Aminophylline enhances resting Ca^{2+} concentrations and twitch tension by adenosine receptor blockade in *Rana pipiens*

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1. We hypothesized that the xanthine aminophylline acts to block adenosine receptors on the surface of skeletal muscle fibres, thereby inhibiting a depressant action of endogenous adenosine. We further hypothesized that this action results in increased concentrations of intracellular resting Ca^{2+} and enhanced twitch tension upon muscle stimulation.

2. Peak twitch tension (P_t) of the semitendinosus muscle in normal frog Ringer solution (NFR) ranged from 6·8 to 9·4 g. Intracellular Ca^{2+} concentrations in control resting fibres ranged from 67 to 70 nM. Aminophylline at 100 μM produced increases of 26 and 22% in P_t and Ca^{2+} concentrations, respectively.

3. The adenosine receptor antagonists 8-phenyltheophylline (8-PT) and 1,3-dipropyl-7-methylxanthine (1,3-d-7-M) both increased P_t by 32% over values in NFR. In addition, 1,3-d-7-M increased resting Ca^{2+} concentrations by 29% over control levels.

4. Adenosine deaminase increased twitch tension and resting intracellular Ca^{2+} concentrations by 22 and 26% over controls, respectively.

5. N^6-(2-phenylisopropyl)adenosine (R-PIA, 1 μM), a potent adenosine analogue, partially blocked both the increase in P_t and intracellular Ca^{2+} concentrations induced by the xanthines, possibly by competing for the adenosine receptor.

6. The data herein provide support for the existence of adenosine receptors on the membranes of skeletal muscle fibres and suggest a possible role for adenosine receptors in the regulation of twitch tension.

The xanthine drugs, particularly aminophylline, have been widely used in the treatment of asthma and other types of respiratory obstruction (Rall, 1982; Viires, Aubier, Murciano, Marty & Pariente, 1983). Although the xanthines were originally thought to act through dilatation of the bronchioles and stimulation of the central nervous system, aminophylline has been shown to increase contractility of the mammalian diaphragm (Aubier, de Troyer, Sampson, Macklem & Roussos, 1981; Howell & Roussos, 1984; Murciano, Aubier, Viires, Mal & Pariente, 1987). Using an isolated frog semitendinosus preparation, we have shown that aminophylline potentiates force generation and power output by a direct effect on the muscle (Ridings, Barry & Faulkner, 1989). These actions occur at low micromolar concentrations, which are within the therapeutic range of the use of these drugs.

Several hypotheses have been proposed to explain the twitch-potentiating effect of xanthines on skeletal muscle. Xanthines may increase twitch force by (a) directly stimulating calcium (Ca^{2+}) release from the sarcoplasmic reticulum, (b) phosphodiesterase inhibition, (c) blocking adenosine receptors on the plasma membrane of muscle fibres, or (d) increasing intracellular Ca^{2+} levels through effects on Ca^{2+} influx and/or Ca^{2+} sequestration in the resting and stimulated muscle fibre (Rall, 1982). However, direct stimulation of Ca^{2+} release from the sarcoplasmic reticulum (hypothesis (a)) requires millimolar concentrations of the drug, which are 10- to 100-fold higher than the therapeutic range (Rall, 1982; Rousseau, LaDine, Liu & Meissner, 1988). Likewise, millimolar concentrations of aminophylline are required for phosphodiesterase inhibition (hypothesis (b)) (Kramer & Wells, 1980; Rall, 1982). Moreover, no relationship has been found between the ability of different xanthines to inhibit phosphodiesterase and to potentiate twitch force. Thus, we first tested the hypothesis that aminophylline increases muscular force by blockade of adenosine receptors on the membrane of muscle fibres, thereby inhibiting a depressant action of endogenous adenosine (hypothesis (c)). The results of these experiments using several adenosine agonists and antagonists are consistent with the interpretation that adenosine receptors are present on skeletal muscle fibres

and that aminophylline may potentiate force production by adenosine receptor blockade.

We also investigated the hypothesis that the potentiating effect of aminophylline on force production results from an increase in resting levels of Ca^{2+} within the muscle fibre (hypothesis (d)). Studies have shown that fura-2 is an excellent dye for the measurement of resting Ca^{2+} levels (Baylor & Hollingsworth, 1987; Klein, Simon, Szücs & Schneider, 1988). The results from fura-2 experiments using adenosine receptor antagonists and agonists were consistent with the interpretation that aminophylline acts through adenosine receptors to increase resting levels of calcium.

METHODS

Small frogs (*Rana pipiens*) were obtained from Kons Scientific (Germantown, WI, USA), housed in a temperature-controlled animal room with 12:12 h light:dark cycle, and fed crickets obtained from Flucker's Crickets (Baton Rouge, LA, USA). Frogs were chilled on ice for 1 h, decapitated, and pithed prior to dissection. For physiological experiments the dorsal head of the semitendinosus muscle was isolated and ligatures (5-0 silk) were tied around proximal and distal tendons. The proximal tendon was tied to a glass hook that was attached to a Kulite B-50 force transducer. The distal tendon of the muscle was tied such that the muscle was mounted between the leads of a pair of platinum field electrodes submerged in frog Ringer solution containing curare at a concentration (10 μM) that blocked neuromuscular transmission. The force transducer was mounted on a Narashige micromanipulator permitting the adjustment of muscle length to the optimum length for the development of maximum twitch tension (L_0). The muscle was stimulated directly at a frequency of 0·01 Hz with supramaximal square wave pulses of 0·2 ms duration. Temperature was maintained at 20 °C with a Neslab Endocal refrigerated circulating bath (RTE-5DD). Maximum isometric twitch tension (P_t) during a contraction was analysed by an IBM AT computer equipped with pCLAMP software (Axon Instruments, Foster City, CA, USA, version 5·5).

Normal frog Ringer solution (NFR) contained (mM): NaCl, 115; Tris, 5; D-glucose, 5; KCl, 2; $CaCl_2$, 1·8. D-Tubocurare (0·01 mM; Sigma) was added to the solution to block neuromuscular transmission. The pH of this and all subsequent solutions was adjusted to 7·2 just prior to use.

Stock solutions of aminophylline (1 mM), adenosine (1 mM), adenosine deaminase (986 units ml^{-1}; Sigma) and N^6-(2-phenylisopropyl)adenosine (R-PIA) (0·1 mM) were prepared in NFR and diluted to the appropriate concentration for a given experiment. A 1 mM stock solution of 1,3-dipropyl-7-methylxanthine (1,3-d-7-M) was prepared in 0·1 M NaOH. All stock solutions and NFR were made fresh weekly.

To obtain control data for a given experiment, the muscle was first immersed in NFR and subjected to the stimulation protocol. Data were collected for eighteen twitches. The bathing solution was then changed by perfusion and the muscle was stimulated either 30 or 60 times at a rate of 0·01 Hz until twitch tension reached a plateau. Data from the last ten plateau twitches were analysed and compared to data from control conditions. At the end of each experimental protocol, muscles were immersed in NFR again for re-analysis of control conditions. If tension fell to more than 10% below original control levels during this wash, the muscle was considered to be unstable and data from it were not used.

For each individual experiment, the average of ten twitches under experimental conditions was then normalized as a percentage of ten twitches in NFR. The data are thus presented as a percentage change from control (mean ± S.E.M.) data for three to twelve experiments in each group. Group data were analysed statistically with an analysis of variance and Tukey's *post hoc* procedure (Devore, 1982). $P \leqslant 0{\cdot}05$ was the criterion used for statistical significance.

For the fura-2 experiments, fibre bundles (10–15 fibres) and single fibres were blunt-dissected from the frog semitendinosus muscle and pinned to the glass bottom of culture dishes, thus allowing the fibres to be kept in frog Ringer solution throughout the experiment while viewing fibres through an inverted microscope. Fura-2 (1 mM, pentapotassium salt, Molecular Probes, Inc., Eugene, OR, USA) was iontophoresed into single fibres using a current of 1 nA delivered by an Axoclamp 2A amplifier (Axon Instruments). Neighbouring non-injected fibres in the same bundle were used for background fluorescence subtraction. The fibre bundle was placed on the stage of a Zeiss IM inverted microscope equipped with a computer-controlled filter wheel, which allowed us to change back and forth between the two excitation wavelengths at a frequency of 100 Hz. When excited with light of 340 nm wavelength, the intensity of the fluorescence increases as the calcium concentration increases. When excited with 380 nm wavelength light, the fluorescence intensity decreases as the calcium concentration increases. By taking the ratio of the fluorescent intensities at the two wavelengths, the internal free calcium concentration can be calculated. Fluorescent video images at each excitation wavelength were acquired using a Dage-MTI SIT camera, enhanced using a computer digital image processing system (Image 1/FL, Universal Imaging Corporation on a Zenith Z386 computer), and stored on computer disks for analysis using the same computer software. Background fluorescence at the two excitation wavelengths were determined, and subtracted from the video signal for each image. Calcium concentration in the muscle fibres was estimated by comparing acquired ratio values with calibrated ratio values.

Calibrated ratios were obtained for each new batch of fura-2. Fura-2 (3 μM), EGTA (10 mM) and known Ca^{2+} concentrations were dissolved in a solution that mimics the internal milieu of the muscle fibre (mM): KCl, 140; NaCl, 10; ATP, 5; Hepes, 10; creatine phosphate, 10; MgCl, 1; pH 7·2. Other soluble factors in the cytoplasm such as lactate, amino acids, inorganic phosphate and calmodulin, were found to have little effect on the fura-2 calibration curves and were therefore not added to the calibration solution (Godt & Maughan, 1988; Williams & Fay, 1990). These solutions were placed on a microscope slide on the stage of the inverted microscope in the identical position to that used for the muscle fibres. The fluorescence ratio at each known Ca^{2+} concentration was obtained and a calibration curve generated. By using signal ratios, the effects on the signals of varying path lengths and fura-2 concentrations were minimized (Grynkiewicz, Poenie & Tsien, 1985; Klein *et al.* 1988). R_{min} and R_{max} values represent, respectively, the fluorescence ratios obtained when the fura-2 contains no bound Ca^{2+} and when the fura-2 is

maximally saturated with Ca^{2+}. Poenie (1990) observed differences in R_{min} and R_{max} values for fura-2 fluorescence when measured in glass capillary tubes and when measured in muscle fibres, probably because the muscle fibre cytoplasm is more viscous than the calibration solutions. Thus, the Image 1/FL software shifts the calibration curve according to the equation:

$$\text{Concentration} = \frac{K_d (F_{min}/F_{max})(R - R_{min})}{(R_{max} - R)},$$

where K_d is the dissociation constant and F_{min} and F_{max} are the fluorescence values obtained at low and high Ca^{2+} concentrations, respectively, measured within a muscle fibre. The ratio $(R - R_{min})/(R_{max} - R)$ is derived from a series of equations that account for background subtraction, gain and offset parameters available through the software program. Since we were interested in the changes in Ca^{2+} concentrations produced in the muscle fibre by aminophylline and other drugs, these measurements were sufficiently accurate for our experiments.

Aminophylline and other drugs were applied to the muscle fibre bundle by perfusion so that the volume of solution bathing the muscle fibres did not change with drug application. Fluorescence ratio values were acquired and stored immediately following administration of aminophylline and every two minutes for 50–70 min thereafter. Changes in Ca^{2+} levels were observed over the identical time period in control muscles that were not exposed to aminophylline, in order to measure any spontaneous changes in Ca^{2+} levels during the course of the experiment.

RESULTS

Effects of aminophylline on P_t and intracellular Ca^{2+} concentrations

At concentrations of 25, 50 and 100 μM, aminophylline significantly enhanced twitch tension by 12 ± 2, 18 ± 3 and $26 \pm 2\%$ ($n = 6$, 6, 12), respectively, over the control twitch tension of $8{\cdot}8 \pm 1{\cdot}6$ g in NFR. The increase in twitch tension was seen within minutes, but peak twitch tension did not reach a plateau until 40 min after the start of the experiment (Fig. 1). The effects of aminophylline were reversed within 50–60 min by returning the muscle to NFR.

The Ca^{2+} indicator dye fura-2 was successfully iontophoresed into single muscle fibres. Unloaded muscle fibres in the same fibre bundle showed minimal background fluorescence. Photobleaching of fura-2 did not occur during the 2 h of experimentation as long as the excitation light beams were turned off when no fluorescence measurements were being made. Moreover, movement artifacts were not a problem in the unstimulated muscle bundle. Intracellular Ca^{2+} concentration in control resting muscle fibres was $67{\cdot}7 \pm 1{\cdot}7$ nM. The values of resting internal free Ca^{2+} measured in the muscle fibres were similar to the values obtained for frog skeletal muscle fibres by other investigators (Klein *et al.* 1988; Iaizzo, Seewald, Oakes &

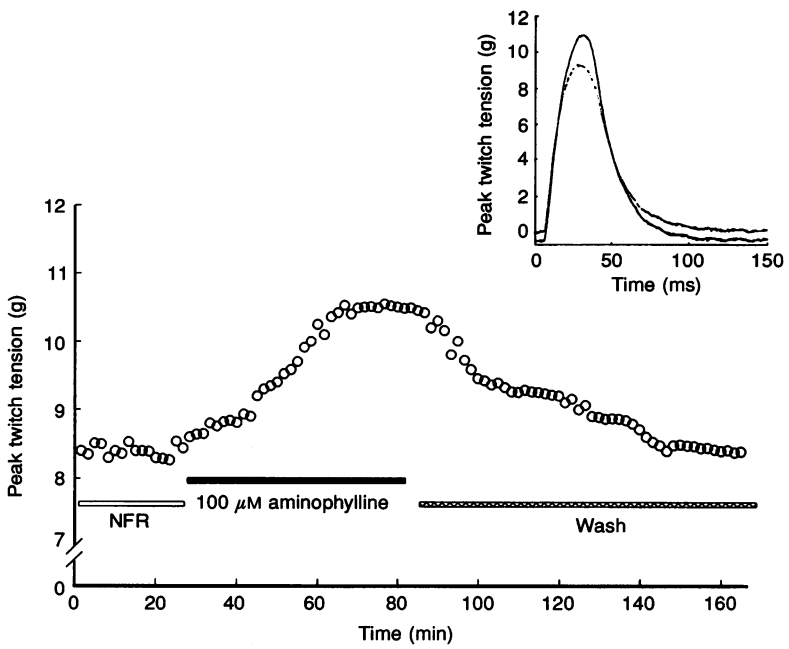

Figure 1. Effects of aminophylline on peak twitch tension
Time course of the effects of 100 μM aminophylline on peak twitch tension in whole frog semitendinosus muscle. Inset, typical twitch trace from contractile properties experiment; peak twitch tension in NFR (interrupted line) and 100 μM aminophylline (continuous line).

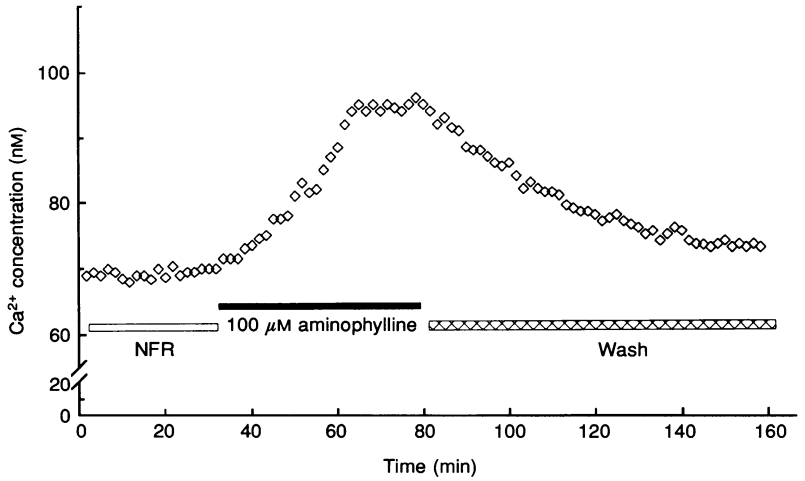

Figure 2. Effects of aminophylline on intracellular Ca^{2+} concentrations
Time course of the effects of 100 μM aminophylline on intracellular Ca^{2+} concentrations in single fibres of frog semitendinosus muscle.

Lehman-Horn, 1989; Himpens, Lydrup, Hellstrand & Casteels, 1990; Klein, Simon & Schneider, 1990; Jacquemond, Csernoch, Klein & Schneider, 1991; Westerblad & Allen, 1991).

Bathing muscle fibres in 100 μM aminophylline increased resting Ca^{2+} levels by $26 \pm 4\%$ ($n = 11$) over the control value of 68 ± 2 nM ($n = 8$) (Figs 2 and 3). The time course of the increase in Ca^{2+} concentration is similar to that of twitch tension (Fig. 2). The effects of aminophylline are also reversed in 50–60 min by returning the muscle to NFR.

Effects of adenosine antagonists and adenosine deaminase on P_t and intracellular Ca^{2+} concentrations

The xanthines have been shown to be adenosine receptor antagonists at low micromolar concentrations, while at higher doses, they inhibit phosphodiesterase and promote release of Ca^{2+} from the sarcoplasmic reticulum (Rall, 1982). Because the increase in twitch tension produced by aminophylline was apparent at low micromolar concentrations, we hypothesized that the action of aminophylline is

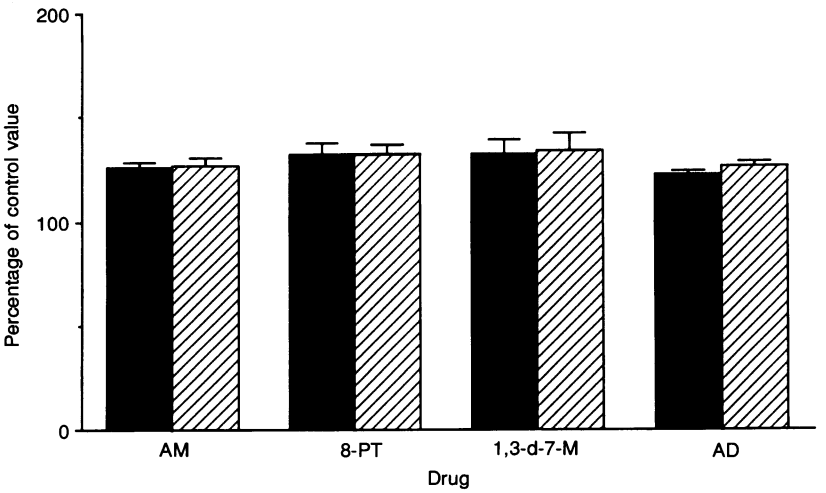

Figure 3. Effects of drugs on whole muscle twitch tension and single fibre intracellular calcium concentration
■, twitch tension; ▨, Ca^{2+} concentration. Data are presented as a percentage of the value obtained in NFR (mean ± S.E.M.) for each individual drug. The number of experiments for each drug is listed in Results. AM, aminophylline (100 μM); 8-PT, 8-phenyltheophylline (100 μM); 1,3-d-7-M, 1,3-dipropyl-7-methylxanthine (100 μM); AD, adenosine deaminase (5 units ml^{-1}).

mediated through blockade of adenosine receptors. To test this hypothesis, the effects on twitch tension of two other adenosine antagonists were studied. 8-Phenyltheophylline (8-PT), an analogue of aminophylline, was chosen because, on other cell types, this drug is a 25 times more specific adenosine receptor blocker than aminophylline (Bruns, Daly & Snyder, 1983). Moreover, this drug does not affect phosphodiesterase activity in brain slices (Smellie, Davis, Daly & Wells, 1979), thus eliminating this pathway as a possible mechanism of action of the drug. From a control twitch tension of 8·4 ± 1·5 g, 8-PT significantly enhanced twitch tension by 18 ± 2, 25 ± 3 and 32 ± 5% at approximate concentrations of 25, 50 and 100 μM ($n = 6$, 6, 12), respectively (Fig. 3). The effects of aminophylline analogues on Ca^{2+} concentration paralleled their effects on twitch tension. From a control resting Ca^{2+} concentration of 70 ± 0·74 nM, 100 μM 8-PT significantly enhanced Ca^{2+} concentration by 32 ± 5% ($n = 4$) (Fig. 3).

Because 8-PT does not completely dissolve at concentrations greater than 10 μM, additional experiments were done using the caffeine analogue 1,3-d-7-M, which is fully soluble in NFR. 1,3-d-7-M is 8- to 40-fold more potent than caffeine at adenosine receptors, depending on the class of adenosine receptor involved (Daly, Padgett & Shamim, 1986). At concentrations of 50 and 100 μM ($n = 6$, 6), 1,3-d-7-M increased twitch tension by 22 ± 2 and 32 ± 7%, respectively, over the control twitch tension of 8·6 ± 1·3 g (Fig. 3). A 100 μM concentration of 1,3-d-7-M also increased Ca^{2+} concentration by 34 ± 8% over the control value of 67 ± 1·5 nM.

An additional series of experiments involved the effects on tension development produced by a reduction of the adenosine concentration in the NFR bath and muscle preparation. Adenosine deaminase was added to the bath to break down endogenous adenosine to inosine, a compound which has no effects on skeletal muscle (Daly, 1982). Adenosine deaminase (0·5 units ml^{-1}) breaks down 0·5 μmoles of adenosine per minute at 25 °C. Thus, application of 0·5 units ml^{-1} adenosine deaminase would be expected to mimic the effects of adenosine antagonists since both treatments will inhibit the actions of adenosine. At a concentration of 0·5 units ml^{-1}, adenosine deaminase significantly increased twitch tension by 22 ± 2% over twitch tension in NFR ($n = 6$) (Fig. 3). In parallel with the physiological experiments, adenosine deaminase increased resting intracellular Ca^{2+} concentrations by 26 ± 2% over the control value of 67 ± 2·4 nM (Fig. 3).

Competition between adenosine and adenosine antagonists

If aminophylline enhances twitch tension by blocking the effects of adenosine, then adenosine would be expected to decrease twitch tension. At a concentration of 100 μM, adenosine decreased twitch tension by less than 5%, which

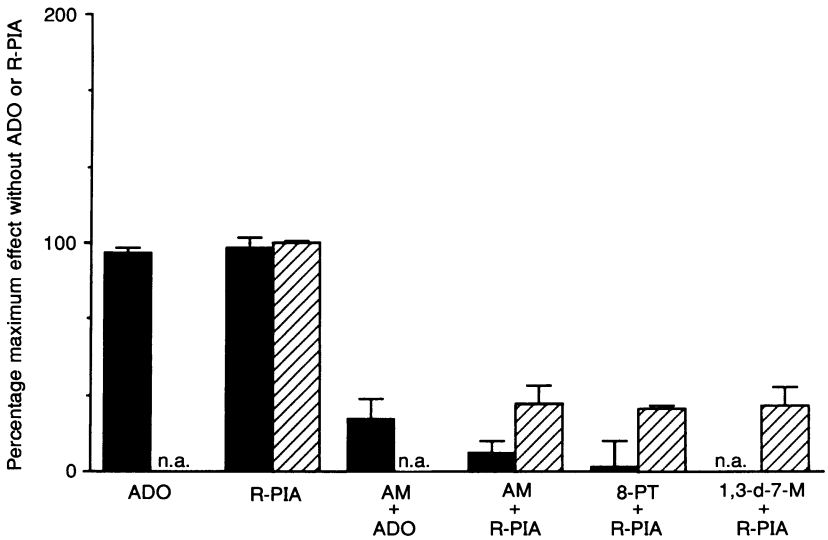

Figure 4. Effects of adenosine and its agonist R-PIA on the actions of xanthine drugs
Adenosine and R-PIA alone produced no effect on twitch tension (■) or resting calcium concentrations (▨) (first 2 sets of columns). However, adenosine and R-PIA inhibited the potentiating effects of the xanthines on both twitch tension and resting Ca^{2+} levels. Data are presented as a percentage of the effects of xanthines in the absence of either adenosine or R-PIA. For example, the effects of aminophylline (third set of bars) are 80% blocked by adenosine. Aminophylline alone (Fig. 3) increased P_t by 26% from 8·8 to 11·1 g. When adenosine was combined with aminophylline in the bath, P_t was 9·3 g, an increase over control of 5%, which is approximately 80% less than aminophylline alone. The number of experiments for each drug is listed in Results. Concentrations of AM, 8-PT and 1,3-d-7-M are as in Fig. 3. ADO, adenosine (100 μM); R-PIA, N^6-(2-phenylisopropyl)adenosine (1 μM).

was not significant ($n = 4$) (Fig. 4). This lack of effect may be due to a saturation of adenosine receptors by endogenous adenosine. If this is the case, adenosine would still be expected to block the effects of aminophylline on twitch tension. Adenosine (100 μM) attenuated by $77 \pm 9\%$ the effects of 100 μM aminophylline ($n = 6$) (Fig. 4). For example, in one experiment aminophylline increased force by 25% in the absence of adenosine and only 5% in the presence of adenosine. Adenosine at 100 μM had a greater effect on lower concentrations of aminophylline, blocking by $85 \pm 3\%$ the effect of 50 μM aminophylline ($n = 4$).

When adenosine is added to the Ringer solution bathing the semitendinosus muscle it may be taken up by muscle and other cells or broken down by the enzyme adenosine deaminase (Daly, 1982). Thus, the concentration of adenosine added to the bath may not be the concentration that reaches the putative adenosine receptors on the muscle fibres. To circumvent this problem, additional experiments were done using the adenosine analogue R-PIA, because it is an inferior substrate for both adenosine uptake systems (Ahlijanian & Takemori, 1986) and adenosine deaminase (Daly, 1982). Like adenosine, R-PIA alone did not have a significant effect on twitch tension ($n = 8$) or Ca^{2+} concentrations ($n = 6$) (Fig. 4). However, R-PIA blocked by 92 ± 5 ($n = 8$) and $72 \pm 8\%$ ($n = 6$) the effects of 100 μM aminophylline on twitch tension and Ca^{2+} concentrations, respectively.

Likewise, R-PIA blocked the potentiating effects of 100 μM 8-PT on twitch tension and Ca^{2+} concentrations by 98 ± 11 and $73 \pm 1\%$, respectively (Fig. 4). R-PIA also blocked the effects of 1,3-d-7-M on Ca^{2+} concentrations by $73 \pm 8\%$.

DISCUSSION

The data herein support the hypothesis that the xanthine drug aminophylline increases peak twitch tension in skeletal muscle. Aminophylline also affects intracellular resting Ca^{2+} concentrations in a manner that parallels the changes in peak twitch tension, both in magnitude and time course. Furthermore, the data are consistent with the interpretation that both of these effects are brought about in part by the blockade of adenosine receptors located on the membrane of skeletal muscle fibres.

Of the four proposed mechanisms of action of aminophylline, two are viable at micromolar concentrations of the drug, which is within the therapeutic range of its use (Rall, 1982). The first mechanism deals with the interaction of adenosine receptors and contractile force. Twitch tension is enhanced by 8-PT and 1,3-d-7-M, which are both known to have a higher affinity than aminophylline for adenosine receptors (Bruns *et al.* 1983; Daly *et al.* 1986). This result is consistent with the interpretation that aminophylline is acting to block an intrinsic inhibitory effect of endogenous adenosine. Furthermore, adenosine deaminase, which depletes adenosine from the bath, mimics the effects of aminophylline.

If aminophylline acts by blocking the effects of endogenous adenosine, there must be a source of adenosine at the surface of the muscle fibres. Circulating adenosine concentrations average 1 μM (Daly, 1982) and increase during muscle contraction in rat (Bockman, Berne & Rubio, 1975) and dog (Tominaga, Curnish, Belardinelli, Rubio & Berne, 1980; Ballard, Cotterrell & Karim, 1989) hindlimb preparations. Adenosine may be released directly from nerve and muscle or produced as a byproduct from the breakdown of ATP that is released both by contracting muscle and the motor nerve terminal (Bockman *et al.* 1975; Silinsky, 1975; Ribeiro & Sebastião, 1987). Adenosine release from contracting skeletal muscle in dog hindlimbs has been reported to increase 36% with a 2 Hz stimulation *in situ* (Tominaga *et al.* 1980) and as much as 12-fold after 10 min of 4 Hz stimulation (Ballard *et al.* 1989). The concentration of adenine nucleotides at the neuromuscular junction reaches as high as 80–100 μM following evoked transmitter release from the motoneuron (Silinsky, 1975). The lack of effect of exogenous adenosine in the confined space of the *in vitro* bath preparation used in this study supports the notion that adenosine levels are increased with contraction.

Poucher, Nowell & Collis (1990) did not observe an increase in twitch tension in cat gracilis muscle in the presence of 8-PT. The differences between their results and those reported here may be explained by differences in species used, *in situ vs. in vitro* experimental preparation, or methods of drug delivery. Moreover, in the cat gracilis muscle 8-PT caused a decrease in blood flow which could in turn reduce muscle twitch tension and counteract any direct potentiating effect of 8-PT on muscle twitch tension.

The inhibitory effects of adenosine are widespread and well documented on a wide variety of tissues (Daly, 1982), but little is known about the direct effect of adenosine on skeletal muscle fibres. The actions of adenosine are mediated by cell-surface receptors in many systems, including liver cells, platelets, glia and neurons (Bockman *et al.* 1975; Van Calker, Müller & Hamprecht, 1979; Londos, Cooper & Wolff, 1980; Snyder, Katims, Annau, Bruns & Daly, 1981; Daly, 1982; Barry, 1988, 1990).

In our experiments neither adenosine nor the agonist R-PIA had any significant effects on twitch tension. These results are probably due to saturation of adenosine receptors by the endogenous adenosine levels. However, it is apparent from our results (Fig. 4) that adenosine and R-PIA significantly attenuate the effects of both aminophylline and 8-PT, possibly by competing for adenosine receptors. These results contrast with those of Supinski, Deal & Kelsen (1986) who reported that the potentiating

effects of theophylline on skeletal muscle contractile force were not blocked by adenosine. They concluded that the effect was not mediated through adenosine receptors. This apparent contradiction can be resolved by noting the difference in incubation time prior to data collection between our experiments and those of Supinski *et al.* (1986). In Supinski's experiments, muscles were incubated in theophylline for only 10 min prior to force measurements. As illustrated in Fig. 1, 10 min would be insufficient time for the maximal effects of the drugs to take effect in our preparation.

Indirect evidence exists for the presence of adenosine receptors on skeletal muscle fibres in humans (Murciano *et al.* 1987). Using drugs known to increase bronchiole dilatation and enhance respiration, these investigators observed increases in diaphragmatic contractility only in the presence of those drugs known to be adenosine antagonists. Challiss, Richards & Budohoski (1992) provided strong evidence for the role of adenosine receptors on the surface of rat soleus muscle in the modulation of insulin action. Both adenosine deaminase and adenosine antagonists increased insulin-dependent metabolic activities in these muscles (Challiss *et al.* 1992), suggesting a depressant action of endogenous adenosine. That both adenosine and R-PIA attenuated the effects of aminophylline in the present experiments is additional evidence for the existence of adenosine receptors on skeletal muscle fibres.

A second proposed mechanism for the action of aminophylline is that the drug alters Ca^{2+} levels within muscle fibres by altering Ca^{2+} influx into the cell, or by altering Ca^{2+} release and/or sequestration into the sarcoplasmic reticulum (Blinks *et al.* 1972). Both resting Ca^{2+} levels and Ca^{2+} influx through voltage-sensitive calcium channels in the T-tubule membranes may be affected by aminophylline. Saadeh, Ayash, Saadeh & Nassar (1985) have shown that resting Ca^{2+} concentrations in rat diaphragm muscle are augmented by 15–100 μM aminophylline through increases in Ca^{2+} uptake in resting muscle fibres. Xanthines may also increase resting intracellular Ca^{2+} concentrations by inhibiting Ca^{2+} sequestration into intracellular organelles or depressing Ca^{2+} pumps (Blinks, Olson, Jewell & Braveny, 1972).

The proposed increase in resting Ca^{2+} levels by aminophylline could explain the effect of aminophylline on twitch tension. It is well-established that the magnitude of P_t is dependent on the amount of Ca^{2+} released from the sarcoplasmic reticulum (Ebashi & Endo, 1968; Endo, 1977). An increased resting level of Ca^{2+} in the sarcoplasm of muscle fibres may facilitate the opening of Ca^{2+} channels in the sarcoplasmic reticulum following membrane depolarization (Endo, Tanaka & Ogawa, 1970; Meissner, Darling & Eveleth, 1986). In calcium channels from the sarcoplasmic reticulum that have been incorporated into lipid bilayers, the probability of opening is increased when Ca^{2+} levels are elevated beyond 100 nM (Ikemoto, Antoniu & Meszaros, 1985; Meissner *et al.* 1986; Smith, Coronado & Meissner, 1986; Ehrlich & Watras, 1988). Thus, a small change in the intracellular Ca^{2+} concentration may not directly open Ca^{2+} channels in the sarcoplasmic reticulum, but may facilitate channel opening upon membrane depolarization, resulting in the observed increase in twitch force. Alternatively, increased resting Ca^{2+} levels may augment Ca^{2+} content in the sarcoplasmic reticulum, resulting in increased Ca^{2+} release upon stimulation.

Several lines of evidence support the hypothesis that the effect of aminophylline on strength is in part a result of increased Ca^{2+} levels. Removal of Ca^{2+} from the media reduces or eliminates the potentiating action of aminophylline on twitch tension in mammalian and frog muscle (Varagic & Kenters, 1978; Aubier *et al.* 1981; Ridings *et al.* 1989) The time course of changes in Ca^{2+} uptake in the present experiment is similar to the time course of the increase in twitch tension due to aminophylline in this and other studies in frog muscle (Ridings *et al.* 1989). That both events are partially blocked by R-PIA is evidence that both actions occur through adenosine receptors. Furthermore, adenosine deaminase, which decreases endogenous adenosine levels, also increased both twitch tension and Ca^{2+} concentration. Because the changes to Ca^{2+} concentration and twitch tension produced by the drugs correlated closely in both time course and magnitude, it is tempting to speculate that these two events might be mechanistically related. Further experiments illustrating that one mechanism cannot occur without the other will be necessary to establish such a relationship.

The slow time course of both twitch tension and Ca^{2+} concentration changes suggests that a second messenger system may be involved in the response of the muscle fibres to aminophylline. Challiss *et al.* (1992) noted a 20 min time to peak in the response of rat skeletal muscle to xanthines, also suggestive of a potential second messenger system. This possibility provides an interesting avenue for future studies.

In summary, our data suggest that aminophylline acts to block adenosine receptors on the surface of skeletal muscle fibres, thereby inhibiting the effects of endogenous adenosine. This action results in increased concentrations of intracellular resting Ca^{2+} and enhanced twitch tension upon muscle stimulation.

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