British Journal of Pharmacology (1997) 121, 1024–1030 

# The involvement of ATP-sensitive potassium channels in $\beta_2$-adrenoceptor agonist-induced vasodilatation on rat diaphragmatic microcirculation

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**1** The effects of glibenclamide (GLB), a blocker of ATP-sensitive potassium ($K_{ATP}$) channels, on diaphragmatic microcirculation in male Sprague-Dawley rats were assessed under basal conditions and after $\beta_2$-adrenoceptor-agonist stimulation. In addition, forskolin was used to bypass $\beta$-adrenoceptors and GTP-binding proteins (G-protein) to explore the possible mechanism of GLB effects. For comparison, the relationships between $K_{ATP}$ channel activity and cyclic GMP-mediated vasodilator responses to acetylcholine (ACh) and sodium nitroprusside (SNP) were also assessed.

**2** Male Sprague-Dawley rats were anaesthetized with urethane and mechanically ventilated. The left hemi-diaphragm of each rat was prepared and microvascular blood flow ($Q_{LDF}$) was recorded with laser-Doppler flowmetry during continuous superfusion with bicarbonate-buffered, prewarmed Ringer solution. The drugs were topically applied to the surface of the hemi-diaphragm.

**3** Salbutamol (0.32–32 $\mu$M), terbutaline (0.32 $\mu$M–0.32 $\mu$M) and forskolin (0.32–10 $\mu$M) each elicited a concentration-dependent increase in $Q_{LDF}$.

**4** Baseline microvascular blood flow was unaffected by a 30 min suffusion of 1 $\mu$M GLB (295$\pm$51 mV vs 325$\pm$62 mV, $P=0.738$).

**5** The vasodilator response elicited by salbutamol (0.32 $\mu$M, 1 $\mu$M and 3.2 $\mu$M), was significantly attenuated by a 30 min superfusion with 1 $\mu$M GLB; this salbutamol-induced vasodilatation was mediated via an interaction with $\beta$-adrenoceptor receptors, as in other experiments it was greatly inhibited by 30-min superfusion with propranolol (10 $\mu$M).

**6** Similarly, following 30-min superfusion with GLB (1 $\mu$M), the terbutaline (1 $\mu$M, 3.2 $\mu$M and 10 $\mu$M)-induced vasodilator response was almost abolished and the vasodilator responses induced by incremental concentrations of forskolin (0.32 $\mu$M, 1 $\mu$M and 3.2 $\mu$M) were also significantly attenuated.

**7** Cromakalim (1.5 $\mu$M, 3 $\mu$M and 3.2 $\mu$M) produced an increase of $Q_{LDF}$ in a dose-dependent manner, which was virtually abolished by GLB (1 $\mu$M). In contrast, the vasodilator responses induced by acetylcholine (32 $\mu$M, 0.1 mM, and 0.32 mM) or sodium nitroprusside (3.2 $\mu$M, 10 $\mu$M and 20 $\mu$M) were independent of GLB (1 $\mu$M).

**8** In conclusion, $K_{ATP}$ channels may be functional, but tonically inactive in the resting diaphragmatic microcirculation and the vasodilator effect of $\beta_2$-adrenoceptor agonists may be partly mediated by $K_{ATP}$ channels; the activation of $K_{ATP}$ channels may involve the accumulation of cyclic AMP in vascular smooth muscle cells.

**Keywords:** Salbutamol; terbutaline; forskolin; cromakalim; acetylcholine; sodium nitroprusside; respiratory muscle; laser-Doppler flowmetry

## Introduction

ATP-sensitive potassium ($K_{ATP}$) channels have been recently identified as important modulators of arteriolar vascular smooth muscle tone (Nelson & Quayle, 1995). Activation of these channels leads to hyperpolarization of vascular smooth muscle and reduces $Ca^{2+}$ entry through voltage-gated $Ca^{2+}$ channels, resulting in relaxation of a variety of blood vessels (Quayle & Standen, 1994). Opening of $K_{ATP}$ channels is regulated by intracellular pH, lactate, $Mg^{2+}$ and nucleotides (Terzic *et al*., 1995). Hence, $K_{ATP}$ channels provide a physiological link between the requirements of tissue metabolism and the regulation of tissue blood flow (Quayle & Standen, 1994). The role of $K_{ATP}$ in modulating blood flow in the diaphragmatic vascular bed is a topic of considerable interest, as the diaphragm is the principle respiratory muscle. It has been demonstrated that $K_{ATP}$ channels play a role in reactive hyperaemia and functional hyperaemia of the canine diaphragm (Comtois *et al*., 1994; Vanelli *et al*., 1994). Whether the studies on the overall change in total diaphragmatic blood fow can be fully applied to the microvascular bed remain in doubt. Because microcirculation is the final arbiter of tissue perfusion adequacy, exploration of the role of $K_{ATP}$ in modulating diaphragmatic microvascular blood flow is of great importance.

Since sympathetic amines influence diaphragm contractility by affecting carbohydrate metabolism (Bowman & Raper, 1964), the few studies performed on the effect of $\beta$-adrenoceptor agonists in the diaphragm have been confined to effects on the contractility and fatigue of the diaphragm (Bowman & Raper, 1964; Aubier *et al*., 1984). To date, there has been no data on the relationship between diaphragmatic microcirculation and $\beta_2$-adrenoceptor agonists. Vasodilatation by $\beta$-adrenoceptor agonists has been traditionally thought to occur through interaction with the $\beta$-adrenoceptor, which is linked to activation of adenylate cyclase and increased formation of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) (Scheid *et al*., 1979). Recently, the traditional view has been challenged by accumulated evidence showing that the hyperpolarization through opening $K_{ATP}$ channels partly mediates vasodilatation induced by $\beta$-adrenoceptor agonists (Quayle & Standen, 1994). Moreover, a variety of endogenous neurohormones, including

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sympathetic amines, have been shown to modulate $K_{ATP}$ channel activity via either a cyclic AMP-dependent mechanism or a direct action of GTP-binding protein (G-protein) (Nelson & Quayle, 1995).

The initial aim of this study was, therefore, to assess the role of $K_{ATP}$ channels in the regulation of diaphragmatic microcirculation under basal conditions and after $\beta_2$-adrenoceptor-agonist stimulation. The rat diaphragm was prepared to allow continuous recording of microcirculatory blood flow by laser-Doppler flowmetry (LDF), and glibenclamide (GLB) was used as a specific pharmacological tool to block $K_{ATP}$ channel activity. Since $K_{ATP}$ channel activity can be modulated by $\beta$-adrenoceptor agonists via either a cyclic AMP-dependent mechanism or a direct action of G-protein, the second aim was to determine the possible mechanism underlying $\beta_2$-adrenoceptor agonist-mediated opening of the $K_{ATP}$ channel activity. For this purpose, the ability of GLB to influence vasodilatation produced by forskolin, a direct stimulator of adenylate cyclase, was assessed.

## Methods

### *Animal preparation*

Male Sprague-Dawley rats (8–10 weeks old; weight 300–350 g) were housed at the Laboratory Animal Center of the College of Medicine at National Cheng Kung University. All animals were acclimatized to a 12 h light: 12 h dark cycle and were maintained on Purina rat chow and tap water *ad libitum*. The animals were fasted overnight but allowed free access to water the day before the experiment.

The animals were initially anaesthetized with intraperitoneal sodium pentobarbitone (30 mg $kg^{-1}$) followed by intravenous 50% (w/v) urethane (1.2–1.5 g $kg^{-1}$) and placed in a supine position on a rodent operating table (Harvard Apparatus, South Natick, MA). After tracheostomy with PE-240 tubing a muscle relaxant (gallamine triethiodide; 60 mg $kg^{-1}$) was administered and the rats were artificially ventilated at tidal volumes between 6 and 7 ml $kg^{-1}$ and a rate of 70 to 80 breaths $min^{-1}$ (model 683, Harvard Apparatus). Adequacy of ventilation was monitored with a microcapnometer (model JS-02262, Polaris, Jerusalem, Israel) through a T-shaped connection to the tracheostomy tube. End-tidal $P_{CO_2}$ was kept at 35 to 40 mmHg. Supplemental $O_2$ was applied to the inspiratory port at a fractional concentration of 40% (balanced air). Mean systemic blood pressure (MAP) was measured with a polyethylene catheter (PE-50) inserted via the right carotid artery and connected to a pressure transducer (model P23 XL, Viggo-Spectramed, Oxnard, CA). The system was filled with heparin/saline (10 iu $ml^{-1}$). A cardiotachometer (model 13-4615-66, Gould Inc., Cleveland, OH) triggered by the pressure signal was used to monitor the heart rate. Another catheter (PE-10) was inserted into the left external jugular vein for administration of fluid and anaesthetic. Normal saline (3–5 ml $h^{-1}$ 100 $g^{-1}$) was infused throughout the experiment via a peristaltic pump (model MS-Reglo, Ismatec, Glattbrugg, Switzerland). Depth of anaesthesia was evaluated hourly by applying pressure to a paw and observing changes in heart rate or blood pressure. When either changed by more than 10% of baseline values, a supplementary dose of urethane (50 mg) was administered intravenously. Rectal temperature was continuously monitored with a thermistor and maintained at 36 to 38°C by a heating lamp and a temperature-regulated bed (model 50-7129, Harvard Apparatus). Arterial blood gas was determined in 200 $\mu$l blood samples, and haematocrit (Hct) was determined in 80 $\mu$l blood samples.

### *Diaphragm preparation*

The techniques used for diaphragm preparation have been described in detail elsewhere (Chang *et al.*, 1995b). Briefly, thoracotomy was performed in the right fifth and sixth intercostal spaces and a 1 cm long segment of the right sixth rib was removed. The diaphragm was separated from the lungs and the mediastinal tissues. An ovoid-shaped stainless steel plate coated with white glossy acrylic was slipped behind the diaphragm to hold the left hemi-diaphragm flat. The plate was fixed to the operating table via a miniature ball-jointed clamp system (model 50-4373, Harvard Apparatus).

Midline and transverse abdominal incisions were made and the ligament between the liver and the central tendon was severed. With the animal in the Trendelenberg position, the upper abdominal wall was folded back and retracted. Abdominal viscera were immobilized by wrapping them with a plastic cast. Superfusion of the abdominal side of the left hemi-diaphragm at a flow rate of 5 ml $min^{-1}$ via a peristaltic pump (model MC-MS/CA/4/8, Ismatec) was begun immediately after exposure, with bicarbonate-buffered Ringer solution. The solution was equilibrated with 5% $CO_2$-95% $N_2$. The temperature of the superfusing fluid was maintained at 37°C, monitored with a thermistor thermometer at the outlet (model 8110-20, Cole-Palmer Instruments, Chicago, IL). A side port connected to a syringe pump (model 55-3333, Harvard Apparatus) was set up for administration of drugs. The infusion rate was set at 1% of the rate of the superfusing fluid, to standardize the final concentration of test agents. The superfusing fluid was continuously removed from the peritoneal cavity via an adjustable vacuum.

### *Laser-Doppler flowmetry*

A commercially available laser-Doppler flowmetry monitor (Laserflo $BPM^2$, Vasamedics, St. Paul, MN) equipped with a small calibre probe (model P 443-3) was used to measure microvascular flow rates. The output signal from the LDF was as a direct current ($Q_{LDF}$, updated 8 times $s^{-1}$). The time constant was set at 1 s.

In all experiments, LDF signals were continuously recorded. The probe, held in an MM-133 micromanipulator (Narishigi Instruments, Tokyo, Japan) was placed perpendicular to the surface of the diaphragm, with the probe tip just touching the water film of the superfusate on the surface of the diaphragm. A site on the left costal diaphragm without visible large vessels was chosen and confirmed by visualization with a long-working-distance stereoscopic zoom microscope (SMZ-1, Nikon, Tokyo, Japan). After stable readings were obtained, the probe was kept in the same fixed position for the duration of the experiments. An average reading time of 30 s was required to provide a stable signal which was independent of vasomotion. At the end of the experiments, the animals were killed with an intravenous injection of saturated potassium chloride. The *postmortem* signal was considered as biological zero and subtracted from the LDF values recorded *in vivo*.

### *Experimental protocols*

Experiments were initiated after a 30- to 45-min stabilization period. Arterial blood gas and haematocrit were determined. The animals used in this study met the following criteria during the stabilization period: (1) MAP > 80 mmHg, (2) pH 7.35 to 7.45, $P_{O_2}$ > 100 mmHg, (3) Hct > 40%, (4) greater than two fold increase in $Q_{LDF}$ compared to baseline values after tropical application of adenosine at 0.1 mM, and (5) no obvious haemorrhage in the muscle tissue under investigation. Ten groups of experiments were performed.

After the period of stabilization, 15 rats (group 1) were used to assess the vasodilator effects of salbutamol, terbutaline and forskolin. Non-cumulative concentration-response curves to one test drug only were obtained in each animal. Five rats received salbutamol at concentrations from 0.32 $\mu$M to 32 $\mu$M, five rats received terbutaline at concentrations from 0.32 $\mu$M to 0.32 mM and five rats received forskolin at concentrations from 0.32 $\mu$M to 10 $\mu$M. Each concentration was continued until a stable response was obtained and a rest period of at least 5 min was allowed for diaphragm recovery following application of

each concentration. Since salbutamol at 0.32 $\mu$M, 1 $\mu$M and 3.2 $\mu$M, terbutaline at 1 $\mu$M, 3.2 $\mu$M and 10 $\mu$M and forskolin at 0.32 $\mu$M, 1 $\mu$M and 3.2 $\mu$M produced dose-dependent vasodilator effects in a similar range, these concentrations were used in the following series of experiments.

Six rats (group 2) were used to investigate the effect of GLB on the vasodilator response to salbutamol. After baseline values had been recorded, salbutamol was applied to the surface of the diaphragm in the order of 0.32 $\mu$M, 1 $\mu$M and 3.2 $\mu$M. After the $Q_{LDF}$ had returned to the baseline value, 1 $\mu$M GLB was applied to the preparation for 30 min by continuous superfusion. Effects of salbutamol at the three concentrations were again recorded while the superfusion of GLB was continued.

Six animals (group 3) were used in a vehicle-control experiment, and four animals (group 4) were used in a time-control experiment. After a first run with salbutamol, as performed in group 2, either the vehicle of GLB or the standard Ringer solution (time-control) was administered for 30 min, and a second run of salbutamol at the same concentrations was performed.

In six rats (group 5), terbutaline, a $\beta_2$-adrenoceptor agonist, at 1 $\mu$M, 3.2 $\mu$M and 10 $\mu$M, was used instead of salbutamol. In a further six rats (group 6), forskolin, an adenylate cyclase stimulator, at 0.32 $\mu$M, 1 $\mu$M and 3.2 $\mu$M, was used. $Q_{LDF}$ was measured by using the group 2 protocol.

In five rats (group 7), the specificity of GLB on $K_{ATP}$ channels was assessed by topical administration of cromakalim, a $K_{ATP}$ opener. The experiments were performed by using the group 2 protocol except that cromakalim at 1.5 $\mu$M, 3 $\mu$M and 4.5 $\mu$M was used in place of salbutamol.

In five rats (group 8), propranolol was used to assess whether salbutamol-induced vasodilatation was mediated through $\beta$-adrenoceptors. Experiments were performed as in group 2, but superfusion with GLB was replaced by superfusion with propranolol 10 $\mu$M for 15 min.

In six rats (group 9) and six other rats (group 10), the relationship between nitric oxide (NO) and $K_{ATP}$ was explored by topical administration of ACh (group 9), an endogenous NO stimulator, and sodium intropusside (SNP, group 10), an exogenous NO donor. The experiments were performed by use of the group 2 protocol, except that ACh, 32 $\mu$M, 0.1 mM and 0.32 mM, and SNP, 3.2 $\mu$M, 10 $\mu$M and 20 $\mu$M, were used in place of salbutamol.

### *Drugs*

Urethane, gallamine triethiodide, dextran 70, salbutamol hemisulphate, terbutaline hemisulphate, forskolin, cromakalim, acetylcholine chloride (ACh), sodium nitroprusside (SNP), GLB, and (±)-propranolol were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). All compounds except urethane, forskolin, cromakalim and GLB were prepared fresh daily in saline and stored on ice during the experiments. Urethane was prepared in saline at a 50% (w/v) concentration. Forskolin was dissolved in a saline solution containing 70% (v/v) alcohol to obtain a stock concentration of 10 mM. Cromakalim was dissolved in a saline solution containing 70% (v/v) alcohol to obtain a stock concentration of 20 mM. GLB was dissolved in a saline solution containing dimethylsulphoxide (DMSO, final concentration 0.1%) and NaOH (final concentration 0.1 mN) to obtain a concentration of 0.1 mM.

### *Data acquisition and statistical analysis*

MAP and $Q_{LDF}$ were fed into the chart recorder (Gould RS 3200 polygraphy) for continuous recording. The output of pressure signals, heart rate and the analogue output from the LDF were directed into a multichannel analogue interphase unit, where the data were sampled at 10 Hz with a 12-bit analogue-to-digital converter (AT codas, Dataq Instrument, Akron, OH) and stored in a personal computer. Recording periods complicated by artefacts were excluded before data analysis. The average LDF signal during a recording time of 30 s was defined as one measurement.

Results are expressed as mean ± s.e.mean. Responses to salbutamol, terbutaline, forskolin, cromakalim, ACh and SNP were measured as the stable increase in $Q_{LDF}$ and expressed as a percentage of baseline values. Baseline $Q_{LDF}$ immediately before the start of suffusion with the vasodilators represented the 100% value for calculation of percentages of the maximal change of $Q_{LDF}$. Differences in baseline values of systemic and microcirculatory physiological variables between groups were analysed for statistical significance by analysis of variance, followed by Student's $t$ test with Bonferroni correction if necessary. Student's $t$ test for paired data was used to analyse the difference between vasodilator responses before and after administration of the vehicle, GLB and propranolol. A probability value of $P<0.05$ was considered statistically significant. The number of observations (measurements) is denoted by $n$.

## Results

The results described below are from experiments carried out on 65 rats that met the inclusion criteria for MAP, arterial blood gases and Hct: MAP was 102 ± 2 mmHg, heart rate was 406 ± 8 beats min$^{-1}$, arterial pH was 7.42 ± 0.01, arterial $P_{O_2}$ was 145.4 ± 4.3 mmHg and arterial $P_{CO_2}$ was 37.5 ± 0.6 mmHg, while Hct was 45 ± 1%. The resting $Q_{LDF}$ was 286 ± 14 mV. Since baseline physiological values and vasodilator responses obtained in animals of group 3 (vehicle-control) and group 4 (time-control) showed no significant difference, the data were pooled for analysis. There were no significant differences in baseline MAP, heart rate or $Q_{LDF}$ between the animals of the eight experimental groups as shown in Table 1.

Figure 1 shows that salbutamol (0.32 $\mu$M – 32 $\mu$M), terbutaline (0.32 $\mu$M – 0.32 mM), and forskolin (0.32 $\mu$M – 10 $\mu$M) each elicited vasodilator responses in a concentration-dependent manner.

Figures 2 and 3 demonstrate that the vasodilator resposes, induced by incremental concentrations of salbutamol (0.32 $\mu$M, 1 $\mu$M and 3.2 $\mu$M), were significantly attenuated after 30 min superfusion with 1 $\mu$M GLB. The baseline microvascular flow was not altered by GLB (295 ± 51 mV vs 325 ± 62 mV, $P=0.738$). In animals in the control group, the vasodilator responses, induced by incremental concentrations of salbutamol, remained the same throughout the experiments (183 ± 13% v s 180 ± 16%, $P=0.479$ for 0.32 $\mu$M; 257 ± 21% vs 233 ± 14%, $P=0.490$ for 1 $\mu$M; and 353 ± 56% vs 382 ± 51%, $P=0.482$ for 3.2 $\mu$M), as illustrated in the top trace of Figure 2.

As can be seen in Figures 2 and 4 terbutaline (1 $\mu$M, 3.2 $\mu$M and 10 $\mu$M) also elicited dose-dependent vasodilator responses and the increase in microvascular flow was nearly abolished by 1 $\mu$M GLB.

**Table 1** Baseline mean systemic arterial blood pressure (MAP), heart rate (HR) and diaphragmatic microvascular blood flow recorded by laser-Doppler flowmetry ($Q_{LDF}$) in the animals of the eight experimental groups

| | *MAP* (mmHg) | *HR* (beats min$^{-1}$) | $Q_{LDF}$ (mV) |
|---|---|---|---|
| Control ($n=10$) | 108 ± 3 | 414 ± 12 | 273 ± 35 |
| Sal + GLB ($n=6$) | 100 ± 4 | 412 ± 16 | 230 ± 40 |
| Terb + GLB ($n=6$) | 107 ± 4 | 425 ± 10 | 268 ± 38 |
| Fosk + GLB ($n=6$) | 101 ± 6 | 415 ± 11 | 284 ± 33 |
| Sal + propranolol ($n=5$) | 98 ± 5 | 403 ± 23 | 244 ± 41 |
| Crom + GLB ($n=5$) | 104 ± 2 | 419 ± 10 | 305 ± 31 |
| ACh + GLB ($n=6$) | 102 ± 6 | 423 ± 7 | 311 ± 26 |
| SNP + GLB ($n=6$) | 100 ± 2 | 391 ± 7 | 235 ± 23 |

Values are mean ± s.e.mean. Sal, salbutamol; GLB, glibenclamide; Terb, terbutaline; Fosk, forskolin; Crom, cromakalim; ACh, acetylcholine; SNP, sodium nitroprusside.

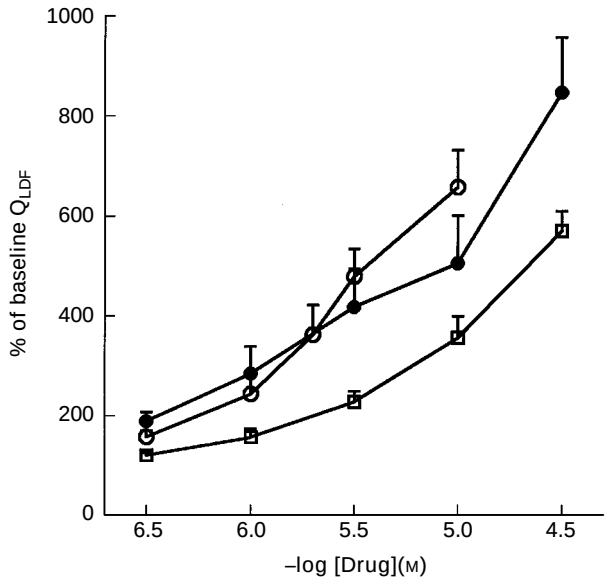


**Figure 1** Dose-response curves of diaphragmatic microcirculatory blood flow measured by laser-Doppler flowmetry ($Q_{LDF}$) to increasing doses of topical application of salbutamol (●, $n=5$), terbutaline (□, $n=6$), and forskolin (○, $n=5$). Responses, shown as a percentage increase of baseline $Q_{LDF}$, are given as the mean from the number of animals shown ($n$); vertical lines show s.e.mean.

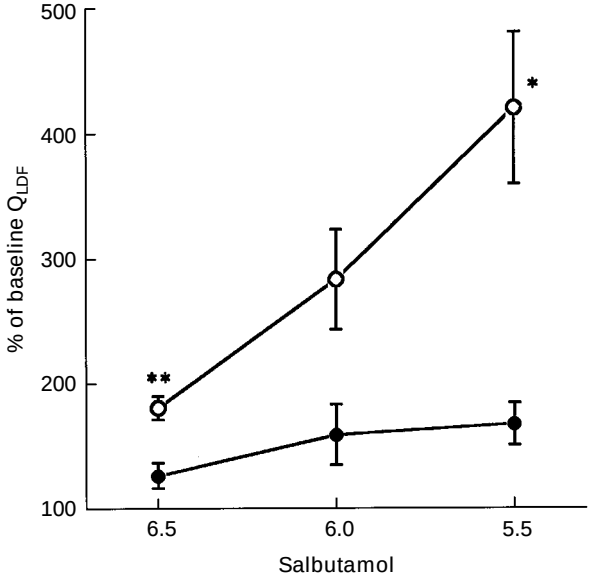


**Figure 3** Dose-response curves of diaphragmatic microcirculatory blood flow, measured by laser-Doppler flowmetry ($Q_{LDF}$), to increasing doses of topical application of salbutamol before (○) and after (●) suffusion of 1 $\mu$M glibenclamide ($n=6$). Responses shown as a percentage increase of baseline $Q_{LDF}$ are given as the means from ($n$) number of animals; vertical lines show s.e.mean. $^*P<0.05$, $^{**}P<0.01$, different responses after 1 $\mu$M GLB.

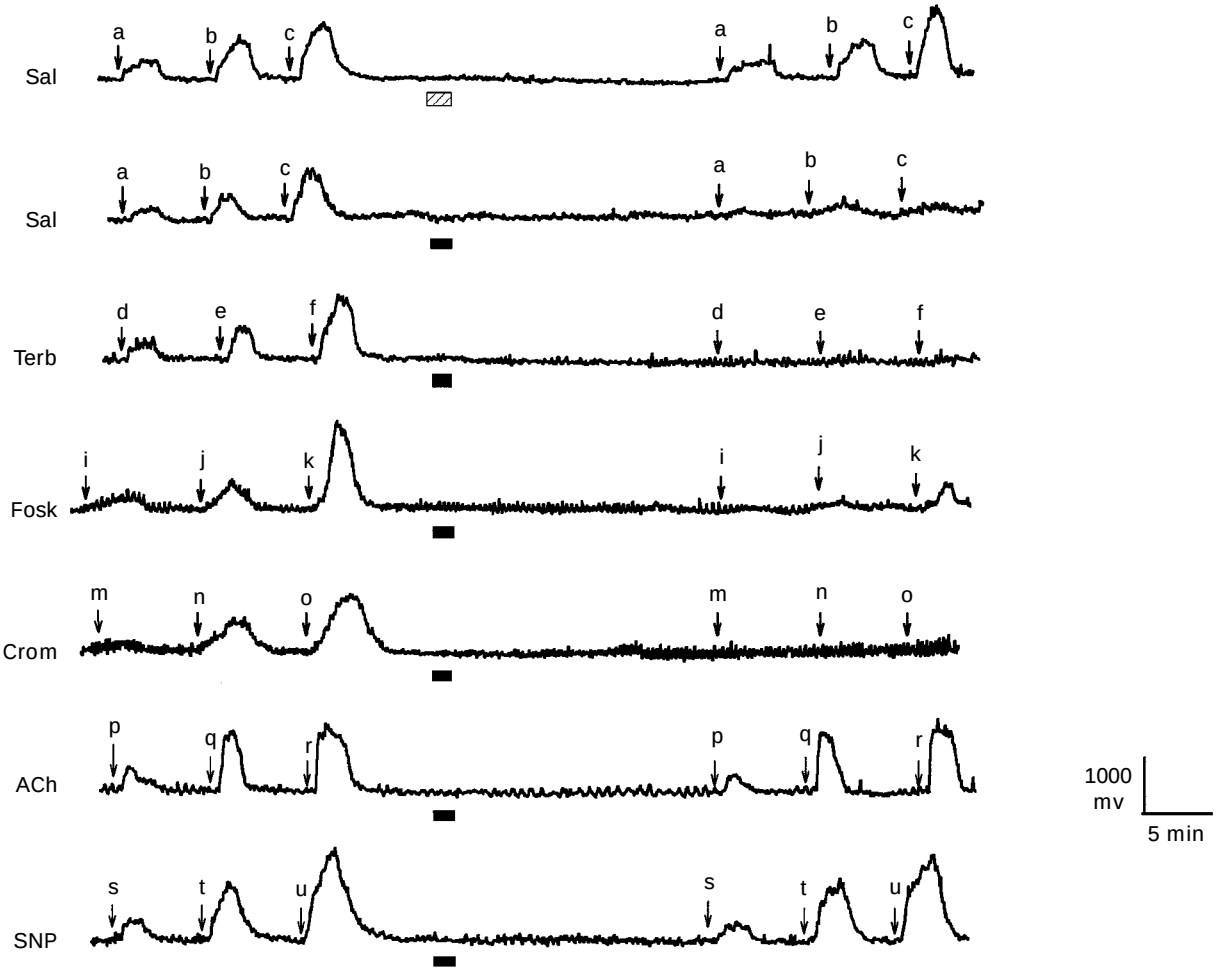


**Figure 2** Tracings showing the effects of 30-min suffusion of glibenclamide (GLB, 1 $\mu$M) or its vehicle on basal blood flow and agonist-induced vasodilator responses, as measured by laser-Doppler flowmetry in rat diaphragms. The time of application of GLB is indicated by the solid bar, and the time of application of its vehicle is indicated by the hatched bar. Arrows indicate the time of administration of salbutamol (Sal) (a) 0.32 $\mu$M, (b) 1 $\mu$M, (c) 3.2 $\mu$M; terbutaline (Terb) (d) 1 $\mu$M, (e) 3.2 $\mu$M, (f) 10 $\mu$M; forskolin (Fosk) (i) 0.32 $\mu$M, (j) 1 $\mu$M, (k) 3.2 $\mu$M; cromakalim (Crom) (m) 1.5 $\mu$M, (n) 3 $\mu$M, (o) 3.2 $\mu$M; acetylcholine (ACh) (p) 32 $\mu$M, (q) 0.1 mM, (r) 0.32 mM; sodium nitroprusside (SNP) (s) 3.2 $\mu$M, (t) 10 $\mu$M, (u) 20 $\mu$M.

Figures 2 and 5 illustrate that the vasodilator responses elicited by incremental concentrations of forskolin (0.32 $\mu$M, 1 $\mu$M and 3.2 $\mu$M) were significantly attenuated by 1 $\mu$M GLB, while Figures 2 and 6 show that GLB (1 $\mu$M) virtually abolished the vasodilator responses induced by cromakalim (1.5 $\mu$M, 3 $\mu$M and 4.5 $\mu$M).

Microvascular blood flow was not affected by 30 min superfusion with 10 $\mu$M propranolol (data not shown), but the vasodilator responses induced by incremental concentrations of salbutamol were significantly suppressed (175$\pm$18% vs 122$\pm$12%, $P<0.05$ for 0.32 $\mu$M; 235%$\pm$29% vs 113$\pm$14%, $P<0.05$ for 1 $\mu$M; and 383$\pm$36% vs 131$\pm$16%, $P<0.05$ for 3.2 $\mu$M).

Figure 7 demonstrates that ACh (32 $\mu$M, 0.1 mM and 0.32 mM) and SNP (3.2 $\mu$M, 10 $\mu$M and 20 $\mu$M) elicited dose-dependent increases in microvascular flow, and that these were not affected by GLB (1 $\mu$M).

Since all drugs were topically administered to the surface of the diaphragm, there was no significant change in MAP following the application of any vasodilator drugs (data not shown).

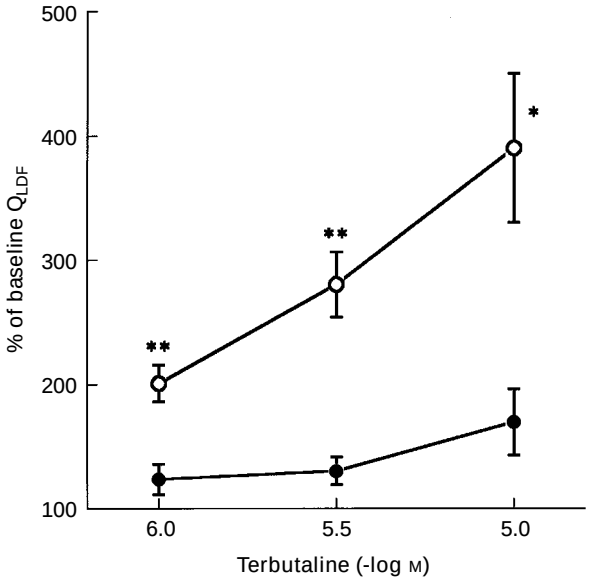


**Figure 4** Dose-response curves of diaphragmatic microcirculatory blood flow, measured by laser-Doppler flowmetry ($Q_{LDF}$), to increasing doses of topical application of terbutaline before (○) and after (●) suffusion of 1 $\mu$M glibenclamide ($n=6$). Responses shown as a percentage increase of baseline $Q_{LDF}$, are given as the means from ($n$) number of animals; vertical lines show s.e.mean. *$P<0.05$, **$P<0.01$, different from responses after GLB.

## Discussion

The results of this study show that (1) cromakalim, an opener of $K_{ATP}$ channels, induced vasodilatation in the diaphragmatic microvascular bed, while GLB, a blocker of $K_{ATP}$ channels, did not affect basal microcirculatory blood flow in the resting diaphragm; (2) vasodilator responses induced by the $\beta_2$-adrenoceptor agonists, salbutamol and terbutaline, were mostly mediated through activation of $K_{ATP}$ channels, as GLB greatly inhibited these responses; (3) forskolin-induced vasodilator responses were also significantly blunted by GLB; (4) vasodilator responses induced by ACh, an endothelium-dependent, NO-mediated agonist, and SNP, an endothelium-independent, NO-mediated agonist, were not affected by GLB.

### *Effects of GLB on basal microcirculatory blood flow*

Recent evidence supports the idea that, depending on the vascular bed studied, $K_{ATP}$ channels may play a role in the maintenance of basal tone (Nelson & Quayle, 1995). In the present study, resting microvascular blood flow was not affected by local application of GLB, suggesting that $K_{ATP}$ channels in our preparations were mostly closed and that $K_{ATP}$ channels probably did not play a significant role in modulating the resting diaphragmatic microcirculation of the anaesthetized rat. Since cromakalim, an opener of $K_{ATP}$ channels, elicited a dose-dependent vasodilator response in the present study, these channels must be present in the resting diaphragmatic microcirculation. Therefore, $K_{ATP}$ channels may be functional, but inactive in this vascular bed. Consistent with our results, GLB

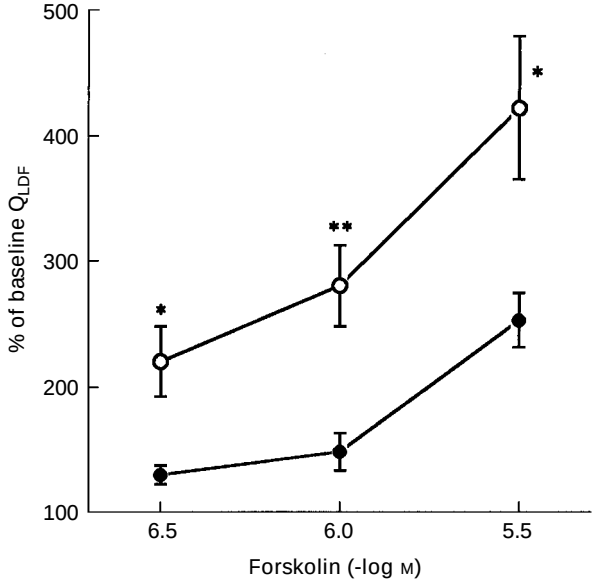


**Figure 5** Dose-response curves of diaphragmatic microcirculatory blood flow, measured by laser-Doppler flowmetry ($Q_{LDF}$), to increasing doses of topical application of forskolin before (○) and after (●) suffusion of 1 $\mu$M glibenclamide ($n=6$). Responses shown as a percentage increase of baseline $Q_{LDF}$, are given as the means from ($n$) number of animals; vertical lines show s.e.mean. *$P<0.05$, **$P<0.01$, different from response after GLB.

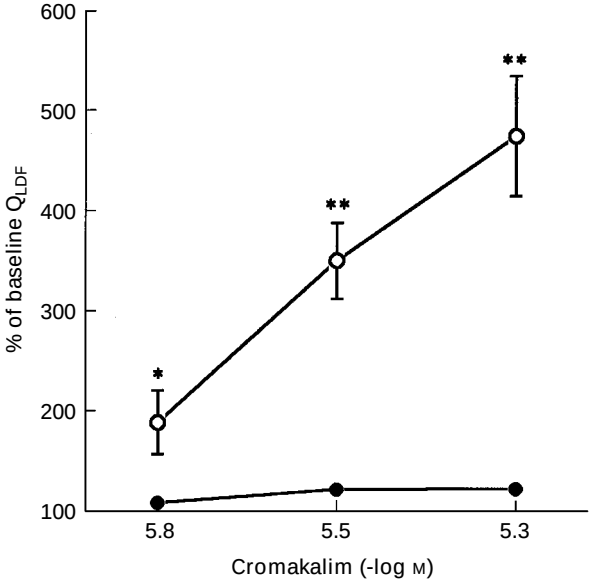


**Figure 6** Dose-response curves of diaphragmatic microcirculatory blood flow, measured by laser-Doppler flowmetry ($Q_{LDF}$), to increasing doses of topical application of cromakalim before (○) and after (●) suffusion of 1 $\mu$M glibenclamide ($n=5$). Responses shown as a percentage increase of baseline $Q_{LDF}$, are given as the means from ($n$) number of animals; vertical lines show s.e.mean. *$P<0.05$, **$P<0.01$, different from response after GLB.

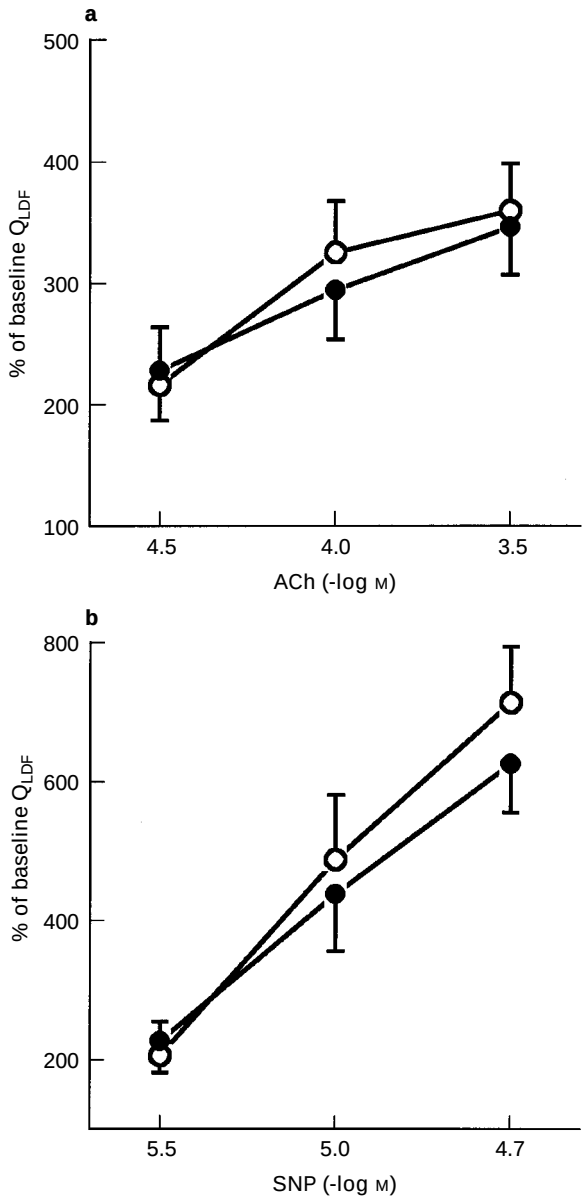


**Figure 7** Dose-response curves of diaphragmatic microcirculatory blood flow, measured by laser-Doppler flowmetry ($Q_{LDF}$), to increasing doses of topical application of (a) acetylcholine (ACh) and (b) sodium nitroprusside (SNP) before (○) and after (●) suffusion of 1 $\mu$M glibenclamide ($n=6$). Responses shown as a percentage increase of baseline $Q_{LDF}$, are given as the means from ($n$) number of animals; vertical lines show s.e.mean.

has been shown not to alter baseline diameters in rat basilar arteries, but to abolish the response of the basilar artery to RP52891, a direct activator of $K_{ATP}$ channels (Faraci & Heistad, 1993). In contrast, a direct intra-arterial infusion of GLB has been shown to reduce resting phrenic arterial blood flow to 50% of control values in canine isolated hemidiaphragm (Vanelli & Hussain, 1994). However, GLB produced only a 5% reduction of canine phrenic arterial blood flow in intact resting diaphragms during spontaneous breathing (Comtois *et al.*, 1994). This discrepancy might be explained by the different species used, the method used to prepare the diaphragm or the technique used for measuring diaphragmatic blood flow.

### *Effects of GLB on microcirculatory blood flow change induced by $\beta_2$-adrenoceptor agonists and forskolin*

In our study, topically administered $\beta_2$-adrenoceptor agonists and forskolin could theoretically have produced dose-dependent vasodilator effects in the diaphragm by affecting vessels of a range of different diameters. However, we really cannot tell from our results the main site of action of these agents. Further studies will be necessary to clarify whether the main site of action of these agents is confined to the microcirculation, as found in canine gracilis muscle and feline hindlimb muscle (Lundvall & Järhult, 1976).

Vasodilatation induced by $\beta$-adrenoceptor agonists has been traditionally thought to occur through interaction with the $\beta$-adrenoceptors, that are linked to activation of adenylate cyclase and increased formation of cyclic AMP (Scheid *et al.*, 1979). Our study showed that salbutamol elicited an increase in diaphragmatic microcirculatory blood flow in a dose-dependent manner. This vasodilator response resulted from a specific pharmacological interaction with the $\beta$-adrenoceptors, as it was almost abolished by propranolol. After local application of GLB, the microcirculatory change stimulated by salbutamol was significantly attenuated. Additionally, terbutaline-induced vasodilator response was also blunted by GLB. These data challenge the traditional view that vasorelaxation induced by $\beta_2$-adrenoceptor agonists is solely mediated via phosphorylation of myosin light chain protein secondary to an accumulation of cyclic AMP in vascular smooth muscle cells. Rather, our results accord with recent electrophysiological evidence from canine saphenous vein that GLB attenuates the hyperpolarization induced by isoprenaline (Nakashima & Vanhoutte, 1994) and suggest that hyperpolarization through opening of $K_{ATP}$ channels mediates vasodilatation induced by $\beta_2$-adrenoceptor agonists.

Similarly, $\beta_1$-adrenoceptor-mediated dilatation of rat basilar artery to noradrenaline was partially inhibited by GLB (Kitazono *et al.*, 1993). Moreover, denopamine, a $\beta_1$-adrenoceptor agonist, elicited an increase in coronary blood flow in the dog which was almost abolished by GLB (Narishige *et al.*, 1994). Moreover, to complicate this issue further, both $\beta_1$- and $\beta_2$-adrenoceptor agonists have been shown to activate $K_{ATP}$ channels in the microcirculatory beds of hamster cheek pouch and cremaster muscle (Jackson, 1993). It may be that differences in species and in the vascular bed studied determine the subtype of $\beta$-adrenoceptor agonists that is coupled to $K_{ATP}$ channel activation.

A variety of endogenous neurohormones including adenosine, vasoactive intestinal peptide, calcitonin-gene related peptide, atrial natriuretic factor, and somatostatin modulate $K_{ATP}$ channel activity via two common pathways: a cyclic AMP-dependent mechanism and a direct action of G-proteins (Quayle & Standen, 1994; Nelson & Quayle, 1995). The cyclic AMP-dependent mechanism implies that cyclic AMP accumulation, following activation of adenylate cyclase either directly or through a receptor on vascular smooth muscle cells, may be linked to the consequent activation of $K_{ATP}$ channels. In our study, forskolin was used to bypass the actions of the $\beta$-adrenoceptors and G-proteins. The vasodilator effect induced by forskolin was attenuated by GLB, suggesting that a mechanism similar to that underlying the link between cyclic AMP elevation and $K_{ATP}$-mediated vasodilatation may be applied to our preparation. Our data are consistent with the observation from rat basilar artery that GLB attenuates vasodilatation induced by forskolin (Kitazono *et al.*, 1993). Moreover, electrophysiological evidence from canine saphenous vein and porcine coronary artery has shown that forskolin produces a hyperpolarization that is attenuated by GLB (Miyoshi & Nakaya, 1993; Nakashima & Vanhoutte, 1994).

Our study also showed that GLB did not affect the vasodilator response induced by ACh. This suggests that cyclic GMP accumulation secondary to the release of endogenous NO is not linked to the activation of $K_{ATP}$ channels, and these channels are, therefore, not involved in prostanoid- and NO-mediated vasodilator response to ACh (Chang *et al.*, 1995a; 1997). On the contrary, smooth muscle hyperpolarizations to ACh in rabbit middle cerebral arteries were abolished by GLB (Standen *et al.*, 1989). The discrepancy has two possible explanations. Firstly, GLB-sensitive smooth muscle hyperpolarization to ACh might not significantly contribute to

acetylcholine-induced vasodilator effects in our preparation. Secondly, hyperpolarization induced by ACh may be mediated through several ion channels in vascular smooth muscle, only one of which is the $K_{ATP}$ channel (McPherson & Angus, 1991; Khan *et al*., 1993).

Our data further showed that the vasodilator effect of SNP, an exogenous donor of NO, was independent of GLB, so excluding the possibility that the abolition of $\beta_2$-adrenoceptor-induced vasodilator response by GLB was due to its non-selective inhibition of vasodilatation. These data strengthen the contention that the cyclic GMP system, as activated by endogenous and exogenous NO, is not coupled to the activation of $K_{ATP}$ channels in the rat diaphragmatic microcirculation. This contention is supported by several observations: charybtoxin and iberiotoxin, specific blockers of calcium-activated potassium channels, significantly inhibit vasodilatation to NO both *in vivo* and *in vitro* (Khan *et al*., 1993; Bolotina *et al*., 1994; Zanzinger *et al*., 1996).

In summary, $K_{ATP}$ channels may be functional, but tonically inactive in the microcirculation of resting the diaphragm and vasodilator effects induced by $\beta_2$-adrenoceptor agonists may be partly mediated by $K_{ATP}$ channels. Furthermore, our data suggest a link between the accumulation of cyclic AMP in vascular smooth muscle cells and the consequent activation of $K_{ATP}$ channels. However, caution should be taken in extending this conclusion to the contracting diaphragm. Further study is needed to explore the relationship between diaphragm fatigue, contractility of the diaphragm and the increased microvascular flow mediated by $K_{ATP}$ channels during $\beta_2$-adrenoceptor stimulation.

This study was funded by National Cheng Kung University Hospital (Tainan, Taiwan) grant NCKUH 86-010.

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*(Received December 18, 1996*
*Revised March 14, 1997*
*Accepted March 17, 1997)*