying theophylline (50 μM); (iii) effect after 19–20 min in theophylline (50 μM) + 2-chloroadenosine (1 μM); (iv) effect after 19–20 min in 2-chloroadenosine (1 μM); (v) 19–20 min after returning to a 2-chloroadenosine-free solution containing theophylline (50 μM). Solutions contained 2.5 mM Mg^{2+} which decreased the twitch response to 66% of its amplitude in the normal bathing solution.

rank order of potency for adenosine and its derivatives was L-PIA, CHA, NECA > 2-chloroadenosine > D-PIA > N^6-methyladenosine, adenosine.

In order to check whether adenosine and its analogues had any direct effect on contraction that could influence significantly the responses to indirect stimulation, experiments were performed with direct stimulation of the muscles in the presence of tubocurarine in a concentration (2×10^{-5} M) that caused complete blockade of neuromuscular transmission. Under these conditions, adenosine and its analogues when applied to muscles in the highest concentration used to test their effects on neuromuscular transmission were devoid of effect on directly evoked twitch responses of the muscles.

Adenosine antagonists

The ability of alkylxanthines (theophylline, 8-PT and isobutylmethylxanthine (IBMX)) to antagonize the effect of 2-chloroadenosine on twitch responses evoked by indirect stimulation of frog-sartorius muscles was investigated. 2-Chloroadenosine was chosen as the agonist because it was the most potent of the easily washed out and commercially available adenosine analogues. In this set of experiments the effect of submaximal concentrations of 2-chloroadenosine in the absence, in the presence and again in the absence of alkylxanthines was always compared sequentially in the same preparation; alkylxanthines were used in concentrations devoid of effect either on e.p.ps or twitch responses evoked by indirect stimulation of the muscles. Figure 4 illustrates the action of 8-PT (2.5 μM) and theophylline (50 μM) against the inhibitory effect of 2-chloroadenosine (1 μM) on the twitch responses evoked by indirect stimulation of two frog-sartorius muscles. As can be seen, both alkylxanthines antagonized the inhibitory effect of 2-chloroadenosine, the antagonism being reversed by washing the xanthines from the tissue baths. After removing the xanthines from the bath, the full effect of 2-chloroadenosine (1 μM) was usually seen within 20–30 min.

A comparison between the antagonistic effect of 8-PT (0.5–2.5 μM) and theophylline (10–50 μM) on 2-chloroadenosine (1μM)-induced inhibition of twitch

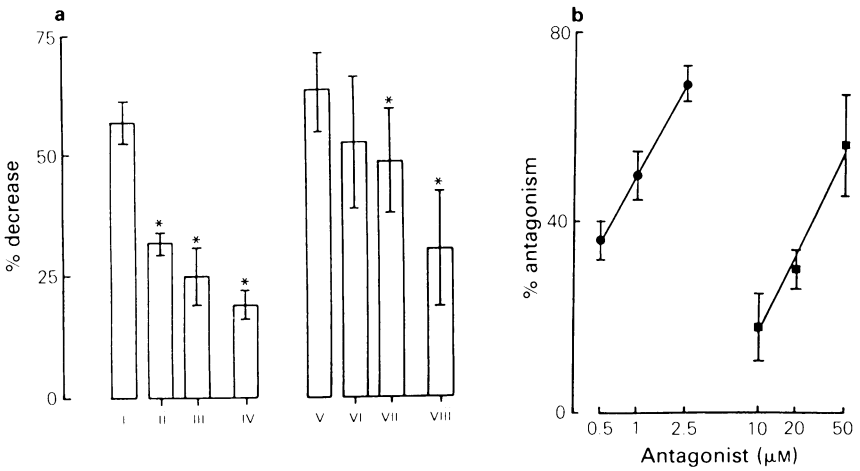

Figure 5 Comparison of the potencies of 8-phenyltheophylline (8-PT) and theophylline in antagonizing the inhibitory effect of 2-chloroadenosine on twitch responses evoked by indirect stimulation of frog-sartorius muscles. (a) The left panel (i–iv) illustrates the antagonism by 8-PT and the right panel (v–viii) the antagonism by theophylline. The ordinates are percentage decreases in twitch tension caused by: (i) and (v) 2-chloroadenosine (1 μM) in the absence of antagonist; (ii), (iii) and (iv) 2-chloroadenosine (1 μM) in the presence of 0.5 μM, 1 μM and 2.5 μM 8-PT respectively; (vi), (vii) and (viii) 2-chloroadenosine in the presence of 10 μM, 20 μM and 50μM theophylline respectively. Solutions contained 2.0–2.5 mM Mg^{2+} which decreased the twitch amplitude to 57.4 ± 2.8% of its value in the normal (Mg^{2+} 1.2 mM) bathing solution. Averaged twitch amplitude in the normal bathing solution: 29 ± 3.8 mN. Each column represents pooled data from 3 experiments. The vertical bars represent ± s.e.mean. * $P < 0.05$ (paired Student's t test) as compared to the effect of 2-chloroadenosine alone in the same experiments. (b) Ordinates: percentage antagonism of the inhibitory action of 2-chloroadenosine (1 μM) caused by different concentrations of 8-PT (●) and theophylline (■). 0% is the effect of 2-chloroadenosine in the absence of antagonist and 100% represents the twitch amplitude in the high Mg^{2+} (2.0–2.5 mM) bathing solutions. Data were obtained from the same experiments as in (a). The vertical bars represent s.e.mean. The regression lines were calculated by the method of least squares (correlation coefficients: 0.999 for (●) and 0.992 for (■)).

responses evoked by indirect stimulation of frog-sartorius muscles is illustrated in Figure 5. 8-PT was a more potent antagonist than theophylline. The concentrations of 8-PT and theophylline that produced 50% antagonism of the effect of 2-chloroadenosine (IC_{50}), calculated from the data shown in Figure 5b, were 1 μM and 40 μM respectively. The antagonism caused by 8-PT could not be attributed to its solvent (methanol 80% v/v with 0.2 M NaOH) since the maximum concentration (0.025% v/v) of solvent present in the 8-PT solutions did not antagonize the inhibitory action of 2-chloroadenosine on twitch responses.

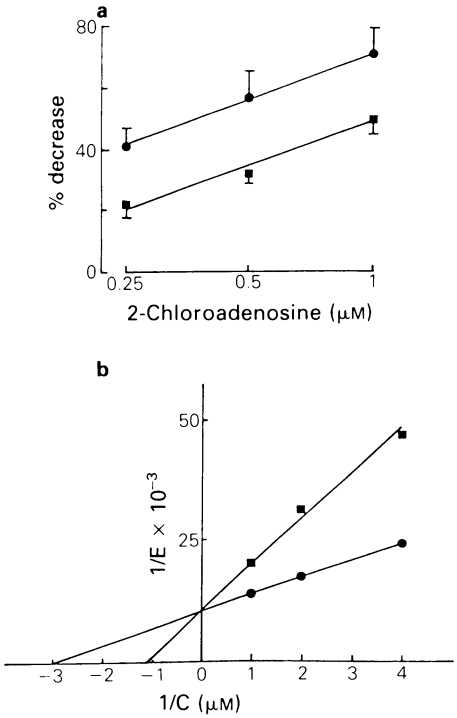

Figure 6 Effect of 8-phenyltheophylline (8-PT) on the concentration-response curve for the inhibitory action of 2-chloroadenosine on the twitch responses evoked by indirect stimulation of frog-sartorius muscles. (a) Percentage decreases in twitch amplitude caused by different concentrations of 2-chloroadenosine in the absence (●) and in the presence (■) of 8-PT (1 μM). The vertical bars represent ± s.e.mean. Each point is the average of 3 experiments. The solutions contained 2.5 mM Mg^{2+} (averaged twitch tension in the high-Mg^{2+} bathing solution was 65 ± 7.7% of the twitch tension in the normal (Mg^{+} 1.2 mM) bathing solution; averaged twitch tension in the normal bathing solution: 33 ± 4.4 mN). (b) Double reciprocal plot of the data shown in (a) where C is the concentration of 2-chloroadenosine and E is the percentage decrease in twitch tension caused by 2-chloroadenosine. The apparent affinity (− 1/x intercept) of 2-chloroadenosine in the absence of 8-PT was 345 nM and in the presence of 8-PT (1 μM) was 869 nM. In (a) and (b) the regression lines were calculated by the method of least squares (correlation coefficients ranged from 0.990 to 0.999).

In the presence of 8-PT (1 μM), the log concentration-response curve for the inhibitory effect of 2-chloroadenosine on twitch amplitude was shifted to the right (Figure 6a). Kinetic analysis by the double-reciprocal plot of the data shown in Figure 6a suggested that the maximal effect of 2-chloroadenosine remains unchanged in the presence of 8-PT but its apparent affinity is decreased by a factor of 2.5 (Figure 6b). In order to test whether the antagonistic action of 8-PT against the inhibitory effect of 2-chloroadenosine on neuromuscular transmission could be surmounted by a supramaximal concentration of 2-chloroadenosine, an experiment was designed in which the effect of 2-chloroadenosine (100 μM) on the amplitude of the twitches of indirectly stimulated muscles was compared in the presence and in the absence of 8-PT (1 μM). In these conditions the effect of 2-chloroadenosine remained quantitatively the same (74% inhibition of twitch amplitude) either in the presence or in the absence of 8-PT.

The ability of IBMX to antagonize the inhibitory action of 2-chloroadenosine on neuromuscular transmission was studied in two experiments. IBMX used in concentrations (10–100 μM) that did not modify the indirectly evoked twitch responses, did not modify the decrease caused by 2-chloroadenosine (1 μM) of the twitch amplitude of indirectly stimulated muscles.

Discussion

The present results show that adenosine and its stable derivatives decreased the amplitude of both e.p.ps and the twitch tension evoked by indirect stimulation of frog sartorius muscle fibres. The rank order of potencies for adenosine and its analogues in decreasing twitch tension was: L-PIA, CHA, NECA > 2-chloroadenosine > D-PIA > N^6-methyladenosine, adenosine.

The inhibitory effects of these substances on twitch responses cannot be attributed to a direct action on contraction since they were devoid of effect on twitch amplitude when the muscles were directly stimulated in the presence of a paralyzing concentration of tubocurarine. The inhibition of neuromuscular transmission caused by adenosine and its analogues should be attributed to a presynaptic action of these substances, i.e., to a decrease in the release of acetylcholine from the motor nerve endings (cf. Ginsborg & Hirst, 1972; Ribeiro & Walker, 1975; Branisteanu *et al.*, 1979; Silinsky, 1980).

In the present study 8-PT and theophylline, but not IBMX, in concentrations that did not modify neuromuscular transmission, antagonized the in-

hibitory effect of 2-chloroadenosine at the neuromuscular junction, 8-PT being a more potent antagonist than theophylline. The present findings showing that the apparent affinity of 2-chloroadenosine was decreased by 8-PT and that the antagonistic action of 8-PT was surmountable by a supramaximal concentration of 2-chloroadenosine, suggest that the antagonism caused by this xanthine is of a competitive nature. This conforms to the idea that the adenosine receptor at the motor nerve endings is a P_1/R-type purinoceptor (Burnstock, 1980; Silinsky, 1980).

R-type adenosine receptors can be classified into A_1 or A_2 types on the basis of the potency of some stable adenosine analogues. The following criteria have been established (see e.g. Daly *et al.*, 1981; Daly, 1983): (1) at the A_2 adenosine receptor the 5′ carboxamides of adenosine, such as NECA, are the most potent adenosine analogues, 2-chloroadenosine has intermediate potency and the less potent agonists are the N^6 substituted compounds L-PIA, CHA and N^6-methyladenosine, whereas at the A_1 adenosine receptor, L-PIA and CHA are the most potent adenosine analogues, 2-chloroadenosine has intermediate potency and the less potent agonists are NECA and N^6-methyladenosine; (2) A_1 adenosine receptors are stereoselective for the PIA isomers, L-PIA being usually more than fifty fold more potent than D-PIA, whereas small stereoselectivity for the PIA isomers is observed at A_2 adenosine receptors; (3) at the A_1 adenosine receptor the concentrations of adenosine analogues required to produce half maximal effects are in the nanomolar range, whereas at the A_2 adenosine receptor they are in the micromolar range.

The present observations that L-PIA and CHA were the most potent agonists and about two hundred fold more potent than N^6-methyladenosine together with the finding that the concentrations of the adenosine analogues required to produce half maximal effects were from the nanomolar to the low micromolar range suggest that the inhibitory effects on neuromuscular transmission caused by adenosine and its derivatives could be mediated by an A_1 adenosine receptor. However, in the present work it was observed that L-PIA was about ten fold more potent that D-PIA and that NECA was one of the most potent adenosine analogues and more potent than 2-chloroadenosine. This indicates that the adenosine receptor at the neuromuscular junction could be an A_2-type of receptor.

Summarizing this, one could say that the receptor which mediates the inhibitory action of adenosine at the neuromuscular junction does not fit the A_1 or the A_2 criteria for adenosine receptor classification. The presence of both A_1 and A_2 adenosine receptors in the same preparation has been postulated (e.g. van Calker *et al.*, 1979; Wojcik & Neff, 1983). If this were the case for the neuromuscular junction, one would expect, on the basis that the A_1-receptor has a higher affinity for adenosine derivatives than the A_2-receptor (see Daly, 1983), a much wider range of effective concentrations for each adenosine agonist than that observed in the present work.

Difficulties in classifying adenosine receptors into the A_1/A_2 nomenclature have also been mentioned in other studies where a functional system rather than a biochemical one (adenylate cyclase activation or inhibition) has been examined pharmacologically. For example the receptors that mediate the effects of adenosine on axons (Ribeiro & Sebastião, 1984), anococcygeus muscle (Stone, 1983), heart (Hughes & Stone, 1983) and postganglionic neurones (Henon & McAfee, 1983) do not exhibit pharmacological profiles in accordance with the A_1/A_2 adenosine receptor classification. Also, the electrophysiological actions of adenosine on the central nervous system (Phillis & Wu, 1981; Dunwiddie & Fredholm, 1984) appear to be mediated by a third, as yet unnamed, extracellular receptor which differs in its pharmacological properties from both the A_1- and A_2-receptors associated with adenylate cyclase.

Several calcium-related mechanisms have been proposed for the inhibition of acetylcholine release by adenosine derivatives: (a) blockade of the voltage-dependent calcium entry (Kuroda, 1978; Ribeiro *et al.*, 1979b; Wu *et al.*, 1982); (b) a decrease in the availability of calcium (Ginsborg & Hirst, 1972) by increasing the rate of calcium uptake into storage sites (Ribeiro & Dominguez, 1978; Branisteanu *et al.*, 1979); (c) a decrease in the apparent affinity for calcium of an intracellular component of the secretory apparatus (Silinsky, 1981; 1984); whether cyclic AMP is involved is an open question, which has recently been discussed (Ribeiro & Sá-Almeida, 1984; Silinsky, 1984).

It is possible that the A_1/A_2 distinction only applies to the adenosine-induced modulation of adenylate cyclase and that adenosine receptors linked to calcium exhibit pharmacological profiles distinct from those of the A_1 or A_2 adenosine receptors. Whether the absence of antagonism by IBMX and a rank order of agonist potencies with L-PIA, CHA and NECA being of similar potency and more potent than 2-chloroadenosine are general characteristics of calcium-linked adenosine receptors, remains to be established.

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