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European Journal of Pharmacology 302 (1996) 79–88

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The effects of pharmacological modulation of K_{ATP} on the guinea-pig isolated diaphragm

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Received 21 September 1995; revised 14 December 1995; accepted 19 December 1995

Abstract

The purpose of the present study was to investigate the functional consequences of K_{ATP} modulation in the normal and the metabolically inhibited guinea-pig isolated diaphragm using the K^+ channel openers cromakalim, pinacidil, RP49356 (*N*-methyl-2-(3-pyridil)-tetrahydrothiopyran-2-carbothiamide-1-oxide) and ZM260384 (2-(2,2-bis(difluoromethyl)-6-nitro-3,4-dihydro-2H-1,4-benzoxazine-4-yl)pyridine-*N*-oxide) and the K^+ channel inhibitors glibenclamide, phentolamine and ciclazindol. All K^+ channel openers accelerated the decline in function induced by intermittent tetanic contractions following metabolic inhibition and delayed the development of contracture. Cromakalim also improved the recovery of twitch tension following 10 min intermittent tetanic stimulation in the hypoxic guinea-pig diaphragm preparation. Of the K^+ channel inhibitors tested, only ciclazindol, at the highest concentration tested (10 μM), significantly delayed the decline in tetanic tension following metabolic inhibition in the guinea-pig isolated diaphragm. None of the inhibitors significantly accelerated the development of contracture. All inhibitors however, antagonised the actions of the K^+ channel opener, cromakalim. The results indicate that opening of K_{ATP} can accelerate the decline in function following metabolic inhibition in the guinea-pig isolated diaphragm. In the absence of K^+ channel openers however, K_{ATP} does not appear to contribute to this decline under the conditions of the present study.

Keywords: K^+ channel opener; K^+ channel inhibitor; K^+ channel, ATP-sensitive; Adenosine; Skeletal muscle; Diaphragm, guinea-pig

1. Introduction

ATP-sensitive K^+ channels (K_{ATP}) have been demonstrated to exist in the sarcolemmal membranes of amphibian and mammalian skeletal muscle (Spruce et al., 1985; Burton et al., 1988). The aim of the present study was to investigate the functional consequences of K_{ATP} modulation in the normoxic and the metabolically inhibited guinea-pig isolated diaphragm.

This study employed the K^+ channel openers cromakalim, pinacidil and RP49356, agents which have previously been shown to open K_{ATP} in skeletal muscle (Weik and Neumcke, 1990). In addition, it was planned to study the effects of the structurally novel fluoroalkyl benzoxazine pyridine-*N*-oxide K^+ channel opener, ZM260384 (2-(2,2-bis(difluoromethyl)-6-nitro-3,4-dihydro-2*H*-1,4-benzoxazine-4-yl)pyridine-*N*-oxide, compound 4 in Russell et al., 1993). It was also planned to study the effects of the adenosine A_1 receptor agonists, *R*(−)-N^6-(2-phenylisopropyl)adenosine (R-PIA), cyclopentyladenosine (CPA) and cyclohexyladenosine (CHA), since in cardiac muscle at least, the adenosine A_1 receptor is coupled to K_{ATP} via a pertussis toxin sensitive G_i protein (Böhm et al., 1986; Kirsch et al., 1990). This study also employed the K_{ATP} inhibitors glibenclamide, phentolamine and ciclazindol. With the exception of glibenclamide (Woll et al., 1989; Quasthoff et al., 1990; Vivaudou et al., 1991; Allard and Lazdunski, 1993; Hussain et al., 1994), these agents have not yet been shown to inhibit skeletal muscle K_{ATP}. Nevertheless, data are available which suggest that these agents are likely to share the ability to inhibit skeletal muscle K_{ATP}. Thus, phentolamine has been shown to inhibit K_{ATP}

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Elsevier Science B.V.
SSDI 0014-2999(95)00872-1

in pancreatic ß-cells (Plant and Henquin, 1990) and ventricular myocytes (Wilde et al., 1994). Phentolamine and ciclazindiol have been shown to inhibit a K^+ channel opener-activated, ATP-sensitive whole cell current in vascular smooth muscle cells (Noack et al., 1992; Ibbotson et al., 1993). Ciclazindol has also been shown capable of antagonising the K^+ channel opener-induced shortening of action potential duration in the guinea-pig isolated papillary muscle (Smith et al., 1993).

A preliminary account of these findings has appeared (Wickenden and Prior, 1994; Wickenden et al., 1994).

2. Materials and methods

Male Dunkin-Hartley guinea-pigs (300–400 g) were killed by cervical dislocation and the right hemi-diaphragm removed. Hemi-diaphragms were spread out on a flat surface in oxygenated Krebs' bicarbonate buffer of the following composition (mM): NaCl, 120; $NaHCO_3$, 25; glucose, 11.2; KH_2PO_4, 1.2; $MgSO_4 \cdot 7H_2O$, 1.2; KCl, 4.7; $CaCl_2$, 2.5; ethylenediaminetetra-acetic acid disodium salt, 0.026. Tissues were cleaned of adhering tissue and two muscle strips (approximately 5–10 mm wide) were cut from the costal portion of the hemi-diaphragm. The diaphragm strips were firmly attached to the lower of two ring electrodes with cotton thread tied around a piece of rib. The preparation was then placed in a 4 ml organ bath containing Krebs' bicarbonate buffer at 37°C and continually gassed with 95% O_2-5% CO_2. The upper end of the preparation was attached to a Dynamometer UF1 isometric force transducer with cotton thread, such that the diaphragm muscle was fixed between ring electrodes situated above and below the tissue. The initial resting tension was set to 5 g (optimal for tension development – data not shown) and tissues were allowed to equilibrate for a period of 1 h, during which the bathing solution was changed every 20 min.

Following the equilibration period, tissues were stimulated to twitch with square wave pulses of 0.5 ms duration at a supramaximal voltage and a frequency of 0.1 Hz. High stimulation voltages ensured direct muscle stimulation. When twitch force was constant (typically 10–15 min), drug(s) or appropriate vehicle was added. Tissues were stimulated for a further 30 min. Twitch tension (g) was measured immediately prior to the addition of drug or vehicle and the effect of drug or vehicle is expressed as percentage inhibition of the initial (pre-drug) twitch tension following 30 min incubation.

2.1. Effects of K^+ channel modulators on twitch tension, the rate of tension decline following metabolic inhibition and the development of contracture

Following the 30 min compound incubation period, stimulation was stopped, the baths were bubbled with 95% N_2-5% CO_2 and the Krebs' bicarbonate buffer changed to a modified Krebs' bicarbonate buffer in which glucose was replaced with an equimolar (11.2 mM) amount of 2-deoxy-D-glucose (grade II, Sigma). Bath P_{O_2} was not measured routinely. However, preliminary determinations revealed that bath P_{O_2} fell from in excess of 500 mm Hg when baths were bubbled with 95% O_2-5% CO_2 to 30–35 mmHg when bubbled with 95% N_2-5% CO_2. Test compound(s) or vehicle was re-administered and the tissues were stimulated to produce tetanic contractions with 330 ms trains of pulses of 0.5 ms duration, supramaximal voltage at 30 Hz, once per second. Every effort was made to ensure the change over time was as short as possible (typically less than 3 min). Stimulation was continued until active force production had declined to zero and a contracture had fully developed (i.e. until basal tension had peaked and begun to fall again).

Tetanic tension (g) was measured at time 0 (i.e. the onset of intermittent tetanic stimulation), and every 30 s for the first 5 min of intermittent tetanic stimulation. Tetanic tension is expressed as a percentage of the initial, pre-drug twitch tension. Time to half maximal decline in tension ($t_{1/2}$) was calculated as the time (min) for tetanic tension to decline to 50% of the tetanic tension determined at time 0. The time (min) for basal tension to increase to a level equivalent to 27.5% of the initial, pre-drug twitch tension, was calculated and is expressed as time to contracture. A contracture equivalent to 27.5% of the initial twitch was a point uniformly reached by all metabolically inhibited preparations and was on the linear rising phase of the contracture response.

2.2. The effect of cromakalim on the recovery of twitch tension following hypoxia

Following the 30 min compound incubation period, stimulation was stopped and the baths were bubbled with 95% N_2-5% CO_2 to create local hypoxia. Cromakalim or vehicle was re-administered and the tissues were stimulated to produce tetanic contractions as described above, for 10 min.

Contractile activity during the hypoxic period was normalised to the pre-drug normoxic twitch contraction and expressed as the area under curve (AUC) ratio according to the following equation: AUC ratio = (area under tension-time curve for 10 min hypoxic tetanic contractions)/(area under tension-time curve for 5 min predrug normoxic twitch contractions).

Following 10 min hypoxic tetanic stimulation, preparations were washed with fresh, oxygenated buffer, drug or vehicle re-administered and the baths bubbled once more with 95% O_2-5% CO_2. The recovery of twitch function was assessed by stimulating the preparations to twitch at a frequency of 0.01 Hz for a further 60 min. Preparations were washed with fresh Krebs' buffer every 20 min and drug or vehicle re-administered after each wash. Twitch

tension during the recovery period was measured and is expressed as a percentage of the initial, pre-drug twitch tension.

Time-matched control experiments were also conducted to monitor any alteration in twitch function over the duration of the experiment. In these experiments, tissues were stimulated to twitch at 0.01 Hz under normoxic conditions throughout.

Only one concentration of drug or a vehicle control were tested in each preparation. All studies employed a 30 min drug equilibration period. All results are expressed as means ± S.E.M for $n \geq 3$ preparations. Stastistical significance was assessed using a *t*-test for unpaired data.

Cromakalim (6-cyano-3,4-dihydro-2,2-dimethyl-trans-4-(2-oxo-1-pyrollidyl)-2*H*-benzo(b)pyran-3-ol), pinacidil (*N*″-cyano-*N*-4-pyridyl-*N*′-1,2,2-trimethylpropylguanidine monohydrate) and ZM260384 (2-(2,2-bis(difluoromethyl)-6-nitro-3,4-dihydro-2*H*-1,4-benzoxazine-4-yl)pyridine-*N*-oxide) were synthesied in the laboratories of the Department of Medicinal Chemistry, Zeneca Pharmaceutical Group, Wilmington, USA and Cardiovascular and Metabolism Research Department, Zeneca Pharmaceuticals, Mereside, Alderley Park, UK. RP49356 (*N*-methyl-2-(3-pyridil)-tetrahydrothiopyran-2-carbothiamide-1-oxide) was a gift from Dr. T. Brown, Rhone Poulenc, Dagenham, Essex, UK. These compounds were made up as 0.01 M stock solutions in 50% dimethylsulphoxide (DMSO)-50% polyethylene glycol 200 (PEG200). Serial 10-fold dilutions were made using the same vehicle. Appropriate dilutions were used such that the final bath concentration of vehicle was always 0.5%DMSO-0.5% PEG. Stock solutions were stored at −20°C.

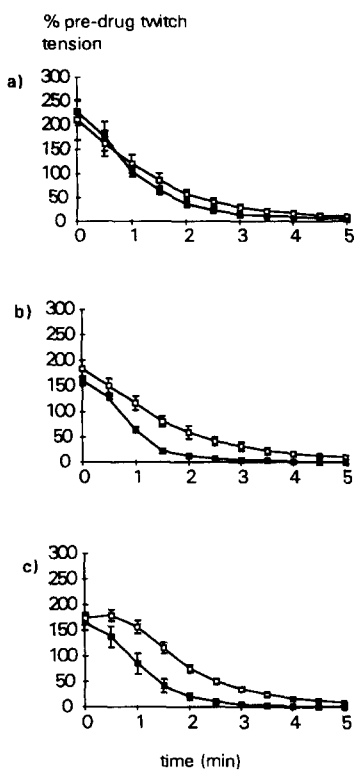

Fig. 1. The effect of cromakalim on the decline of tetanic tension following metabolic inhibition in the guinea-pig isolated diaphragm. The decline in tetanic tension following metabolic inhibition was measured in the presence of (a) 10 μM, (b) 30 μM (c) 100 μM cromakalim (■) and vehicle (□). Symbols represent the means of 4–6 experiments. Vertical bars represent the S.E.M.

Nitrendipine and glibenclamide were purchased from the Sigma Chemical Company, Poole, UK and were made up as 0.01 M stock solutions in DMSO. Serial 10-fold dilutions were made using the same vehicle. Appropriate dilutions were used such that the final bath concentration of vehicle was always 1%. Solutions were made up fresh every day.

R(−)-N^6-(2-Phenylisopropyl)adenosine (R-PIA), cyclopentyladenosine (CPA) and cyclohexyladenosine (CHA) were purchased from Research Biochemicals International, St. Albans, UK. These compounds were made up as 0.01 M solutions in DMSO.

Phentolamine HCl was purchased from the Sigma Chemical Company and ciclazindol was a gift from Wyeth Laboratories, Taplow Maidenhead, UK. These compounds were made up as 0.01 M stock solutions in distilled water and made up fresh each day.

3. Results

3.1. Effects of K^+ channel modulators on twitch tension and tetanic tension

Initial twitch tension was variable between groups. Mean initial twitch tension was 14.5 g ($n = 27$ groups; range 3.4 g–25.1 g). Tension variability most probably represents differences in the size of preparations and the alignment of muscle fibres with the force transducer. Since there appeared to be no obvious relationship between initial tension and any experimental parameter such as rate of tension decline or contracture, no preparations were excluded from the study on the basis of initial tension. Twitch tension declined during the first 10–15 min stimulation. Following this initial decline however, twitch tension was relatively stable. Intermittent tetanic stimulation, following metabolic inhibition generated initial tetanic tensions typically 120–220% of those generated by twitch stimulation (Fig. 1).

Neither the K^+ channel openers, cromakalim (10–100 μM), pinacidil (10–100 μM), RP49356 (10–100 μM) and ZM260384 (0.03–1.0 μM), nor the Ca^{2+} antagonist, nitrendipine (10 μM) inhibited twitch tension or initial tetanic tension in the guinea-pig isolated diaphragm following 30 min incubation (data not shown). At the highest concentration tested however, ZM260384 (10 μM) significantly inhibited twitch contraction (inhibition of twitch was 43.2 ± 1.0% ($n = 4$) and 16.7 ± 1.8% ($n = 12$) in the presence of ZM260384 (10 μM) and vehicle, respectively;

$P < 0.05$, 2-tailed unpaired t-test) and initial tetanic tension (initial tetanic tension was 76.0 ± 12.0% ($n = 4$) and 170.5 ± 20.3% ($n = 12$) of pre-drug twitch tension in the presence of ZM260384 (10 μM) and vehicle, respectively; $P < 0.05$, 2-tailed unpaired t-test).

Glibenclamide (0.01–1 μM) had no effect on twitch tension in the guinea-pig isolated diaphragm. The highest concentration of glibenclamide tested (10 μM) however, significantly inhibited twitch (inhibition of twitch was 38.9 ± 4.4% ($n = 5$) and 27.0 ± 2.1% ($n = 12$) in the presence of glibenclamide (10 μM) and vehicle, respectively; $P < 0.05$, 2-tailed unpaired t-test). Glibenclamide similarly inhibited initial tetanic tension only at the highest concentration tested (initial tetanic tension was 107.9 ± 8.0% ($n = 5$) and 130.0 ± 5.2% ($n = 12$) of pre-drug twitch tension in the presence of glibenclamide (10 μM) and vehicle, respectively; $P < 0.05$, 2-tailed unpaired t-test).

Phentolamine (10–30 μM) was also without significant effect on twitch tension. However, twitch tension in vehicle-treated tissues appeared to decline to a greater extent than in phentolamine (100 μM)-treated tissues (16.8 ± 1.9% compared to 1.4 ± 3.0%, Table 1; $P < 0.05$, 2-tailed unpaired t-test). A similar trend toward increased tension was also seen for the effects of phentolamine (10–100 μM) on the initial tetanic tension. However, these changes did not prove statistically significant.

Table 1
The effect of K^+ channel openers, a Ca^{2+} channel antagonist and K^+ channel inhibitors on the time to half maximal decline in tetanic tension ($t_{1/2}$, min) in the guinea-pig isolated diaphragm

Compound	$t_{1/2}$ (min)			P
	Concurrent vehicle control (n)	Drug-treated (n)	% Vehicle	
Cromakalim				
10 μM	1.19 ± 0.18 (5)	0.92 ± 0.14 (5)	77.3 ± 11.8	ns
30 μM	1.34 ± 0.07 (4)	0.88 ± 0.10 (4)	65.9 ± 5.3	a
100 μM	1.89 ± 0.14 (6)	1.01 ± 0.12 (6)	53.7 ± 7.9	b
Pinacidil				
10 μM	1.54 ± 0.08 (5)	1.28 ± 0.10 (5)	83.5 ± 7.0	ns
30 μM	1.17 ± 0.06 (3)	1.09 ± 0.13 (4)	92.9 ± 11.0	ns
100 μM	1.29 ± 0.17 (4)	0.86 ± 0.04 (4)	67.0 ± 2.9	a
RP49356				
10 μM	1.58 ± 0.08 (4)	1.80 ± 0.04 (5)	114 ± 2.6	ns
30 μM	1.52 ± 0.11 (4)	1.43 ± 0.07 (6)	94 ± 4.5	ns
100 μM	1.70 ± 0.08 (4)	1.31 ± 0.03 (5)	75.7 ± 1.8	a
ZM260384				
0.03 μM	0.97 ± 0.07 (12)	1.08 ± 0.05 (4)	110.7 ± 4.9	ns
0.1 μM		0.96 ± 0.11 (8)	98.5 ± 11.2	ns
0.3 μM		0.76 ± 0.04 (8)	78.5 ± 4.3	a
1 μM		0.61 ± 0.05 (8)	63.1 ± 5.7	b
10 μM		0.40 ± 0.04 (4)	41.2 ± 4.2	b
Nitrendipine				
1 μM	1.24 ± 0.02 (3)	1.22 ± 0.08 (4)	98.0 ± 6.2	ns
10 μM		1.17 ± 0.11 (4)	94.5 ± 8.8	ns
Glibenclamide				
0.01 μM	1.52 ± 0.05 (6)	1.54 ± 0.04 (6)	102.0 ± 3.0	ns
0.1 μM	1.28 ± 0.13 (6)	1.38 ± 0.11 (6)	108.3 ± 8.6	ns
1 μM		1.46 ± 0.08 (6)	114.2 ± 6.4	ns
10 μM		1.49 ± 0.05 (5)	116.2 ± 4.0	ns
Phentolamine				
10 μM	0.82 ± 0.03 (4)	0.86 ± 0.05 (4)	104.7 ± 5.8	ns
30 μM		0.87 ± 0.02 (6)	106.1 ± 2.1	ns
100 μM		0.88 ± 0.03 (6)	107.9 ± 3.3	ns
Ciclazindol				
0.1 μM	0.94 ± 0.04 (15)	0.95 ± 0.08 (6)	101.1 ± 8.1	ns
1 μM		1.01 ± 0.05 (6)	108.2 ± 5.1	ns
10 μM		1.23 ± 0.05 (6)	131.2 ± 5.9	b

Figures are means ± S.E.M. Figures in parentheses indicate number of preparations. [ns] $P > 0.05$, [a] $P < 0.05$, [b] $P < 0.01$ 1-tailed unpaired t-test vs. vehicle control. In the absence of a number, the concurrent vehicle control $t_{1/2}$ is the last value quoted.

Ciclazindol (0.1–10 μM) had no significant effect on twitch tension or initial tetanic tension in the guinea-pig isolated diaphragm.

3.2. Effects of K^+ channel modulators on the rate of tension decline following metabolic inhibition and the development of contracture

Tetanic tension rapidly declined to zero over the first 5 min of stimulation following metabolic inhibition (Fig. 1). The time for half maximal decline in tetanic tension ($t_{1/2}$) was variable from experiment to experiment (see column headed 'concurrent vehicle control, $t_{1/2}$ min', Table 1). The reasons for this variability were not entirely clear. Every effort was made to minimise the intra-group variability by the use of concurrent vehicle controls, using tissues from sex-, age- and weight-matched animals.

The K^+ channel openers cromakalim (Fig. 1), pinacidil, RP49356 and ZM260384 promoted the decline in tension induced by intermittent stimulation and metabolic inihibition. When compared to concurrent vehicle control values, cromakalim (30–100 μM), pinacidil (100 μM), RP49356

Table 2
The effect of K^+ channel openers, a Ca^{2+} channel antagonist, adenosine A_1 receptor agonists and K^+ channel inhibitors on the time to contracture (time to contracture, min) in the guinea-pig isolated diaphragm

Compound	Time to contracture (min)			P
	Concurrent vehicle control (n)	Drug-treated (n)	% Vehicle	
Cromakalim				
10 μM	23.2 ± 0.6 (5)	27.6 ± 3.0 (4)	119.1 ± 13.0	
30 μM	26.3 ± 2.5 (4)	38.8 ± 0.8 (4)	147.5 ± 3.0	a
100 μM	27.5 ± 3.0 (6)	61.2 ± 3.6 (6)	222.7 ± 13.1	b
Pinacidil				
10 μM	19.2 ± 1.5 (5)	26.0 ± 2.2 (5)	135.4 ± 11.4	a
30 μM	20.1 ± 2.2 (3)	32.1 ± 2.6 (4)	159.6 ± 12.7	a
100 μM	18.9 ± 2.6 (4)	32.7 ± 2.6 (4)	173.0 ± 13.9	b
RP49356				
10 μM	16.3 ± 1.0 (4)	22.0 ± 1.9 (5)	135.0 ± 11.6	a
30 μM	18.0 ± 0.7 (4)	27.0 ± 1.4 (6)	150.0 ± 8.1	b
100 μM	17.2 ± 0.6 (4)	32.8 ± 0.8 (5)	190.4 ± 5.0	b
ZM260384				
0.03 μM	18.5 ± 2.8 (12)	23.0 ± 2.2 (4)	124.6 ± 11.9	ns
0.1 μM		24.5 ± 2.9 (8)	132.7 ± 15.8	ns
0.3 μM		28.0 ± 1.8 (8)	151.7 ± 9.7	b
1 μM		30.8 ± 2.7 (8)	166.6 ± 14.4	b
10 μM		42.4 ± 5.8 (4)	229.6 ± 31.2	b
Nitrendipine				
1 μM	20.7 ± 3.2 (3)	22.6 ± 2.7 (4)	107.9 ± 12.7	ns
10 μM		23.1 ± 3.5 (4)	110.6 ± 16.8	ns
CPA (10 μM)	23.3 ± 1.5 (7)	22.8 ± 1.9 (4)	97.6 ± 8.3	ns
CHA (10 μM)	22.1 ± 2.0 (4)	19.3 ± 2.6 (4)	87.1 ± 11.6	ns
R-PIA (1 μM)	23.3 ± 1.5 (7)	22.6 ± 2.2 (4)	97.1 ± 9.4	ns
Glibenclamide				
0.01 μM	22.7 ± 1.2 (6)	22.5 ± 2.0 (6)	99.4 ± 8.7	ns
0.1 μM	22.9 ± 1.4 (6)	20.3 ± 1.0 (6)	88.7 ± 4.4	ns
1 μM		22.8 ± 0.5 (6)	99.6 ± 2.0	ns
10 μM		24.4 ± 1.8 (5)	106.7 ± 7.7	ns
Phentolamine				
10 μM	17.1 ± 0.2 (4)	17.6 ± 1.5 (4)	102.7 ± 8.6	ns
30 μM		17.6 ± 1.4 (6)	102.9 ± 8.4	ns
100 μM		21.7 ± 1.6 (6)	127.1 ± 9.6	a
Ciclazindol				
0.1 μM	20.3 ± 0.7 (18)	23.3 ± 1.1 (6)	114.7 ± 5.2	a
1 μM		19.5 ± 1.2 (6)	96.0 ± 5.8	ns
10 μM		23.5 ± 0.6 (6)	116.0 ± 3.1	b

Figures are means ± S.E.M. Figures in parentheses indicate number of preparations. CPA, cyclopentyladenosine; CHA, cyclohexyladenosine; R-PIA $R(-)$-N^6-(2-phenylisopropyl)adenosine. ns $P > 0.05$, a $P < 0.05$, b $P < 0.01$ 1-tailed unpaired t-test vs. vehicle control. In the absence of a number, the concurrent vehicle control time to contracture is the last value quoted.

(100 μM) and ZM260384 (0.3–10 μM) significantly reduced the $t_{1/2}$ (Table 1). ZM260384 appeared considerably more potent than cromakalim. Unlike the K^+ channel openers, the Ca^{2+} channel antagonist nitrendipine (1–10 μM) had no effect on $t_{1/2}$ (Table 1).

All inhibitors showed a trend towards increasing the $t_{1/2}$. However, only ciclazindol (10 μM) significantly delayed $t_{1/2}$ when compared to $t_{1/2}$ in concurrent vehicle control experiments (Table 1).

Continued stimulation in metabolically inhibited preparations led to the development of a contracture. Cromakalim (30–100 μM), pinacidil (10–100 μM), RP49356 (10–100 μM) and ZM260384 (0.3–10 μM) all significantly delayed time to contracture (Table 2). ZM260384 appeared 30–100-fold more potent than cromakalim at delaying time to contracture in the metabolically inhibited guinea-pig diaphragm. Nitrendipine (1–10 μM) had no effect on time to contracture following metabolic inhibition (Table 2).

The adenosine A_1 receptor agonists, CPA (10 μM), CHA (10 μM) and R-PIA (1 μM) did not delay time to contracture (Table 2).

Glibenclamide (0.01–10 μM) and phentolamine (10–30 μM) had no effect on time to contracture (Table 2). The highest concentration of phentolamine (100 μM) produced a 27% delay in time to contracture (Table 2). Ciclazindol (0.1–10 μM) also tended to delay time to contracture, however these effects were small and not concentration-related (Table 2).

3.3. The interaction between K^+ channel inhibitors and openers

Glibenclamide (0.01–1 μM), phentolamine (10–100 μM) and ciclazindol (0.1–10 μM) all inhibited the cromakalim (100 μM)-induced reduction in $t_{1/2}$ in a concentration-related manner (Fig. 2). However, the inhibition of the cromakalim-induced reduction in $t_{1/2}$ by ciclazindol (0.1–10 μM) appeared incomplete. Nevertheless, in each case, the $t_{1/2}$ in the presence of cromakalim (100 μM) alone was significantly less than the $t_{1/2}$ in the presence of cromakalim (100 μM) and the highest concentration of inhibitor.

Glibenclamide (0.01–1 μM), phentolamine (10–100 μM) and ciclazindol (0.1–10 μM) also inhibited the cromakalim (100 μM)-induced delay in time to contracture in a concentration-related manner (Fig. 2). The inhibition of the cromakalim-induced delay in time to contracture by both phentolamine (10–100 μM) and ciclazindol (0.1–10 μM) appeared incomplete. This may reflect the tendency of both these compounds to delay time to contracture per se (see Table 2). Time to contracture in the presence of cromakalim (100 μM) alone however, was significantly greater than the time to contracture in the presence of cromakalim (100 μM) and the highest concentration of each inhibitor.

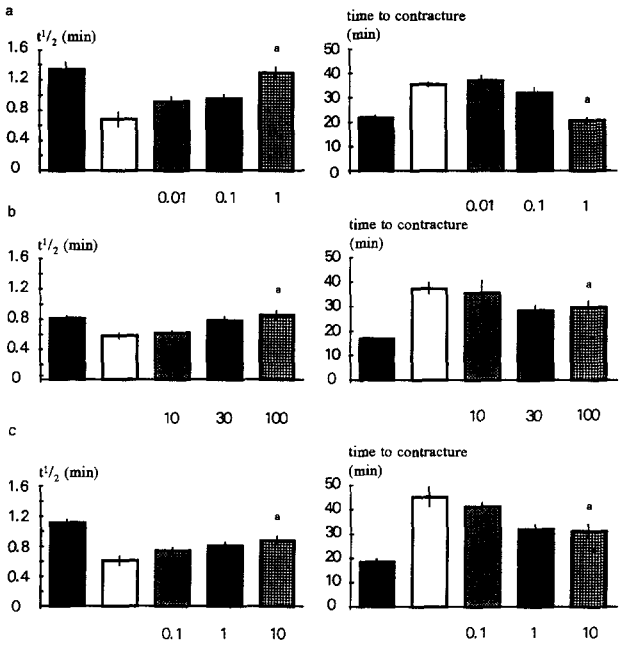

Fig. 2. The effect of (a) glibenclamide, (b) phentolamine and (c) ciclazindol on cromakalim (100 μM)-induced reduction in $t_{1/2}$ (left-hand panel) and on cromakalim (100 μM)-induced delay in time to contracture (right-hand panel) in the metabolically inhibited guinea-pig diaphragm. Solid bars indicate values measured in the presence of vehicle alone. Open bars represent values measured in the presence of cromakalim (100 μM). Shaded bars represent values measured in the presence of cromakalim (100 μM) and K^+ channel inhibitor at the concentration shown. Bars represent the means of > 4 experiments. Vertical lines represent the S.E.M. [a] $P < 0.05$ cromakalim alone vs. cromakalim + inhibitor (1-tailed unpaired t-test).

Glibenclamide (1 μM) also antagonised ZM260384-induced inhibition of twitch tension in the normoxic guinea-pig isolated diaphragm (Fig. 3).

3.4. The effect of cromakalim on the recovery of twitch tension following hypoxia

Pre-drug twitch tension was 12.7 ± 0.9 g ($n = 6$) in the vehicle group and 11.7 ± 1.3 g ($n = 10$) in the cromakalim

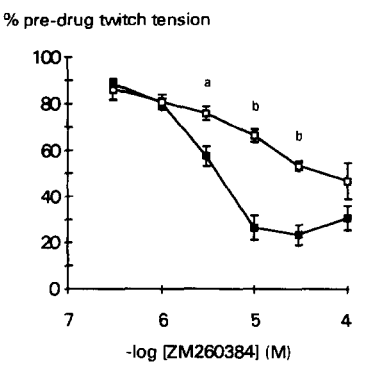

Fig. 3. The effect of ZM260384 on twitch tension in the normoxic guinea-pig isolated diaphragm in the presence of vehicle (■) or glibenclamide (1 μM; □). Symbols represent the mean of 4 experiments. Vertical bars represent the S.E.M. [a] $P < 0.05$, [b] $P < 0.01$, 2-tailed, unpaired t-test.

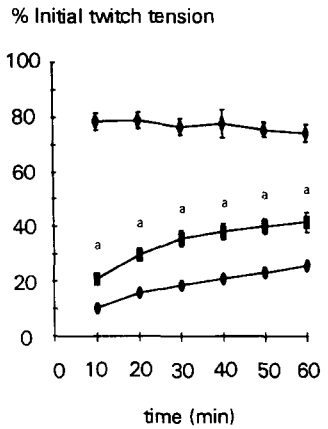

Fig. 4. The effect of cromakalim on the recovery of twitch function following 10 min hypoxia in the guinea-pig isolated diaphragm. The recovery of twitch tension was measured following 10 min hypoxia in the presence of cromakalim (100 μM, ■) and vehicle (●) in the guinea-pig isolated diaphragm. ♦ denotes time-matched normoxic control. Symbols represent the mean of 4–10 experiments. Vertical bars represent the S.E.M. [a] $P < 0.01$ 1-tailed unpaired t-test cromakalim vs. vehicle.

group. These values were not significantly different. Cromakalim (100 μM) had no effect on twitch tension (data not shown) as described previously. The AUC ratio was 1.51 ± 1.0 ($n = 6$) and 1.17 ± 0.13 ($n = 10$) in vehicle- and cromakalim (100 μM)-treated tissues, respectively. The AUC ratio was significantly smaller (1-tailed unpaired t-test) in cromakalim-treated tissues, indicating that cromakalim (100 μM) reduced function during the hypoxic period.

On reoxygenation, twitch tension recovered slowly and incompletely over 60 min. Twitch tension was significantly greater in the cromakalim-treated group at all time points (Fig. 4). In time-matched control experiments without hypoxia, twitch tension declined to $74.3 \pm 3.1\%$ ($n = 4$) of initial twitch tension.

4. Discussion

The aim of the present study was to investigate the functional consequences of K_{ATP} modulation in normoxic and metabolically inhibited guinea-pig isolated diaphragm.

4.1. Effects of K^+ channel openers

At concentrations that were without effect on twitch function in normoxic muscle, the structurally diverse K^+ channel openers cromakalim, pinacidil, RP49356 and ZM260384 accelerated the decline in function following metabolic inhibition (hypoxia and complete metabolic inhibition) in the guinea-pig isolated diaphragm. This K^+ channel opener-induced early contractile failure was associated with a delay in the development of contracture in the metabolically inhibited guinea-pig diaphragm. The effects of the K^+ channel openers were not shared by the L-type Ca^{2+} channel antagonist, nitrendipine, or by the adenosine A_1-receptor agonists, R-PIA, CPA and CHA. The K^+ channel opener, cromakalim, improved post-hypoxic recovery of function in the guinea-pig isolated diaphragm. The present study confirms the findings of Wesselcouch et al. (1993), who reported that the K^+ channel openers, cromakalim, pinacidil and P-1075, accelerated the decline in function and improved functional recovery in the hypoxic rat extensor digitorum longus muscles, and of Gurden and Hart (1995) who reported that cromakalim accelerated the decline in function in the isolated, hypoxic rabbit tenuissimus muscle.

4.2. The role of K_{ATP} in the action of the K^+ channel openers

The finding that the structurally diverse K_{ATP} inhibitors glibenclamide, phentolamine and ciclazindol antagonised the ability of cromakalim to promote the decline in function and to delay the development of contracture, in a concentration-dependent manner, strongly supports a role for K_{ATP} in the actions of cromakalim in the metabolically inhibited guinea-pig diaphragm. Although phentolamine and ciclazindol are also capable of inhibiting voltage-gated K^+ channels (Ibbotson et al., 1993), this property is not shared by glibenclamide and as such this property is unlikely to underlie the findings of the present study. Although the contribution of K_{ATP} to the effects of the other K^+ channel openers was not tested in the present study, the similarity in the profile of activity of pinacidil, RP49356 and ZM260384 to that of cromakalim suggests that K_{ATP} also plays a predominant role in the effects of these agents. The finding that unlike cromakalim, ZM260384 inhibited twitch tension in normoxic muscle raises the possibility that ZM260384, at high concentrations at least, may posses an additional property. However, this effect of ZM260384 was also antagonised by glibenclamide suggesting that inhibition of twitch is more likely to be a consequence of the ability of efficacious openers to open K_{ATP} in the presence of high levels of intracellular ATP.

The finding that glibenclamide was capable of antagonising the effects of cromakalim in skeletal muscle supports the findings of Benton and Haylett (1992), Wesselcouch et al. (1993) and Gurden and Hart (1995). Interestingly though, phentolamine failed to antagonise levcromakalim in the study of Benton and Haylett (1992). The reason for the difference between the findings of Benton and Haylett and the findings of the present study are not clear. It may relate to the short incubation period (approximately 5 min) used in the study of Benton and Haylett (1992), the fact that phentolamine was added after the effect of levcromakalim was established or to differences in the pharmacology of K_{ATP} in amphibian and mammalian skeletal muscle.

4.3. The basis of the selective action of K^+ channel openers on the metabolically inhibited diaphragm

The basis of the apparently selective inhibition of function in the metabolically inhibited diaphragm is not entirely clear from the present study. K^+ channel openers may reduce the K_i for K_{ATP} inhibition by ATP in skeletal muscle as has been described in cardiac muscle (Thuringer and Escande, 1989; Ripoll et al., 1990). This would have the effect of shifting the channel open probability vs. [ATP] relationship to the right, increasing both the threshold [ATP] at which channels first open and the channel open probability for a given [ATP]. In normal guinea-pig diaphragm, levels of ATP may be sufficiently high to ensure channels remain maximally inhibited, despite the presence of all but the highest concentrations of the most potent K^+ channel openers (e.g ZM260384). Under normal, physiological conditions therefore, K^+ channel openers would be unlikely to exert any functional effects. However, as the levels of ATP fall as a result of mechanical activity and metabolic inhibition, K_{ATP} would open earlier and to a greater extent in the presence of K^+ channel openers than in thier absence. K^+ channel openers could therefore be expected to exert functional effects under conditions of ATP depletion. Some support for this hypothesis has appeared recently. Hussain et al. (1994) reported that the ability of BRL38227 (levcromakalim) to activate K_{ATP} in mouse skeletal muscle fibres was reduced in the presence of high (> 1 mM) ATP. Such an explanation is also commonly used to rationalise the ischaemia-selective effects of K^+ channel openers in cardiac muscle (Ripoll et al., 1990).

4.4. The mechanism by which K^+ channel openers inhibit function in the metabolically inhibited diaphragm

K_{ATP} activation in skeletal muscle, under suitable conditions, clearly leads to inhibition of contractile activity. The basis of this inhibition probably involves a reduction in Ca^{2+} availability. The mechanism by which K_{ATP} opening limits Ca^{2+} availability is not clear. Simple hyperpolarisation-induced inhibition of Ca^{2+} influx through L-type Ca^{2+} channels is unlikely to underlie the effects of K^+ channel openers since the effects of K^+ channel openers were not shared by the Ca^{2+} antagonist, nitrendipine. A direct effect on the sarcoplasmic reticulum to inhibit Ca^{2+} release is also unlikely, since although electrically induced Ca^{2+} transients can be inhibited by cromakalim in metabolically compromised skeletal muscle fibres (Burton and Smith, 1994), caffeine-induced Ca^{2+} transients are unaffected by cromakalim under the same conditions (Briggs, Sturgess, BoSmith – personal communication). It seems therefore, that cromakalim must act to inhibit excitation-contraction coupling in some way. One potential mechanism by which K^+ channel openers could inhibit excitation-contraction coupling could be by promoting K^+ accumulation in the limited extracellular space provided by the t-tubule system, inducing localised membrane depolarisation and localised Na^+ channel inactivation. This would result in a reduction of the action potential amplitude and reduce the propagation of the action potential down the t-tubule system.

4.5. The mechanism by which K^+ channel openers protect against contracture and improve functional recovery

In the present study K^+ channel opener-induced early contractile failure was associated with a delay in the onset of contracture or improved functional recovery post-hypoxia. Since the most likely cause of contracture is a failure of cross bridge detachment due to ATP depletion (Koretsune and Marban, 1990), it seems that K^+ channel openers, by inducing early contractile failure, preserve ATP levels for longer during times of metabolic inhibition. In support of this contention, early contractile failure in the guinea-pig isolated peroneus longus has been shown to be associated with preservation of tissue ATP (Wickenden et al., 1995). Such an 'energy-sparing' effect is also the simplest explanation for the ability of cromakalim to improve functional recovery post-hypoxia in the guinea-pig isolated diaphragm.

4.6. The lack of effect of adenosine A_1 receptor agonists in the diaphragm

In cardiac muscle, adenosine activates K_{ATP} via an adenosine A_1 receptor linked to a pertussis toxin sensitive G_i protein (Böhm et al., 1986; Kirsch et al., 1990). More recently evidence has emerged to suggest that adenosine also activates K_{ATP} in skeletal muscle membrane patches (Barrett-Jolley et al., 1995), and it has been proposed that adenosine generated locally as a result of ATP consumption, may act as an endogenous K_{ATP} opener in skeletal muscle (Marshall et al., 1993). In the present study however, the adenosine A_1 receptor agonists R-PIA, CHA and CPA were without effect in the guinea-pig isolated diaphragm, at concentrations of agonists that were at least an order of magnitude greater than their respective pD_2 values in guinea-pig heart preparations (Ueeda et al., 1991) The reasons for this apparent discrepancy are unclear. Low adenosine A_1 receptor number, receptor down-regulation as a result of high endogenous adenosine levels, species and/or muscle differences cannot be discounted.

4.7. K^+ channel inhibitors

Neither glibenclamide, phentolamine nor ciclazindol had any consistent, concentration-related effects on twitch tension in the normoxic guinea-pig diaphragm preparation. Glibenclamide and phentolamine failed to significantly delay the decline in tetanic tension following metabolic inhibition in the guinea-pig diaphragm preparation. Ci-

clazindol delayed the decline in function only at the highest concentration tested. None of the K^+ channel inhibitors tested accelerated the onset of contracture in the metabolically inhibited guinea-pig diaphragm.

Similar findings have been reported recently by Wesselcouch et al. (1993), who reported that glibenclamide did not affect the loss of twitch force in the hypoxic isolated rat extensor digitorum longus preparation, by Light et al. (1994) who reported that glibenclamide did not affect the decline in tension in the normoxic, isolated frog sartorius preparation, and by Gurden and Hart (1995), who reported that glibenclamide was without effect on the decline in function in the isolated, hypoxic rabbit tenuissimus muscle.

4.8. Possible explanations for the lack of effect of K^+ channel inhibitors per se

The conditions of the present study are likely to induce a marked decline in intracellular ATP concentration. Clearly, the drop in intracellular ATP concentration is sufficient to induce contracture. Furthermore, other factors such as ADP, H^+ etc. (Davies, 1990; Vivaudou et al., 1991; Allard and Lazdunski, 1992) are known to influence channel activity in skeletal muscle. It seems highly likely therefore, that K_{ATP} channels will be activated under the conditions of the present study. The inability of K^+ channel inhibitors to modify muscle function in the present study should probably therefore be interpreted as either an inability of the K^+ channel inhibitors to inhibit K_{ATP} under the conditions of study or an inability of K_{ATP} opening to influence mechanical activity under the conditions of study, rather than a failure of K_{ATP} to open.

The inability of glibenclamide to inhibit K_{ATP} under conditions of severe metabolic stress has previously been reported by Findlay (1993), who concluded that glibenclamide was unable to inhibit the opening of K_{ATP} during the final stages of metabolic stress. Patch clamp electrophysiology studies are clearly required to determine whether the K^+ channel inhibitors retain their ability to inhibit K_{ATP} under the conditions of the present study. However, all agents antagonised the effects of cromakalim under identical conditions of metabolic inhibition. Furthermore, Castle and Haylett (1987) have demonstrated that glibenclamide is capable of inhibiting a component of K^+ efflux associated with complete metabolic exhaustion and contracture in the isolated frog sartorius muscle. Collectively, these findings suggest that the K^+ channel inhibitors retain some activity that is consistent with K_{ATP} inhibition under conditions of extreme metabolic inhibition.

The most likely explanation for the findings of the present study therefore is that K_{ATP} opens but that this opening is unable to modify function under the conditions of the present study. One of the mechanisms by which K_{ATP} opening could conceivably inhibit function is by promoting K^+ accumulation as discussed above. In vitro evidence suggests that the extent to which K^+ accumulation contributes to the decline in muscle force varies depending on the mode of stimulation. Thus, K^+ accumulation and the associated failure of action potential propagation is thought to be a major contributor to the loss of force that occurs during continuous high frequency stimulation (Lännergren and Westerblad, 1986, 1987) but this mechanism is not thought to contribute significantly to the decline in function seen on intermittent stimulation (Westerblad and Lännergren, 1986; Westerblad et al., 1990). Clearly, studies investigating the effect of K^+ channel inhibitors in skeletal muscle stimulated continually at high frequency will be of interest.

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