J. Pharm. Pharmacol. 1993, 45: 1060–1066
Received November 2, 1992

Effects of Alkylxanthines on Contractility of Diaphragm Fibres Isolated from Normal and Sensitized Guinea-pigs

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Abstract—This study investigates the effects of alkylxanthines on twitch tension generated by electrical stimulation (supramaximal pulses, 0·2 ms duration, 1 Hz) of diaphragm muscle fibres isolated from normal and actively-sensitized guinea-pigs. Caffeine, theophylline and theobromine increased, in a concentration-dependent manner (50–500 μM), twitch tension in normal and sensitized diaphragm. Caffeine (500 μM) enhanced contractility to a greater extent than theophylline or theobromine. Twitch potentiation by caffeine (500 μM) was significantly greater in sensitized diaphragm. Verapamil (0·1–100 μM) did not alter twitch contractions in the absence or presence of alkylxanthines in normal or sensitized strips. Dantrolene (0·01–100 μM) depressed, in a concentration-dependent fashion, twitch contractions of normal and sensitized diaphragm. The inhibitory concentration 50% (expressed as $-\log IC50$) was $6{\cdot}78 \pm 0{\cdot}13$ in normal tissues and $6{\cdot}15 \pm 0{\cdot}11$ in sensitized tissues ($n=6$ in each group; $P<0{\cdot}05$). Exposure to Ca^{2+}-free, EGTA (0·1 mM)-containing medium, depressed twitch contraction of normal diaphragm to a lesser extent than that of sensitized diaphragm. Methylxanthines reversed depression of twitch contractions produced by exposure to dantrolene (5 μM) or a Ca^{2+}-free medium. Adenosine (1–1000 μM) was without effect whereas enprofylline (50–500 μM) enhanced diaphragm contractility in normal tissues. This indicates that blockade of adenosine receptors is not involved in the inotropic effect of alkylxanthines in guinea-pig diaphragm. Results from this study suggest that alkylxanthines enhance diaphragm contractility in the guinea-pig by releasing intracellular Ca^{2+} and promoting extracellular Ca^{2+} entry through verapamil-insensitive pathways. An alteration of Ca^{2+} movements and stores may be present in the sensitized diaphragm.

Alkylxanthines, particularly theophylline and its derivatives, benefit patients with airway obstruction (Rall 1990). This effect has been primarily attributed to bronchodilatation (Weinberger & Riegelman 1974). However, several studies suggest that mechanisms other than relaxation of airway smooth muscle are relevant to the clinical improvement produced by theophylline compounds. An enhancement in the contractility of the respiratory muscles is claimed as one such mechanism (Aubier 1987). Thus, theophylline or aminophylline, at plasma concentrations within the therapeutic range (10–20 mg L^{-1}, 55–110 μM) augmented diaphragm contractility in anaesthetized dogs, normal subjects, and patients with obstructive pulmonary disease (Aubier & Roussos 1985; Aubier 1987).

By contrast, supratherapeutic concentrations of methylxanthines are needed to potentiate twitch tension in isolated preparations of diaphragm. This has been attributed to problems of drug diffusion through large muscle strips. Viirès et al (1986) have demonstrated that therapeutic concentrations of theophylline increase contractility of single diaphragm fibres making this in-vitro model preferable to classical preparations. The precise mechanism of this direct inotropic effect of methylxanthines is presently unknown, but several studies indicate that it is mediated through alterations in transmembrane and intracellular Ca^{2+} movements (Aubier & Roussos 1985).

Most of the in-vitro work on the effects of methylxanthines on diaphragm contractility has been carried out in the rat (Varagic & Kentera 1978; Viirès et al 1986, 1988; Delbono & Kotsias 1988; Kolbeck & Speir 1989) and less often in other animal species such as the hamster (Wittmann & Kelsen 1982; Esau 1988), the mouse (Kimura et al 1985) or the guinea-pig (Supinski et al 1986). However, studies using the guinea-pig diaphragm would be particularly interesting since similarities in fibre composition with the human diaphragm have been reported (Lieberman et al 1973). In addition, the guinea-pig isolated trachea is a preparation frequently used to assess bronchodilators, and active sensitization of the guinea-pig is a common model of allergic asthma.

We have demonstrated the existence of non-specific hyperreactivity to spasmogens (Morcillo et al 1984), diminished efficacy of verapamil (Perpiñá et al 1987) and altered ^{45}Ca movements (Perpiñá et al 1991) in sensitized guinea-pig airway muscle. Sources of Ca^{2+} for caffeine- and cooling-induced contraction differ in normal and sensitized guinea-pig trachea (Ortiz et al 1988, 1991). These results suggest that the relative contribution of extracellular and intracellular Ca^{2+} to muscle activation differs in normal and sensitized tissues. To our knowledge, the pharmacological reactivity of sensitized diaphragm in-vitro has not yet been studied; hence, we were interested in finding whether hyper-activity to inotropic agents and differences in Ca^{2+} movements exist in sensitized compared with normal diaphragm strips.

Materials and Methods

Tissue preparation

Guinea-pigs of either sex, 350–400 g, were randomly allocated into two groups, normal (nonsensitized) and sensitized. On day 0 the animals were injected subcutaneously with 0·25 mL Freund's complete adjuvant plus 1·25 μg g^{-1} bovine serum albumin (BSA) dissolved in 0·25 mL saline. On day 2 and day 4 the animals received the same amount of Freund's complete adjuvant and BSA by the intramuscular

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route. The animals were used for experiments on days 21 to 25. The nonsensitized group was subjected to the same protocol but received only saline. The animals were killed by stunning and exsanguination and the diaphragm with adjacent rib sections was rapidly removed and placed in a dissection dish containing a physiological salt solution (PSS; composition in mM: NaCl 137, KCl 4, $MgCl_2$ 1, KH_2PO_4 1, $NaHCO_3$ 12, $CaCl_2$ 2, glucose 6·5) aerated with 5% CO_2 in O_2 (pH 7·4).

A bundle of intact costal diaphragm fibres was obtained and then microscopically dissected to yield strips 1 mm wide and 1·5 cm long as previously reported by Viirès et al (1986). Strips were mounted in 5-mL tissue chambers containing PSS bubbled with 5% CO_2 in O_2 (pH 7·4) and maintained at 37°C. Field stimulation of tissue strips was carried out by means of platinum ring electrodes located at the top and bottom of the preparation. Slightly supramaximal, monophasic square wave pulses, 0·2 ms in duration, were delivered at a frequency of 1 Hz with a Grass S88 stimulator (Viirès et al 1986). Isometric force developed in response to electrical stimulation was measured with a high sensitivity transducer (Hewlett Packard FTA 100-1) connected through an amplifier (HP 88053) to a Philips PM 8222 recorder. After a 20-min equilibration period, tissue length was adjusted by means of a micrometer (HP FTA 1011) to yield maximal isometric tension (L_0). All subsequent studies were performed at this length. The strip was stimulated with single pulses (3 min^{-1}) and a further equilibration period permitted (about 30 min) to obtain stable twitch contractions of the preparation before any pharmacological intervention.

Experimental protocol

Prior experiments demonstrated that addition to the bath of pancuronium (1–10 μM), neostigmine (0·1–1 μM) or tetrodotoxin (1 μM) did not change peak diaphragm twitch tension in preparations from normal and sensitized guinea-pigs (data not shown).

After stable twitch contractions of preparations from normal and sensitized guinea-pigs had been attained, caffeine, theophylline or theobromine was cumulatively added to the bath in concentrations ranging from 50 to 500 μM. Concentrations above 500 μM were not tested since previous experiments showed that these concentrations resulted in increases in the resting tension of the preparation (data not shown).

After an initial concentration-effect curve for one of these methylxanthines had been obtained, the tissues were allocated randomly in equal numbers to test or time-matched control groups and a second concentration-effect curve was generated after the tissue had been equilibrated in PSS for 30 min. Dantrolene (5 μM) or verapamil (10 μM) was present in the test tissues for 20 min before and during the construction of the second concentration-effect curve. The control tissues were treated similarly but were not exposed to the antagonist. Additional experiments were carried out following the same protocol except that after the first concentration-effect curve the test tissues were exposed to a Ca^{2+}-free PSS containing EGTA (0·1 mM) for 15 min before and throughout the second concentration-effect curve.

In separate experiments, cumulative concentration-effect curves for dantrolene (0·01–100 μM) or verapamil (0·1–100 μM) were constructed in normal and sensitized tissues. At the end of control and test experiments in normal and sensitized preparations, BSA (1 mg mL^{-1}) was added to the bath to confirm the existence of an antigen-induced response of sensitized strips and the lack of responsiveness of normal tissues.

In an additional set of experiments with normal tissues, adenosine (1–1000 μM) was cumulatively added to the bath. In other experiments, a cumulative concentration-effect curve for enprofylline (50–500 μM) was obtained in normal tissues; second curves for enprofylline were constructed in the absence (time matched control) or presence of verapamil (10 μM), dantrolene (5 μM) or Ca^{2+}-free EGTA (0·1 mM)-containing medium as previously outlined.

Experiments in diaphragm and soleus isolated from guinea-pig and rat

Guinea-pigs, 350–400 g, and Wistar rats, 250–300 g, of either sex were killed by stunning and exsanguinated. Diaphragm and soleus were obtained and prepared to measure isometric force (twitch) as described above. After a 30-min equilibration period, during which time twitch contractions had stabilized, the PSS was substituted for a Ca^{2+}-free, EGTA (0·1 mM)-containing solution while the muscle strips were continuously stimulated and twitches recorded for 30 min (Viirès et al 1988).

Drugs and solutions; statistical analysis of results

The following substances were used: adenosine (Sigma, Madrid), BSA (fraction V, fatty acid free; Sigma), caffeine (Sigma), dantrolene disodium (Alonga, Barcelona), enprofylline (AB Draco/Astra), ethyleneglycol-bis-(β-aminoethyl-ether)-*N*-*N*′-tetraacetic acid (EGTA, Sigma), Freund's complete adjuvant (Difco, Detroit, MI), neostigmine methyl sulphate (Roche), pancuronium bromide (Sigma), theobromine (Sigma), theophylline (Sigma), tetrodotoxin (Sigma), (±)-verapamil hydrochloride (Biosedra-Knoll, Madrid).

Drug concentrations are expressed in terms of the final molar (M) bath concentration of the active species. Drugs were dissolved in PSS except theobromine which was dissolved in carmellose (0·1% w/v, Sigma). Addition of the vehicles to the bath did not alter isometric force (twitch).

After the equilibration period and determination of L_0, baseline force output was measured before any pharmacological intervention and expressed as mg of force. The effects of drug addition to the bath tissues are expressed as percent of baseline. The wet tissue weight was measured and differences were not found between normal and sensitized tissues, and between time-matched controls and test tissues (data not shown).

Data are presented as means ± s.e.m. Statistical analysis of the results was performed by analysis of variance followed by Duncan's test. Differences were considered significant when $P < 0·05$.

Results

Baseline values and responses to BSA

The mean maximal twitch tension generated by strips of normal diaphragm was 84 ± 7 mg (n = 24) and that obtained

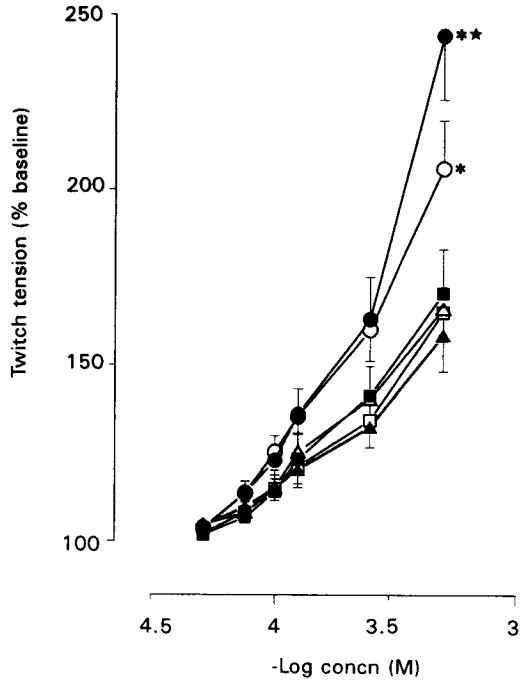

FIG. 1. The effect of caffeine (○, ●), theophylline (□, ■) and theobromine (△, ▲) on twitch contraction of diaphragm muscle fibres isolated from normal (open symbols) and sensitized (closed symbols) guinea-pigs. Data are mean ± s.e.m. for six different animals at each concentration. *$P < 0.05$ compared with theophylline or theobromine. ★$P < 0.05$ compared with normal preparations.

in strips of sensitized diaphragm was 122 ± 13 mg (n = 24) ($P < 0.05$).

Only the sensitized diaphragm strips reacted after addition to the bath of BSA (1 mg mL^{-1}) with a contracture (increase of resting tension of 63·9 ± 16·2 mg, n = 24) without potentiation of twitch contraction; normal diaphragm was not responsive to BSA (1 mg mL^{-1}).

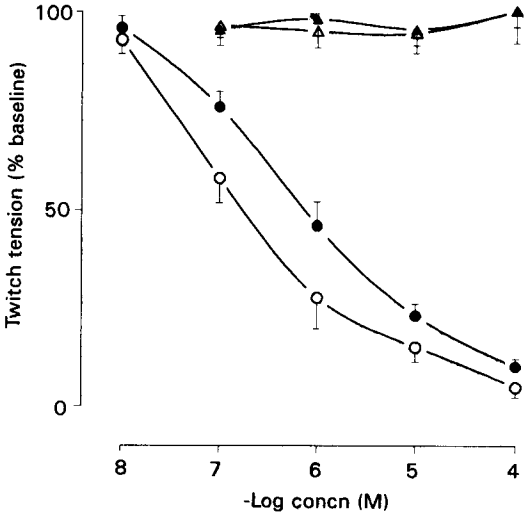

FIG. 2. The effect of verapamil (△, ▲) and dantrolene (○, ●) on twitch contraction of diaphragm muscle fibres isolated from normal (open symbols) and sensitized (closed symbols) guinea-pigs. Data are mean ± s.e.m. for six different animals at each concentration.

Responses to caffeine, theophylline and theobromine in normal and sensitized tissues

Caffeine, theophylline and theobromine augmented the peak diaphragm twitch tension in a concentration-related fashion (50–500 μM) (Fig. 1). Caffeine (500 μM) enhanced diaphragm tension in normal and sensitized tissues to a greater extent than the same concentration of theophylline and theobromine. Twitch potentiation by caffeine (500 μM) was significantly greater in sensitized compared with normal tissues (Fig. 1).

Effects of verapamil and dantrolene on the responses to caffeine, theophylline and theobromine

Verapamil, in concentrations up to 100 μM, did not significantly alter twitch contraction in normal or sensitized tissues (Fig. 2). Concentration-effect curves for caffeine, theophylline and theobromine generated in the presence of verapamil (10 μM) did not significantly differ from those obtained in time-matched control preparations. The concentration-effect curves are not shown but responses (expressed as % of baseline) to caffeine (500 μM) in the absence (time-matched control) or presence of verapamil (10 μM) were, respectively, 209 ± 7 vs 206 ± 10 in normal tisues and 247 ± 9 vs 243 ± 9 in sensitized tissues; those to theophylline (500 μM) were 159 ± 6 vs 178 ± 4, and 189 ± 8 vs 179 ± 6 in sensitized tissues; and those to theobromine (500 μM) were 153 ± 7 vs 164 ± 7 in normal, and 183 ± 9 vs 173 ± 10 in sensitized tissues (n = 6 in each group).

Dantrolene (0·01–100 μM) produced a concentration-related inhibition of twitch tension which was virtually abolished with concentrations of 100 μM (Fig. 2). The inhibitory concentration 50% (expressed as −log IC50) was 6·78 ± 0·13 (n = 6) in normal tissues and 6·15 ± 0·11 (n = 6) in sensitized strips ($P < 0.05$ vs normal). Therefore, greater concentrations of dantrolene were needed to obtain 50% inhibition of twitch contraction in sensitized compared with normal diaphragm. A concentration of dantrolene of 5 μM which produced 75–80% inhibition of twitch contraction in normal and sensitized tissues, was selected for further experiments.

Cumulative addition to the bath of increasing concentrations (50–500 μM) of caffeine, theophylline or theobromine in the presence of dantrolene (5 μM) resulted in a concentration-dependent twitch potentiation from the depressed values imposed by dantrolene (Fig. 3). The examination of the concentration-effect curves for caffeine, theophylline and theobromine in the presence of dantrolene shows that the efficacy of methylxanthines to reverse the depressant effect of dantrolene was similar in sensitized and normal tissues except for the lower segment of the concentration-effect curve for theobromine (Fig. 3).

Responses to caffeine, theophylline and theobromine in a Ca^{2+}-free medium

After 15 min of exposure to a Ca^{2+}-free EGTA (0·1 mM)-containing solution, a small decrease in twitch amplitude was observed (the twitch amplitude amounted to 81 ± 3% of control i.e. 19% decrease; n = 6) in normal diaphragm. The same protocol produced a greater decrease of twitch amplitude in sensitized tissue (63 ± 6% of baseline i.e. 37% decrease; n = 6; $P < 0.05$ vs normal tissue). Prior experiments

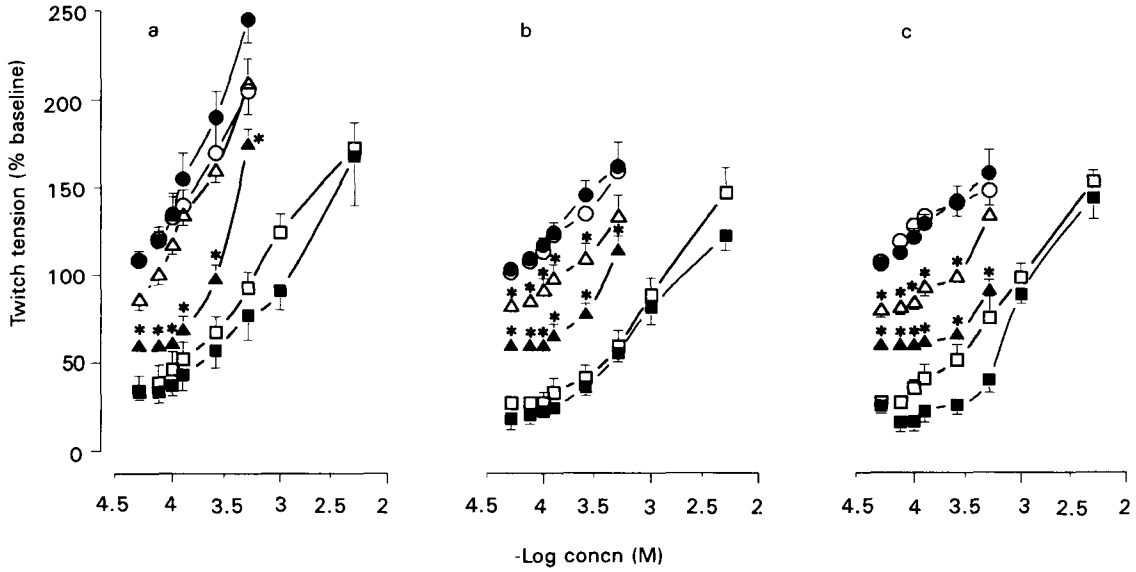

FIG. 3. The effect of caffeine (a), theophylline (b) and theobromine (c) on twitch contraction of diaphragm muscle fibres from normal (open symbols) and sensitized (closed symbols) guinea-pigs. Responses were obtained in the absence (time-matched control ○, ●) or presence of either dantrolene (5 μM □, ■) or a Ca^{2+}-free medium EGTA (0·1 mM △, ▲). Data are mean ± s.e.m. for six different animals at each concentration. $^*P < 0.05$ compared with their corresponding values in normal (○) or sensitized (●) tissues.

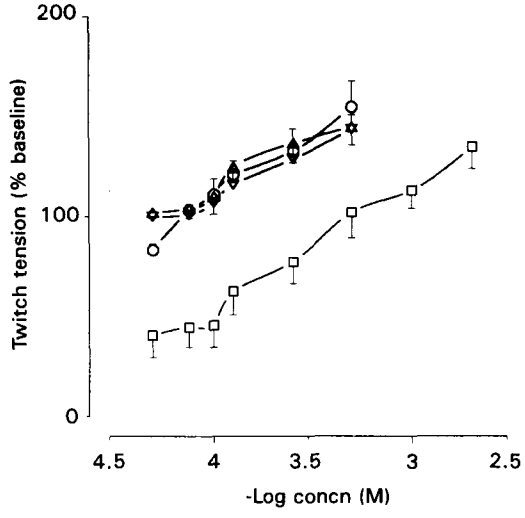

FIG. 4. The effect of enprofylline on twitch contraction of guinea-pig isolated diaphragm in the absence (time-matched control △) or presence of verapamil (10 μM ▽), dantrolene (5 μM □) or a Ca^{2+}-free EGTA (0·1 mM)-containing medium (○). Data are mean ± s.e.m. for six different animals at each concentration.

carried out in paired strips but in the absence of any drug treatment demonstrated that incubation with Ca^{2+}-free EGTA (0·1 mM) solution for 30–40 min (the time required to produce a complete concentration-effect curve to methylxanthines) did not result in additional changes of twitch tension compared with 15 min exposure (data not shown).

In the normal diaphragm, the enhancing effect of caffeine on twitch tension was not altered after incubation in a Ca^{2+}-free medium. In contrast, responses to theophylline and theobromine were partly attenuated in Ca^{2+}-free media (Fig. 3). In sensitized tissues subjected to extracellular Ca^{2+} deprivation the concentration-effect curves for methylxanthines started from a lower value of twitch tension and lay to the right of those generated in time-matched control tissues (Fig. 3).

Effects of adenosine and enprofylline in normal diaphragm

Adenosine (1–1000 μM) had no significant effect on diaphragm tension. Enprofylline (50–500 μM) elicited a concentration-related enhancement of twitch contractions (Fig. 4). The concentration-effect curve for enprofylline did not significantly differ from those for theophylline and theobromine shown in Fig. 1. Neither verapamil (10 μM) nor incubation in a Ca^{2+}-free medium altered the concentration-effect curve for enprofylline (Fig. 4). Enprofylline reversed the inhibitory effect produced by dantrolene (5 μM) in a concentration-dependent manner (Fig. 4).

Effect of extracellular Ca^{2+} withdrawal on twitch tension in diaphragm and soleus of guinea-pig and rat

Preparations of diaphragm (normal, i.e. nonsensitized) from guinea-pig and rat behaved in a strikingly different manner when exposed to a Ca^{2+}-free EGTA (0·1 mM)-containing solution (Fig. 5). For the rat diaphragm, exposure to a Ca^{2+}-free medium was followed by a rapid decrease in twitch tension with virtually complete abolition of the response within 20 min. Guinea-pig diaphragm exposed to Ca^{2+}-free medium showed a much slower rate of twitch decay that reached a plateau around 80% of baseline within 15 min.

In contrast, the time course of the twitch decay for guinea-pig and rat soleus muscles was similar, with a rapid decline of twitch tension to a plateau around 40% of baseline within 30 min of Ca^{2+}-free exposure (Fig. 5).

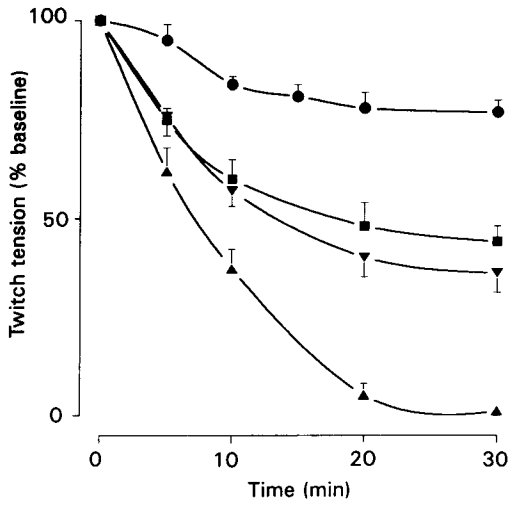

FIG. 5. Time course of decay in twitch tension during exposure to Ca^{2+}-free EGTA (0·1 mM)-containing solution. ● % changes of diaphragm fibre preparations from normal guinea-pigs; ■ % changes of soleus fibre preparations from normal guinea-pigs; ▼ % changes of soleus fibre preparations from normal rats; ▲ % changes of diaphragm fibre preparations from normal rats. Points are mean ± s.e.m. for six different animals in each group.

Discussion

Enhancement of diaphragm contractility by alkylxanthines

The present study shows that methylxanthines (caffeine, theophylline and theobromine) augment contractility of diaphragm muscle fibres isolated from normal and actively sensitized guinea-pigs. Another xanthine derivative, enprofylline, also increased twitch contractions of normal diaphragm. These results are consistent with previous findings for theophylline using the same experimental model in normal (nonsensitized) rats (Viirès et al 1986). The use of this model was intended to overcome the problem of inhomogeneous drug diffusion encountered in large muscle strips.

This study also confirms and extends previous reports showing that caffeine, theophylline and aminophylline enhance diaphragm contractility in isolated preparations from several animal species not subjected to immunization (Varagic & Kentera 1978; Wittmann & Kelsen 1982; Kimura et al 1985; Supinski et al 1986; Sherman et al 1988). The observed increase of diaphragm tension by alkylxanthines was concentration-dependent but it did not approach a plateau in the upper range of concentrations studied. This impeded the calculation of their relative potencies as inotropic agents based on determinations of concentrations producing half of the maximal effect. Supinski et al (1986) did not find a plateau of the response of theophylline added in concentrations up to 1 mM to guinea-pig isolated diaphragm. By contrast, effects of theophylline on twitch tension generated by rat isolated diaphragm fibres reached a plateau at a drug concentration of 0·33 mM (Viirès et al 1986). Sherman et al (1988) found no plateau for aminophylline (up to 2 mM) in rat isolated hemidiaphragm. There is no apparent explanation for this difference among studies.

In this model, therapeutic concentrations of theophylline and enprofylline or concentrations of caffeine easily achievable in-vivo effectively enhanced diaphragm contractility in-vitro. However, when the therapeutic range is corrected for the presence of plasma proteins, to which in man 40% of theophylline and 17% of caffeine are bound (Hendeles 1985), the resulting concentrations of alkylxanthines ($\leqslant 50$ μM) produced a rather modest or no enhancement of diaphragm contractility compared with that attainable with higher concentrations (this study) and it could explain discrepancies among clinical reports (Aubier et al 1981; Moxham et al 1985).

Mechanism of the enhancement of diaphragm contractility by alkylxanthines. Effects of Ca^{2+}-free medium and calcium antagonists

The intropic effect elicited by alkylxanthines on the isolated diaphragm results from a direct action (Viirès et al 1986; this study). The precise mechanism by which xanthine derivatives directly influence diaphragm contractility is unclear. Alkylxanthines inhibit phosphodiesterase enzyme activity and increase intracellular concentrations of cyclic (c)AMP (Bergstrand 1985). However, there is no relationship between the effectiveness of a given alkylxanthine in raising cAMP concentration and its ability to increase twitch tension in the rat isolated diaphragm (Kramer & Wells 1980). Caffeine, for example, is a weaker inhibitor of phosphodiesterase, but more efficacious than theophylline in increasing force (Supinski et al 1984; this study).

The intracellular action of methylxanthines is affected by differences in physicochemical properties and its ability to penetrate into the cell (Bianchi 1962). The rank order of lipid solubility for methylxanthines is caffeine > theophylline > theobromine (Budavari et al 1989). Therefore, the difference between caffeine and the other xanthines could be related to their different abilities to cross the plasmalemma. A water-soluble xanthine derivative has not been used in these experiments since Small et al (1988) have shown that one such compound also crosses the cell membrane.

It has been suggested that the actions of methylxanthines on respiratory skeletal muscle contractility may be produced via an action on plasmalemmal adenosine receptors (Fredholm 1980). Theophylline is a potent antagonist of adenosine and this autacoid decreases Ca^{2+} influx in frog skeletal muscle (Prosdocini & Bianchi 1981). However, adenosine has no effect on diaphragm contractility even at high concentrations (Supinski et al 1986; this study). Furthermore, enprofylline is a poor adenosine antagonist (Persson et al 1986) but produced a concentration-dependent increase in twitch tension similar to that obtained with theophylline. Therefore, the apparent efficacy of alkylxanthines on diaphragm contractility does not correlate with their relative potency as phosphodiesterase inhibitors or as adenosine blockers.

The positive inotropic effect of alkylxanthines may be due to a direct effect on Ca^{2+} movements involved in the excitation-contraction coupling. Caffeine has been shown to increase Ca^{2+} entry into skeletal muscle cells (Isaacson & Sanders 1967) and to increase the release of Ca^{2+} in isolated sarcoplasmic reticulum (Katz et al 1977). Theophylline also releases Ca^{2+} from the sarcoplasmic reticulum but an increased influx of labelled Ca^{2+} has not been demonstrated (Bianchi & Narayan 1981). Recent findings reinforce the possibility of a membrane site of action for these drugs. Theophylline hyperpolarizes hamster (Esau 1988) and rat

(Delbono & Kotsias 1988) diaphragm muscle. The mechanism of this effect is unknown but it may result from stimulation of Na^+-K^+ pump or activation of Ca^{2+}-dependent K^+ channels.

Intracellular Ca^{2+} stores are considered as the major source of free Ca^{2+} available to skeletal muscle contractile proteins. In contrast, the relevance of transmembrane Ca^{2+} influx differs among muscle-types and animal species. Thus, the response to external Ca^{2+} deprivation was similar in soleus muscle fibres isolated from rat and guinea-pig but marked differences appeared for diaphragm. Rat diaphragm fibres were found to be highly dependent on extracellular Ca^{2+} for contraction (abolition of twitch contraction after 30 min in Ca^{2+}-free medium) confirming a previous study by Viirès et al (1988), whereas guinea-pig diaphragm fibres were only partly dependent (20% decrease of twitch tension after 30 min in Ca^{2+}-free medium). Verapamil did not affect twitch contraction indicating that Ca^{2+} entry through voltage-dependent channels sensitive to verapamil is not involved. Dantrolene depressed twitch contraction indicating that intracellular Ca^{2+} movements affected by this agent (Putney & Bianchi 1974; Morgan & Bryant 1977) are involved in force generation by the guinea-pig diaphragm. The possible contribution of transmembrane Ca^{2+} entry sensitive to dantrolene (Morgan & Bryant 1977; Salata & Jalife 1982) should also be considered. The inhibition of twitch contraction by dantrolene was reversed by alkylxanthines (Herbette et al 1982; this study).

The concentration-effect curves to alkylxanthines obtained in guinea-pig diaphragm strips were not affected by verapamil. Responses to caffeine were not impaired when generated in a Ca^{2+}-free medium and those to theophylline and theobromine were only partly attenuated in Ca^{2+}-free medium. This indicates that extracellular Ca^{2+} is not essential to express the positive inotropic effect of alkylxanthines in the guinea-pig diaphragm.

This is in contrast with observations on the effects of verapamil or hypocalcaemia on the in-vivo effects of methylxanthines in the canine diaphragm (Aubier et al 1983, 1985) and with the influence of verapamil or a Ca^{2+}-free solution on the in-vitro effects of methylxanthines in the rat (Varagic & Kentera 1978; Saadeh et al 1985; Kolbeck & Speir 1989) and hamster (Esau 1988) isolated diaphragm. These differences may be explained by the small effect of extracellular Ca^{2+} removal on the contractility of the guinea-pig diaphragm. Although similarities exist in fibre composition between guinea-pig and human diaphragms (Lieberman et al 1973) extrapolation of results to man must be cautious. The influence of extracellular Ca^{2+} deprivation or calcium-channel blockers on the effects of methylxanthines on transdiaphragm pressure in man has not yet been explored.

Responses in the sensitized diaphragm

Diaphragm strips from sensitized guinea-pigs contracted in response to BSA (1 mg mL^{-1}) without change in twitch amplitude. To our knowledge, a Schultz–Dale reaction in skeletal muscle isolated from sensitized animals has not been previously described and the mechanism underlying this immunologically-induced contracture of the guinea-pig diaphragm remains to be elucidated. Immediate hypersensitivity reactions have been previously shown in guinea-pig isolated heart actively sensitized to ovalbumin and attributed to cardiac mast cell degranulation and release of mediators (Liebig et al 1975; Andjelkovic & Zlokovic 1982).

The main differences found in this study in the pharmacologic reactivity of sensitized diaphragm compared with normal tissues are greater baseline values of twitch tension in sensitized tissues, greater reliance on extracellular Ca^{2+} for contraction, a lowered potency of dantrolene to depress twitch contraction, and a greater enhancing effect of caffeine (500 μM) on diaphragm contractility.

Collectively these findings suggest the existence of an alteration of Ca^{2+} homeostasis in the sensitized diaphragm. The mechanism by which external Ca^{2+} removal inhibits skeletal muscle contraction is unclear. Extracellular Ca^{2+} does not directly serve as an initiator but it is required to maintain surface membrane stores of initiating Ca^{2+} in the t-tubule (Frank 1980). Diaphragm muscle from sensitized guinea-pigs appears more sensitive to depletion of this surface Ca^{2+} store after exposure to a Ca^{2+}-free medium.

In conclusion, the present study shows that methylxanthines enhance contractility of diaphragm fibres isolated from normal and sensitized guinea-pigs but an alteration in Ca^{2+} mobilization may be present in sensitized diaphragm. The clinical implications of these findings remain to be demonstrated.

Acknowledgements

This work was supported by Grants FAR90-0680 and FAR91-0875 from C.I.C.Y.T. (Ministerio de Industria y Energia), Spain. The authors thank Mr P. Santamaría for valuable technical assistance.

References

Andjelkovic, I., Zlokovic, B. (1982) Protective effects of glucagon during the anaphylactic response in guinea-pig isolated heart. Br. J. Pharmacol. 76: 483–489

Aubier, M. (1987) Effect of theophylline on diaphragmatic muscle function. Chest 92: 27S–31S

Aubier, M., Roussos, C. (1985) Effect of theophylline on respiratory muscle function. Chest 88: 91S–97S

Aubier, M., Troyer, A. D., Sampson, M., Macklem, P. T., Roussos, C. (1981) Aminophylline improves diaphragmatic contractility. N. Engl. J. Med. 305: 249–252

Aubier, M., Murciano, D., Viirès, N., Lecocgnic, Y., Pariente, R. (1983) Diaphragmatic contractility enhanced by aminophylline: role of extracellular calcium. J. Appl. Physiol. 54: 460–464

Aubier, M., Viirès, N., Piquet, J., Murciano, D., Blanchet, F., Marty, C., Gherardi, R., Pariente, R. (1985) Effects of hypocalcemia on diaphragmatic strength generation. J. Appl. Physiol. 58: 2054–2061

Bergstrand, H. (1985) Xanthines as phosphodiesterase inhibitors. In: Andersson, K. E., Persson, C. G. A. (eds) Anti-asthma Xanthines and Adenosine. Excerpta Medica, Amsterdam, pp 16–22

Bianchi, C.P. (1962) Kinetics of radiocaffeine uptake and release in frog sartorius. J. Pharmacol. Exp. Ther. 138: 41–47

Bianchi, C. P., Narayan, S. (1981) Possible role of the transverse tubules in accumulating calcium released from the terminal cisternae by stimulation and drugs. Can. J. Physiol. Pharmacol. 60: 503–507

Budavari, S., O'Neil, M.J., Smith, A., Heckelman, P.E. (eds) (1989) The Merck Index. 11th edn, Merck & Co Inc, Rahway, NJ, USA

Delbono, O., Kotsias, B. A. (1988) Hyperpolarizing effect of aminophylline, theophylline and cAMP on rat diaphragm fibers. J. Appl. Physiol. 64: 1893–1899

Esau, S. A. (1988) Interaction of theophylline, verapamil and

diltiazem on hamster diaphragm muscle force in vitro. Am. J. Physiol. 254: C365–C371

Frank, G. B. (1980) The current view of the source of trigger calcium in excitation-contraction coupling in vertebrate skeletal muscle. Biochem. Pharmacol. 29: 2399–2406

Fredholm, B. B. (1980) Are methylxanthine effects due to antagonism of endogenous adenosine? Trends Pharmacol. Sci. 1: 129–132

Hendeles, L. (1985) Update on the pharmacodynamics and pharmacokinetics of theophylline. Chest 88: 103S–111S

Herbette, L., Messineo, F. C., Kataz, A. M. (1982) The interaction of drugs with the sarcoplasmic reticulum. Ann. Rev. Pharmacol. Toxicol. 22: 413–434

Isaacson, A., Sanders, A. (1967) Quinine and caffeine effects on ^{45}Ca movements in frog sartorius muscle. J. Gen. Physiol. 50: 2109–2128

Katz, A. M., Repke, D. I., Hasselbach, W. (1977) Dependence of ionophore- and caffeine-induced calcium release from sarcoplasmic reticulum vesicles on external and internal calcium ion concentrations. J. Biol. Chem. 252: 1983–1949

Kimura, I., Kimura, M., Kimura, M. (1985) Modification by dantrolene, procaine and suxamethonium of caffeine-induced changes in aequorin luminescence transients and twitch tensions of directly-stimulated diaphragm muscle of mouse. Br. J. Pharmacol. 86: 319–326

Kolbeck, R. C., Speir, W. A. (1989) Diltiazem, verapamil and nifedipine inhibit theophylline-enhanced diaphragmatic contractility. Am. Rev. Respir. Dis. 139: 139–145

Kramer, G. L., Wells, J. N. (1980) Xanthines and skeletal muscle: lack of relationship between phosphodiesterase inhibition and increased twitch tension in rat diaphragm. Mol. Pharmacol. 17: 73–78

Lieberman, D. A., Faulkner, J. A., Craig, A. B., Maxwell, L. C. (1973) Performance and histochemical composition of guinea-pig and rat hemidiaphragm. J. Appl. Physiol. 34: 233–237

Liebig, R., Benauer, W., Pescar, B. A. (1975) Prostaglandin slow-reacting substance, and histamine release from anaphylactic guinea-pig hearts, and its pharmacological modifications. Naunyn Schmiedebergs Arch. Pharmacol. 289: 65–76

Morcillo, E. J., Perpiñá, M., Esplugues, J. (1984) Hyperresponsiveness to autacoids and autonomic drugs in lung parenchymal strips from sensitized guinea-pigs. Am. Rev. Respir. Dis. 129: 948–951

Morgan, K. G., Bryant, S. H. (1977) The mechanism of action of dantrolene sodium. J. Pharmacol. Exp. Ther. 201: 138–147

Moxham, J., Miller, J., Wiles, C. M., Morris, A., Green, M. (1985) Effect of aminophylline on the human diaphragm. Thorax 40: 288–292

Ortiz, J. L., Cortijo, J., Morcillo, E. J., Sanz, C., Perpiñá, M., Esplugues, J. (1988) The spasmogenic effect of caffeine in trachealis isolated from control and actively sensitized guinea-pigs. Eur. J. Pharmacol. 158: 243–249

Ortiz, J. L., Cortijo, J., Sanz, C., De Diego, A., Esplugues , J., Morcillo, E. (1991) Cooling-induced contraction of trachea isolated from normal and sensitized guinea-pigs. Naunyn Schmiedebergs Arch. Pharmacol. 343: 418–426

Perpiñá, M., Cortijo, J., Sanz, C., Esplugues, J., Morcillo, E. J. (1987) Active sensitization discriminates between groups of calcium antagonists in lung parenchymal strips. Bull. Eur. Physiopathol. Respir. 23: 255–260

Perpiñá, M., Cortijo, J., Fornás, E., Palau, M., Ortiz, J. L., Morcillo, E. J. (1991) Hyperreactivity and ^{45}Ca movements in sensitized guinea pig tracheal muscle. Eur. Respir. J. 4: 450–455

Persson, C. G. A., Andersson, K. E., Kjellin, G. (1986) Effects of enprofylline and theophylline may show the role of adenosine. Life Sci. 38: 1057–1072

Prosdocini, M., Bianchi, C. P. (1981) Effect of adenosine on oxygen uptake and electrolyte content of frog muscle. J. Pharmacol. Exp. Ther. 218: 87–96

Putney, J. W., Bianchi, C. P. (1974) Site of action of dantrolene in frog sartorius muscle. J. Pharmacol. Exp. Ther. 189: 202–209

Rall, T. W. (1990) Drugs used in the treatment of asthma. The methylxanthines, cromolyn sodium and other agents. In: Rall, T. W., Nies, A. S., Taylor, P. (eds) The Pharmacological Basis of Therapeutics. 8th edn., Pergamon Press. New York, pp 618–637

Saadeh, G. M., Ayash, R. E., Saadeh, F. M., Nassar, C. (1985) Effect of aminophylline on calcium transport across the rat diaphragm. Gen. Pharmacol. 16: 541–543

Salata, J. J., Jalife, J. (1982) Effects of dantrolene sodium on the electrophysiological properties of canine cardiac Purkinje fibers. J. Pharmacol. Exp. Ther. 220: 157–166

Sherman, M. S., Aldrich, T. K., Chaudhry, I., Nagashima, H. (1988) The effect of digoxin on contractility and fatigue of isolated guinea-pig and rat hemidiaphragms. Am. Rev. Respir. Dis. 138: 1180–1184

Small, R. C., Boyle, J. P., Cortijo, J., Curtis-Prior, P. B., Davies, J. M., Foster, R. W., Hofer, P. (1988) The relaxant and spasmogenic effects of some xanthine derivatives acting on guinea-pig isolated trachealis muscle. Br. J. Pharmacol. 94: 1091–1100

Supinski, G. S., Deal, E. C., Kelsen, S. G. (1984) The effects of caffeine and theophylline on diaphragm contractility. Am. Rev. Respir. Dis. 130: 429–433

Supinski, G. S., Deal, E. C., Kelsen, S. G. (1986) Comparative effects of theophylline and adenosine on respiratory skeletal and smooth muscle. Am. Rev. Respir. Dis. 133: 809–813

Varagic, V. M., Kentera, D. (1978) Interactions of calcium, dibutyryl cyclic AMP, isoprenaline and aminophylline on the isometric contraction of the isolated hemidiaphragm of the rat. Naunyn Schmiedebergs Arch. Pharmacol. 303: 47–53

Viirès, N., Aubier, M., Murciano, D., Marty, C., Pariente, R. (1986) Effects of theophylline on isolated diaphragmatic fibers. A model for pharmacologic studies on diaphragmatic contractility. Am. Rev. Respir. Dis. 133: 1060–1064

Viirès, N., Murciano, D., Seta, J. P., Durenil, B., Pariente, R., Aubier, M. (1988) Effect of Ca^{2+} withdrawal on diaphragmatic fiber tension generation., J. Appl. Physiol. 64: 26–30

Weinberger, M., Riegelman, S. (1974) Rational use of theophylline for bronchodilatation. N. Engl. J. Med. 291: 151–153

Wittmann, T. A., Kelsen, S. G. (1982) The effect of caffeine on diaphragmatic muscle force in normal hamsters. Am. Rev. Respir. Dis. 126: 499–504