1-[*N,O*-Bis-(5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), an inhibitor of calcium-dependent camodulin protein kinase II, inhibits both insulin- and hypoxia-stimulated glucose transport in skeletal muscle

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Previous studies have indicated a role for calmodulin in hypoxia- and insulin-stimulated glucose transport. However, since calmodulin interacts with multiple protein targets, it is unknown which of these targets is involved in the regulation of glucose transport. In the present study, we have used the calcium-dependent calmodulin protein kinase II (CAMKII) inhibitor 1-[*N*,*O*-bis-(5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62) to investigate the possible role of this enzyme in the regulation of glucose transport in isolated rat soleus and epitrochlearis muscles. KN-62 did not affect basal 2-deoxyglucose transport, but it did inhibit both insulin- and hypoxia-stimulated glucose transport activity by 46 and 40 % respectively. 1-[*N*,*O*-Bis-(1,5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-04), a structural analogue of KN-62 that does not inhibit CAMKII, had no effect on hypoxia- or insulin-stimulated glucose transport. Accordingly, KN-62 decreased the stimulated cell-surface GLUT4 labelling by a similar extent as the inhibition of glucose transport (insulin, 49 % and hypoxia, 54 %). Additional experiments showed that KN-62 also inhibited insulin- and hypoxia-stimulated transport by 37 and 40 % respectively in isolated rat epitrochlearis (a fast-twitch muscle), indicating that the effect of KN-62 was not limited to the slow-twitch fibres of the soleus. The inhibitory effect of KN-62 on hypoxia-stimulated glucose transport appears to be specific to CAMKII, since KN-62 did not inhibit hypoxia-stimulated ^{45}Ca efflux from muscles pre-loaded with ^{45}Ca, or hypoxia-stimulated glycogen breakdown. Additionally, KN-62 affected neither insulin-stimulated phosphoinositide 3-kinase nor Akt activity, suggesting that the effects of KN-62 are not due to non-specific effects of this inhibitor on these regions of the insulin-signalling cascade. The results of the present study suggest that CAMKII might have a distinct role in insulin- and hypoxia-stimulated glucose transport, possibly in the vesicular trafficking of GLUT4.

Key words: Akt kinase, GLUT4, photolabelling, PI 3-kinase.

INTRODUCTION

It has been well known for the past 19 years that insulin stimulates glucose transport in skeletal muscle by causing the translocation of GLUT4 glucose transporters to the plasma membrane [1]. Muscular contraction or hypoxia (which acts via the same mechanism as muscle contraction), however, represents a second mechanism for stimulation of glucose transport in muscle [2,3]. This is evidenced by the fact that the combined effects of contraction/hypoxia and insulin on glucose transport are additive to each other [4]. Although insulin and muscle contraction/hypoxia stimulate glucose transport by causing translocation of GLUT4 to the plasma membrane, these stimuli act via different mechanisms. Insulin is known to stimulate glucose transport through activation of the tyrosine kinase activity of its own receptor, and subsequent phosphorylation and activation of downstream effectors, whereas the mechanism by which muscle contraction/hypoxia stimulates GLUT4 translocation remains largely unknown [4,5]. Wortmannin, an inhibitor of phosphoinositide 3-kinase (PI-3K), inhibits glucose transport when stimulated by insulin, but it does not inhibit contraction- or hypoxia-stimulated glucose transport [6]. This indicates that contraction and hypoxia do not stimulate glucose transport via PI-3K [5]. Much accumulated evidence has implicated an elevation in intracellular calcium in the stimulation of glucose transport by contraction or hypoxia [7–10]. In general, the effects of an elevation in intracellular calcium are mediated by calcium-binding proteins, such as calmodulin [11,12]. It has been shown that an antagonist of calmodulin (CGS 9343B) inhibits both contraction/hypoxia- and insulin-stimulated glucose transport [10]. However, since calcium/calmodulin interacts with multiple protein targets, the identity of the signalling molecule involved in contraction- or insulin-stimulated glucose transport remains unknown from these studies.

Calcium-dependent calmodulin protein kinase II (CAMKII) is a calcium/calmodulin-activated protein kinase that is activated by the rise in cytoplasmic calcium that follows membrane depolarization, and is expressed in a variety of tissues [13,14]. Additionally, CAMKII has also been shown to be involved in regulated exocytosis in neuronal cell lines [15]. Since CAMKII

Abbreviations used: ATB-BMPA, 2-*N*-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis-(D-mannos-4-yloxy)-2-propylamine; CAMKII, calcium-dependent calmodulin protein kinase II; 2DG, 2-deoxyglucose; IRS-1, insulin-receptor substrate 1; KHB, Krebs–Henseleit buffer; KN-04, 1-[*N*,*O*-bis-(1,5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine; KN-62, 1-[*N*,*O*-bis-(5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine; PI-3K, phosphoinositide 3-kinase.

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has been detected in skeletal muscle, its ability to be activated by a rise in cytoplasmic calcium, and its role in regulated exocytosis, makes it an attractive signalling molecule to mediate the effects of contraction/hypoxia on glucose transport [16,17]. A specific inhibitor of CAMKII, 1-[*N*,*O*-bis-(5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), has been described previously [18,19]. This inhibitor binds to the calmodulin-binding site of CAMKII, and consequently does not inhibit other classes of calmodulin-sensitive kinases [19]. Therefore the first purpose of the present study was to evaluate the role of CAMKII in hypoxia-stimulated glucose transport using KN-62.

Whether calcium/calmodulin plays a role in insulin-stimulated glucose transport is not as clear as it is for contraction, and is somewhat controversial [8,9,20–22]. Nevertheless, it has been shown that calmodulin antagonists also inhibit insulin-stimulated glucose transport [9,10]. Furthermore, it has also been shown that the insulin receptor tyrosine kinase is capable of phosphorylating calmodulin *in vitro*, and that calmodulin interacts with insulin-receptor substrate 1 (IRS-1) [23], Thus it is possible that activation of CAMKII might play a role in insulin-stimulated glucose transport; therefore an additional purpose of the present study was to evaluate the role of CAMKII in insulin-stimulated glucose transport using the CAMKII inhibitor KN-62.

EXPERIMENTAL

Animals

Specific pathogen-free male Wistar rats weighing 100–125 g were obtained from Charles River Laboratories (Boston, MA, U.S.A.). Upon arrival, rats were housed four to a cage in a temperature-controlled animal room maintained on a 12:12 h light–dark cycle. The rats were fed *ad lib*. on NIH standard chow and water.

Muscle preparation

Rats in the post-prandial state were anaesthetized with 5 mg/100 g body-weight sodium pentobarbital. Epitrochlearis or soleus muscles were dissected out, blotted on gauze, and transferred to 25-ml Erlenmeyer flasks containing 2 ml of Krebs–Henseleit buffer (KHB) containing 0.1% (w/v) BSA, 32 mM mannitol, 8 mM glucose and either no or 13.3 nM insulin. Hypoxic muscles were placed in similar medium containing KHB that had been previously gassed with N_2/CO_2 (19:1). The flasks were incubated in a shaking water bath maintained at 35 °C for 1 h, and were continuously gassed with O_2/CO_2 (19:1) (normoxic muscles) or N_2/CO_2 (19:1) (hypoxic muscles). Soleus muscles were split before incubation in order to allow for proper diffusion of substrate in these muscles [24], and were incubated in the same medium as the epitrochlearis muscles. Before incubation under basal conditions (no additions), or with insulin or hypoxia, split soleus muscles were allowed to recover (pre-incubation) for 1 h in the absence of insulin or hypoxia [24]. KN-62 and [1-[*N*,*O*-bis-(1,5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-04) were dissolved in DMSO, and when present in the incubations, a similar quantity of DMSO was added to the control muscles. The concentration of DMSO added to the incubation medium never exceeded 0.5%. When KN-62 was present during the pre-incubation with insulin or hypoxia, it was also present during the incubation with insulin.

Measurement of glucose transport activity

Glucose transport activity was measured using 2-deoxyglucose (2DG), as described in detail previously [25]. Following the above incubations, muscles were blotted and transferred to flasks containing 1.5 ml of KHB containing 1 mM 2-deoxy-[1,2-^{3}H]-glucose (1.5 mCi/mmol) and 39 mM [1-^{14}C]mannitol (8 μCi/mmol), and the same additions as in the previous incubation. The flasks were incubated at 29 °C for 20 min and were continuously gassed with O_2/CO_2 (19:1). After the incubations, the muscles were frozen between tongs cooled to the temperature of liquid nitrogen, and stored at −70 °C until they were processed for measurement of 2DG transport. Frozen muscles were homogenized in 1 ml of 0.6 M perchloric acid. Homogenates were centrifuged at 5000 ***g***, and aliquots of the supernatant were counted for radioactivity in a liquid-scintillation counter.

2-*N*-4-(1-Azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis-(D-mannos-4-yloxy)-2-propylamine (ATB-BMPA) photoaffinity labelling of isolated muscles

Isolated muscles were photolabelled as described previously [25]. Following the incubation and wash steps described above, isolated soleus muscles were incubated in 1 ml of KHB buffer, which contained insulin, but no inhibitor, at the same concentration as in the previous incubation, and 1 mCi/ml ATB-BMPA for 5 min at 25 °C. Each muscle was then irradiated for 2×2 min intervals in a photometer lamp. The muscles were manually turned over between intervals. Following irradiation, the muscles were blotted and frozen between liquid-nitrogen-cooled clamps. Muscles were kept stored at −70 °C until processed.

Muscles were processed for determination of labelling as described previously [25]. Briefly, Thesit (Boehringer Mannheim, Indianapolis, IN, U.S.A.)-solubilized crude membranes were coupled to an immunocomplex consisting of anti-(C-terminus GLUT4) serum and Protein A–Sepharose overnight. The immunocomplex was washed and the antibody conjugate was lysed from the Protein A–Sepharose with SDS/urea sample buffer.

The immunoprecipitated GLUT4 transporters were subjected to SDS/PAGE, and the gels were stained with Coomassie Blue, destained and sliced into 8-mm slices. The slices were dried, solubilized in 30% (v/v) H_2O_2 and 2% (v/v) ammonium hydroxide, and radioactivity was counted in a liquid-scintillation counter. The levels of radioactivity associated with each peak were obtained by integrating the radioactivity under the peak and subtracting the average background activity of slices on the left-hand side of the peak. Total radioactivity was standardized by expression in units of d.p.m./mg of wet weight.

Measurement of ^{45}Ca depletion

Soleus muscles were loaded with ^{45}Ca for 60 min at 35 °C in 2 ml of KHB containing 8 mM glucose, 32 mM mannitol and 1 μCi/ml of ^{45}Ca, as described by Youn et al. [9] and Clausen et al. [20]. Following the initial 1-h incubation, the muscles were then washed for 1 h with non-radioactive medium, which was changed every 20 min. After the wash period, some of the muscles were frozen; these muscles served as zero-time controls. The remaining muscles were incubated in the presence or absence of 10 μM KN-62, and with or without hypoxia. At 20-min intervals, the muscles were transferred to fresh medium. At the end of the incubation period, the muscles were frozen in tongs cooled to the temperature of liquid nitrogen and stored at −70 °C. Frozen muscles were homogenized in 1 ml of 0.6 M perchloric acid, the homogenates were centrifuged at 5000 ***g*** for 10 min, and aliquots of the supernatant were counted for radioactivity by liquid-scintillation counting.

Measurement of muscle glycogen

Muscles incubated as described above were frozen between tongs cooled to the temperature of liquid nitrogen and stored at −70 °C until processed. Muscles were homogenized in 1 ml of 0.6 M perchloric acid while standing on ice in Kontes (Vineland, NJ, U.S.A.) glass-on-glass grinding tubes. Glycogen was digested with amyloglucosidase (Boehringer Mannheim), and glucose was measured in the supernatant using the hexokinase/glucose-6-phosphate dehydrogenase method, as described by Passonneau and Lauderdale [26].

Measurement of PI-3K activity

Soleus muscles were incubated as described above in the presence or absence of 13.3 nM insulin or 13.3 nM insulin plus KN-62. Muscles were frozen between liquid-N_2-cooled tongs, and then processed as described previously [27]. Briefly, frozen muscles were homogenized in ice-cold lysis buffer [50 mM Hepes (pH 7.2)/2 mM EDTA/30 mM sodium pyrophosphate/1 % (v/v) Triton X-100/10 % (v/v) glycerol/10 mM NaF/150 mM NaCl/2 mM Na_3VO_4/5 μg/ml leupeptin/1.5 mg/ml benzamidine/0.5 mg/ml pepstatin A/2 μg/ml aprotinin/1 mM AEBSF (Pefabloc, Boehringer Mannheim)/10 μg/ml antipain] and mixed end-over-end for 45 min at 4 °C. The lysates were then centrifuged at 35000 ***g***. A portion of the resultant supernatant was used for determination of protein concentration using the bicinchoninic acid procedure (Pierce, Rockford, IL, U.S.A.), and the remainder of the supernatant was stored at −80 °C.

PI-3K activity was measured as described previously [27]. A 1-mg aliquot of soleus supernatant was incubated with affinity-purified anti-IRS1 antibody (Upstate Biotechnology Inc., Lake Placid, NY, U.S.A.) and Protein A–Sepharose. The immune pellets were then washed three times with buffer 1 [PBS (pH 7.5)/1 % Nonidet P40/100 μM Na_3VO_4], three times with buffer 2 [100 mM Tris/HCl (pH 7.5)/500 mM LiCl/100 μM Na_3VO_4] and twice with buffer 3 (10 mM Tris/HCl (pH 7.5)/100 mM EDTA/100 μM Na_3VO_4]. IRS1-associated PI-3K activity was measred for each sample as described by Goodyear et al. [27]. Briefly, the immunopellet was resuspended in 50 μl of the final wash buffer containing 20 μg of phosphatidyinositol (Avanti Polar Lipids, Albaster, AL, U.S.A.) and 100 mM $MgCl_2$. The reaction was initiated at room temperature by the addition of 5 μl of a phosphorylation mixture containing 880 μM ATP and 20 μCi of [γ-^{32}P]ATP (Amersham, Arlington Heights, IL, U.S.A.). After 20 min of continuous vortex-mixing, the reaction was stopped by the sequential addition of 8 M HCl and chloroform/methanol (1:1, v/v). The reaction mixture was vortex-mixed for 5–10 min, and then centrifuged at 2000 ***g*** for 3–5 min; 30 μl of the organic phase containing the reaction products was spotted on to an aluminium-backed silica TLC plate pretreated with 1 % (v/v) potassium oxalate solution. The products were resolved in chloroform/methanol/water/ammonium hydroxide (60:47:11.3:2, by vol.) solution and detected by autoradiography. The spots corresponding to the 3′-monophosphorylated products were excised from the TLC plate and counted for radioactivity by liquid-scintillation counting.

Akt immunoprecipitation

Isolated muscles were incubated under the experimental conditions described above. Following incubation, the muscles were trimmed of their tendons, blotted and frozen between tongs cooled to the temperature of liquid nitrogen. Muscles were kept stored at −80 °C until processed.

Lysates were prepared from the incubated muscles (prepared as described above) for the PI-3K assays. Lysates were centrifuged at 18000 ***g*** for 15 min, and the protein concentration of the supernatant was determined by using the bicinchoninic acid method, using crystalline BSA as a standard. Aliquots of the supernatant corresponding to 2 mg of protein were immunoprecipitated for 2 h with 5 μl of affinity-purified anti-Akt antibody (raised against 16 amino acids of the C-terminus of rat Akt 2). Protein A–agarose (50 μl; Life Technologies, Gaithersburg, MD, U.S.A.) was then added, and the samples were rotated for 1 h. The samples were spun down at 15000 ***g*** to pellet the immunocomplexes. Immunopellets were then washed three times with ice-cold lysis buffer, and three times with 1 × kinase buffer [20 mM Hepes (pH 7.2)/5 mM $MgCl_2$].

Akt kinase assay

Akt activity was measured as described previously [28]. Briefly, the immunopellet was resuspended in 30 μl of kinase assay buffer [20 mM Hepes (pH 7.2)/5 mM $MgCl_2$/1 mM dithiothreitol/2 μM ATP/0.2 mM EGTA/2 μg per reaction (rxn) protein kinase inhibitor (PKI; Sigma cat. no. P0300)/25 μg/rxn histone 2B/5 μCi/rxn [γ-^{32}P]ATP (5000 Ci/mmol)]. The samples were incubated at 30 °C for 30 min, and the reaction was terminated by the addition of 20 μl of 4 × Laemmli sample buffer, with subsequent boiling for 2 min. The samples were then centrifuged to pellet the agarose, and the supernatant was loaded on an SDS/12.5 % polyacrylamide gel. The resolved proteins were then transferred to 0.45 μm nitrocellulose in a Hoefer Tank Blotter (Hoefer Instruments, San Francisco, CA, U.S.A.). The nitrocellulose was then exposed to a PhosphorImager cassette (Molecular Dynamics, Sunnyvale, CA, U.S.A.) for 48 h. All samples were prepared relative to the insulin-stimulated samples (i.e. insulin was set to 100 %).

Akt Western blotting

Akt immunoprecipitated samples were prepared for SDS/PAGE by the addition of 2 × Laemmli sample buffer, and boiled for 2 min. The samples, together with molecular-mass markers (Sigma, St Louis, MO, U.S.A.), were loaded on an SDS/7.5 % polyacrylamide gel. Resolved samples were transferred to a PVDF membrane (Bio-Rad, Hercules, CA, U.S.A.). The membranes were rinsed with water and blocked in 5 % (w/v) non-fat dry milk in Tween Tris-buffered saline (TTBS), pH 7.5, for 2 h. The membranes were rinsed in TTBS, and incubated overnight with anti-Akt antibody. The membranes were rinsed in TTBS and incubated in horseradish peroxidase-coupled goat anti-rabbit antibody (Cappel, Durham, NC, U.S.A.) for 2 h. The membranes were then rinsed in TTBS, and resolved bands were detected using an enhanced chemiluminescence system (Amersham).

Statistical analysis

The data were analysed by analysis of variance to test the effect of different treatments (basal, basal plus KN-62, with insulin, with insulin plus KN-62, with hypoxia, and with hypoxia plus KN-62) on muscle glucose uptake, muscle glycogen and glucose transporter distribution. When a significant F ratio was obtained, the Newman–Keuls *post hoc* test was employed to identify statistically significant differences ($P < 0.05$) between the values of the means.

RESULTS

2DG transport

Initially we performed experiments based on concentration dependency to determine the concentrations of KN-62 that inhibited maximally hypoxia and insulin-stimulated 2DG transport in isolated rat soleus strips (Figure 1). Maximal inhibition of hypoxia-stimulated 2DG transport had already been achieved with a concentration of KN-62 of 1 μM, whereas insulin-stimulated 2DG transport required 100 μM KN-62 for maximal inhibition. Because concentrations of KN-62 higher than 10 μM have been shown to have non-specific effects on other kinases [18], we chose to use a concentration of 10 μM for all other experiments.

We then compared the effects of KN-62 on 2DG transport in isolated soleus strips with those in intact epitrochlearis muscles (Figures 2a and 2b). In isolated soleus muscles, 10 μM KN-62 inhibited hypoxia-stimulated 2DG transport by 40%, and insulin-stimulated 2DG transport by 46%. KN-04, a structural analogue of KN-62 that does not inhibit CAMKII, did not significantly affect 2DG transport in isolated soleus muscles (Table 1). Similarly, in isolated epitrochlearis muscles, KN-62 inhibited hypoxia-stimulated 2DG transport (with 38% inhibition) and insulin-stimulated 2DG transport (with 37% inhibition) to a similar extent as that found in soleus muscle (Table 2). Although basal 2DG transport was slightly lowered by KN-62 in the soleus and epitrochlearis muscles, this effect was not statistically significant.

Cell-surface GLUT4 labelling

In order to examine the role of GLUT4 translocation in the effect of KN-62 on hypoxia and insulin-stimulated 2DG transport, we measured cell-surface GLUT4 photolabelling in isolated soleus muscle strips (Figures 2A and 2B). In isolated soleus muscles, 10 μM KN-62 inhibited both the hypoxia- and insulin-stimulated increases in cell-surface GLUT4 to a slightly greater extent (54 and 49% for hypoxia and insulin respectively) than the inhibition of 2DG transport (40% and 46% for hypoxia and insulin respectively); however, the differences in the percentage inhibitions for either condition between 2DG transport and photolabelling were not statistically significantly different.

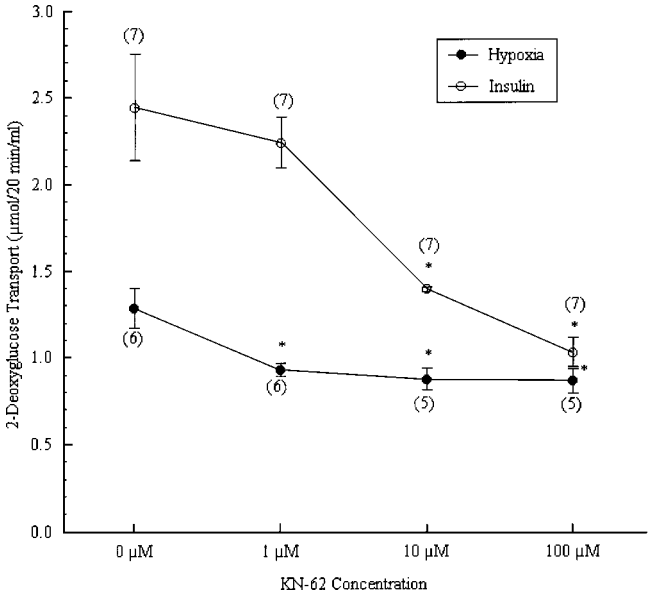

Figure 1 Concentration dependency of effects of KN-62 on hypoxia- and insulin-stimulated 2DG transport in isolated rat soleus muscles

Muscles were incubated for 1 h at 35 °C in KHB containing 0.1% (w/v) BSA, 32 mM mannitol and 8 mM glucose. Muscles were then incubated in the presence or absence of hypoxia or maximal insulin (13.3 nM), and increasing concentrations of KN-62, as noted. The incubated muscles were washed and 2DG transport was measured as described in the Experimental section. Values are given as means ± S.E.M. Numbers in parentheses indicate the number of observations. *Significant difference compared with the corresponding condition in the absence of KN-62 ($P < 0.05$).

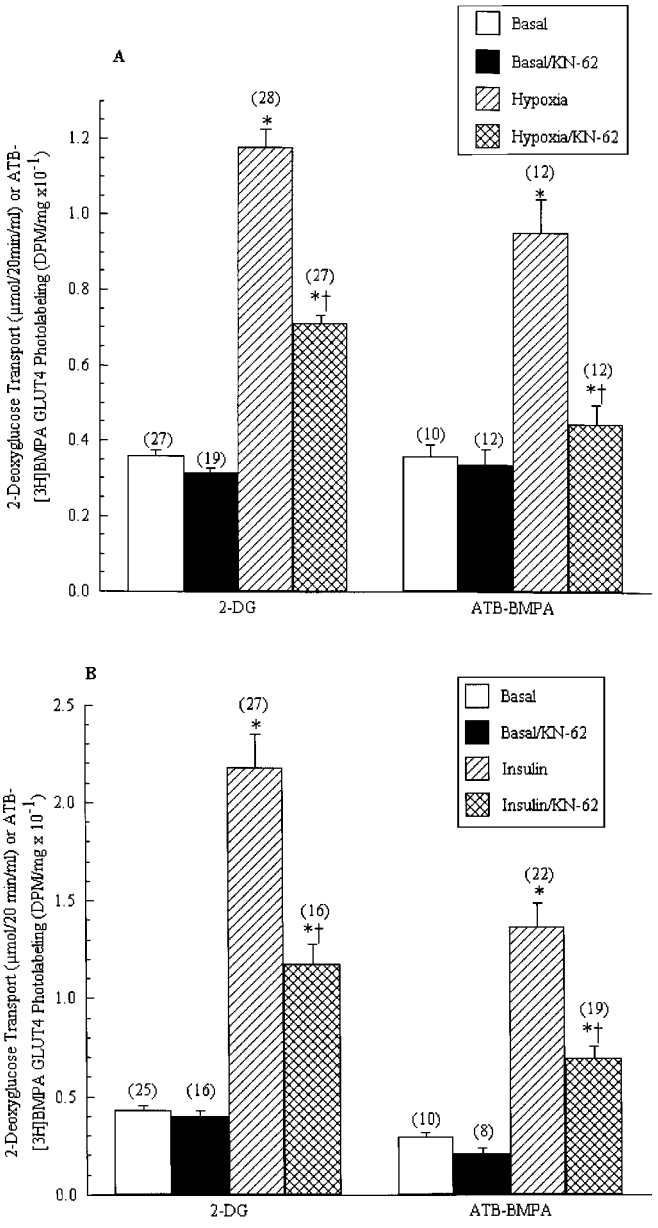

Figure 2 Hypoxia (A)- and insulin (B)-stimulated 2DG transport and cell-surface GLUT4 photolabelling in isolated rat soleus muscles

Muscles were incubated as described in the legend to Figure 1. The incubated muscles were then used either for 2DG transport or ATB-BMPA cell-surface GLUT4 photolabelling, as described in the Experimental section. Values given are means ± S.E.M. Numbers in parentheses indicate the number of observations. * Significant difference from basal ($P < 0.05$); † significant difference from the corresponding condition minus KN-62 ($P < 0.05$).

Table 1 The effect of KN-62 and KN-04 on hypoxia- and insulin-stimulated 2DG transport and glycogen levels in isolated rat soleus muscles

Muscles were incubated for 1 h in KHB containing 0.1% BSA, 8 mM glucose, 32 mM mannitol, and plus or minus hypoxia, maximal insulin (13.3 nM), and either 10 μM KN-04 or KN-62. Muscles were then washed, and 2DG transport and glycogen levels were measured as described in the Experimental section. The numbers of observations are indicated in parentheses. Values shown are means $\pm$ S.E.M. ND, not determined.

	Condition		
Muscle parameter measured	Basal	+ Hypoxia	+ Insulin
(a) 2DG transport (μmol/20 min per ml)			
No further addition	0.237 $\pm$ 0.021 (21)	0.831 $\pm$ 0.060 (19)*	1.605 $\pm$ 0.202 (6)*
+ KN-04	ND	0.762 $\pm$ 0.053 (15)	1.628 $\pm$ 0.186 (5)
+ KN-62	0.222 $\pm$ 0.085 (9)	0.616 $\pm$ 0.104 (5)*†‡	0.939 $\pm$ 0.103 (5)*†‡
(b) Glycogen (μmol/g)			
No further addition	16.70 $\pm$ 1.00 (28)	7.83 $\pm$ 0.64 (19)*	20.85 $\pm$ 3.00 (11)*
+ KN-62	13.77 $\pm$ 0.88 (13)	6.93 $\pm$ 1.23 (5)*	19.92 $\pm$ 2.93 (10)*

* Significant difference from corresponding basal ($P < 0.05$); † significant difference from corresponding condition minus KN-62 or KN-04 ($P < 0.05$); ‡ significant difference from corresponding condition plus KN-04 ($P < 0.05$).

Table 2 The effect of KN-62 and KN-04 on hypoxia- and insulin-stimulated 2DG transport and glycogen levels in isolated rat epitrochlearis muscles

Muscles were incubated for 1 h in KHB containing 0.1% (w/v) BSA, 8 mM glucose, 32 mM mannitol, and plus or minus hypoxia, maximal insulin (13.3 nM), and either 10 μM KN-04 or KN-62. Muscles were then washed, and 2DG transport and glycogen were measured as described in the Experimental section. The numbers of observations are indicated in parentheses. Values shown are means $\pm$ S.E.M. ND, not determined.

	Condition		
Muscle parameter measured	Basal	+ Hypoxia	+ Insulin
(a) 2DG transport (μmol/20 min per ml)			
No addition	0.191 $\pm$ 0.021 (20)	0.698 $\pm$ 0.084 (16)*	0.844 $\pm$ 0.053 (17)*
KN-04	ND	0.642 $\pm$ 0.033 (7)	0.744 $\pm$ 0.053 (7)
KN-62	0.146 $\pm$ 0.020 (21)	0.430 $\pm$ 0.033 (11)*†‡	0.534 $\pm$ 0.032 (11)*†‡
(b) Glycogen (μmol/g)			
No addition	28.77 $\pm$ 2.10 (15)	7.48 $\pm$ 1.55 (12)*	31.92 $\pm$ 1.61 (16)
+ KN-62	26.84 $\pm$ 2.79 (15)	4.15 $\pm$ 1.00 (8)*	27.62 $\pm$ 3.09 (7)

* Significant difference from corresponding basal ($P < 0.05$); † significant difference from corresponding condition minus KN-62 ($P < 0.05$); ‡ significant difference from corresponding condition plus KN-04 ($P < 0.05$).

Muscle glycogen content

In order to assess if KN-62 had a non-specific effect on glycogen breakdown via inhibition of phosphorylase kinase (another calmodulin-sensitive enzyme), we measured the glycogen content of isolated soleus strips and epitrochlearis muscles (Tables 1 and 2). KN-62 had no effect on hypoxia-stimulated glycogen breakdown in either muscle type. Furthermore, KN-62 had no effect on glycogen levels in basal or insulin-stimulated soleus or epitrochlearis muscles (Tables 1 and 2).

^{45}Ca efflux

Since KN-62 has been shown to inhibit calcium channels in some cell types, we measured ^{45}Ca release from soleus muscle strips that were pre-loaded with ^{45}Ca. KN-62 had no effect on hypoxia-stimulated ^{45}Ca release (Figure 3). If one assumes a single exponential decrease in ^{45}Ca content, as described previously by Clausen et al. [20,29] and Youn et al. [9], then the content of ^{45}Ca after incubation is equal to ^{45}Ca content before incubation $\times$ $e^{-(\text{fractional efflux rate} \times 60\ \text{min})}$. Hypoxia in both the presence and absence of KN-62 increased the fractional efflux rate by approx. 2-fold (control, 0.41%; hypoxia, 0.8%; hypoxia plus KN-62, 0.9%).

Insulin-stimulated Akt and PI-3K activity

Since PI-3K has previously been shown to be necessary for insulin-stimulated GLUT4 translocation, we also measured insulin-stimulated PI-3K activity in the presence and absence of KN-62. KN-62 had no effect on insulin-stimulated PI-3K activity (in units of c.p.m./20 min per mg of enzyme: basal, 933 $\pm$ 226; insulin, 3585 $\pm$ 533; insulin + KN-62, 3573 $\pm$ 546). Moreover, to look at a further downstream member of the insulin-signalling cascade, we also measured insulin-stimulated Akt activity. Akt activity in insulin-stimulated muscles was unaffected by KN-62 (in relative PhosphorImager units: basal, 58.5 $\pm$ 3.9; insulin, 100 $\pm$ 4.9; insulin $-$ KN-62, 91.0 $\pm$ 2.5,). Additionally, KN-62 did not affect the insulin-induced shift in electrophoretic mobility observed in the Akt band on SDS/PAGE (results not shown).

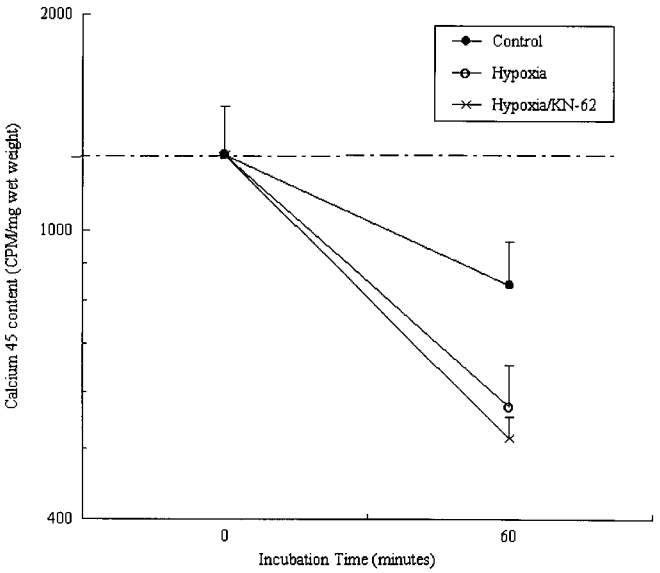

Figure 3 ^{45}Ca efflux from isolated rat soleus muscles

Muscles were initially incubated for 1 h in KHB as described in the legend to Figure 1, except that the KHB contained 1 μCi/ml ^{45}Ca. The muscles were then washed extensively and processed as described in the Experimental section. The dot-dashed line represents ^{45}Ca content before incubation. The *y*-axis is plotted on a logarithmic scale, where the slope of each line represents the fractional ^{45}Ca efflux rate under each respective condition. Values given are means $\pm$ S.E.M for six muscles per group.

DISCUSSION

Many previous studies have indicated a role for calmodulin in the signalling of both insulin- and contraction-stimulated glucose transport. The results of the present study support this hypothesis in skeletal muscle. Previous studies that suggested a role for calmodulin in activation of glucose transport by these stimuli used the calmodulin antagonists W-7 or CGS 9343B [10,21]. However, these compounds have non-specific effects on other protein kinases and, because calmodulin interacts with multiple protein targets, it is unclear which of these proteins were inhibited by these antagonists [10,11,23,30]. In the present study, we have investigated the role of calmodulin in the stimulation of glucose transport by hypoxia and insulin by utilizing the CAMKII inhibitor KN-62, which, unlike CGS 9343B or W-7, inhibits calmodulin-sensitive kinases by binding to the calmodulin-binding site of the kinase, rather than to calmodulin itself [18]. Since the interaction of calmodulin and each target enzyme is thought to be unique, this gives the advantage of inhibiting only one enzyme, rather than a whole series of calmodulin-sensitive signalling processes [11].

Despite the relatively large amount of information known about insulin signalling of glucose transport, little is known about the mechanism whereby contraction or hypoxia activates glucose transport, although previous studies have pointed to the involvement of a rise in intracellular calcium in mediating this effect [4,7,8,21]. Thus the activation of CAMKII by the rise in cytoplasmic calcium that occurs following membrane depolarization made this kinase an attractive candidate as a mediator of hypoxia/contraction-stimulated glucose transport in muscle [13, 31,32]. In agreement with this hypothesis, 10 μM KN-62 inhibited hypoxia-stimulated 2DG transport by 40 % in isolated soleus muscles. Furthermore, this effect was not limited to the slow-twitch fibres of the soleus, as KN-62 inhibited 38 % of hypoxia-stimulated 2DG transport in the epitrochlearis muscle, which is comprised exclusively of fast-twitch fibres.

Although previous studies have suggested that a rise in intracellular calcium plays a role in the stimulation of glucose transport by hypoxia/contraction [7,9,21], it is not clear whether calcium/calmodulin plays a role in insulin stimulation of glucose transport [8,9,22,29]. We therefore reasoned that, since CAMKII is activated by a rise in intracellular calcium, KN-62 would only inhibit the hypoxia/contraction-stimulated pathway of glucose transport [13,32]. In contrast with this hypothesis, 10 μM KN-62 inhibited insulin-stimulated glucose transport activity by 46 % in soleus and 37 % in epitrochlearis muscles. In support of these data, several previous studies have suggested interactions between calmodulin and the insulin-signalling cascade via phosphorylation of calmodulin by the receptor tyrosine kinase, by association of the IRS proteins with calmodulin, or direct interaction with/activation of PI-3K [23,33–36]. Nevertheless, it is unclear from these data how CAMKII could be involved in insulin signalling. It could be speculated that phosphorylation of calmodulin by the insulin receptor tyrosine kinase, or interaction of calmodulin with IRS-1, could lower its threshold for activation by calcium, thereby allowing for calmodulin's activation at a basal cellular calcium level, and its subsequent binding to and activation of CAMKII [35,37]. This remains to be determined by further studies; however, these data do provide evidence of an interaction between CAMKII and a common step in insulin and contraction-/hypoxia-signalling that leads to stimulation of glucose transport.

It is interesting to note that hypoxia-stimulated 2DG transport appears to be more sensitive to inhibition by KN-62 than insulin-stimulated transport. A 1 μM concentration of KN-62 inhibited hypoxia-stimulated 2-DG transport by 27 %, but did not significantly affect insulin-stimulated 2DG transport. This would seem to suggest that CAMKII might have a greater involvement in hypoxia/contraction-stimulated transport than in insulin-stimulated transport. However, higher concentrations of KN-62 did not produce further inhibition of hypoxia-stimulated glucose transport in the soleus, whereas insulin-stimulated transport was further inhibited. Moreover, in isolated epitrochlearis muscles (a fast-twitch muscle in which transport is less sensitive to stimulation by insulin than the soleus [38]), 10 μM KN-62 inhibited hypoxia- and insulin-stimulated transport by a similar extent (38 % compared with 37 %). Therefore a more likely hypothesis is that CAMKII might play an equal role in both hypoxia/contraction- and insulin-stimulated glucose transport, but that this was not observed in the soleus muscle, owing to its lower capacity for hypoxia-stimulated transport.

The results of the present study would seem to conflict with those of Youn et al. [21], who found that the calmodulin antagonist W-7 inhibited submaximal, but not maximally insulin-stimulated, glucose transport. Additionally, W-7 had no effect on either submaximal or maximal hypoxia-stimulated transport. However, as mentioned previously, W-7 is a general calmodulin antagonist, which exhibits non-specific effects on other kinases [10], whereas KN-62 binds to the calmodulin-binding site of CAMKII alone. Therefore W-7 will affect various calmodulin sensitive processes throughout the cell, while KN-62 will not [18]. The action of W-7 and KN-62 on muscle calcium levels also differs: while W-7 increases intracellular calcium levels [21], KN-62 does not seem to alter this parameter, as evidenced by the lack of an effect of KN-62 on glycogen concentration in basal epitrochlearis or soleus muscles. Moreover, our results are in agreement with the those of Shashkin et al. [10], who used a more specific calmodulin antagonist (CGS 9343B) and found that,

similar to our results, this compound inhibited both insulin- and hypoxia-stimulated glucose transport. Therefore we believe the differences between our results and those of Youn et al. [21] are due to the non-specific nature of W-7.

The mechanism whereby inhibition of CAMKII would inhibit glucose transport could involve inhibition of GLUT4 translocation, or alterations in its transport activity. However, previous work from our laboratory using the ATB-BMPA glucose transporter photoaffinity label has shown that both the hypoxia- and insulin-stimulated increases in glucose transport in skeletal muscle can be explained by corresponding increases in cell-surface GLUT4 [25]. Consistent with our previous findings, increases in glucose transport activity in the present study are accompanied by increases in cell-surface GLUT4. In turn, the inhibitory effects of KN-62 are accompanied by decreases in the cell-surface photolabelling of GLUT4, although the inhibition of photolabelling is somewhat greater than the inhibition of glucose transport activity. This discrepancy could reflect differences in the sensitivities of the two methods and/or differential effects of the interactions between GLUT4 and transportable (2DG) and non-transportable (ATB-BMPA) substrates. Although previous studies have shown that it is possible to photolabel GLUT4 contained in occluded vesicles that have no transport activity [39], it is unlikely that KN-62 could inhibit photolabelling without a corresponding inhibition of glucose transport activity, since the photolabel binds to the same site as glucose. A second possibility would be that KN-62 prevented the interaction of the photolabel with the GLUT4 transporters. However, this is also unlikely, since KN-62 was not included in the incubation medium with the photolabel. Finally, it is also possible that the KN-62-mediated inhibition of GLUT4 translocation is compensated for by an increase in GLUT4 activity, which, in turn, causes a mismatch between glucose transport and cell-surface labelling. Which of these possibilities is correct remains to be determined by further investigation; however, we believe that our data suggest a semi-quantitative inhibition of hypoxia- and insulin-stimulated GLUT4 translocation with CAMKII inhibition

It is possible that the effects of KN-62 on hypoxia- and insulin-stimulated glucose transport activity are mediated by non-specific effects on other protein kinases or ion channels. Owing to the fact that several studies have shown that KN-62 blocks calcium channels in other cell types [40,41], it was conceivable that the effects of KN-62 on hypoxia-stimulated glucose transport were due to an inhibition of sarcoplasmic-reticulum calcium-release channels, similar to the effect of dantrolene, which inhibits hypoxia/contraction-stimulated glucose transport [7,21]. However, KN-62 did not influence hypoxia-stimulated ^{45}Ca release, which suggests that KN-62 has no effect on hypoxia-stimulated sarcoplasmic-reticulum calcium release [9,21,22]. Furthermore, KN-62 did not influence hypoxia-stimulated glycogen depletion, indicating that KN-62 is not inhibiting phosphorylase kinase via a non-specific interaction with this calmodulin-binding site of this enzyme.

With regard to insulin-stimulated glucose transport, we have also failed to find a non-specific effect of KN-62 on two important parts of the insulin-signalling pathway (Akt or PI-3K activity). A second possibility for a non-specific inhibition of insulin-stimulated transport would be that KN-62 caused a sustained increase in intracellular calcium levels (which have been shown to inhibit insulin-stimulated glucose transport) via inhibition of the sarcoplasmic-reticulum Ca^{2+}-ATPase pump [21,42]. However, the fact that KN-62 did not alter basal glycogen levels argues against this interpretation, since a sustained elevation of intracellular calcium would be expected to cause glycogen depletion via activation of phosphorylase [7,42]. Therefore, although we have not measured hypoxia- or insulin-stimulated CAMKII activity in skeletal muscle, we believe these data argue for a specific effect of KN-62 on CAMKII (or a CAMKII-like enzyme) that is involved in the stimulation of skeletal-muscle glucose transport by hypoxia/contraction and insulin.

There are two possible sites at which CAMKII or a similar calmodulin-dependent protein kinase could function in insulin- and hypoxia-stimulated GLUT4 translocation: vesicle docking and fusion, or inhibition of translocation itself. With regard to vesicle docking and fusion, several recent studies have shown that calmodulin is involved in regulated secretion in neurons [43–45], possibly at the level of the interaction between various vesicle-soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors (v-SNAREs) and target-soluble (t)-SNAREs, which comprise the core of the exocytic fusion machine [43,44,46]. Moreover, Colombo et al. [44] have shown that inhibitors of CAMKII directly inhibit endosome fusion in an *in vitro* fusion assay. A second possibility is that CAMKII is involved in regulation of GLUT4 vesicle translocation. Therefore it is of interest to note that our data are strikingly similar to those of Schweitzer et al. [15], who showed that KN-62 inhibited approx. 50 % of the carbachol-induced secretion of noradrenaline (norepinephrine) from PC-12 cells, and concluded that noradrenaline release contained CAMKII-dependent and -independent components [15]. Furthermore, these authors hypothesized that KN-62 inhibited the release of a reserve population of neurotransmitter vesicles that were anchored to the cytoskeleton [15]. In neuronal cells, the major substrate of CAMKII is synapsin, whose function is to 'tether' neurosecretory vesicles to the cytoskeleton. Upon phosphorylation of synapsin by CAMKII, the tether is released and the neurosecretory vesicles translocate to the plasma membrane, where they dock and fuse with the membrane [47]. Although there is no synapsin in skeletal muscle, a homologous protein, CLIP 170, has been cloned from other non-neuronal cell types [48,49]. Whether a similar protein exists in skeletal muscle remains to be determined.

In the light of these data, it is interesting to note that Oattey et al. [50] have recently shown that there appears to be a portion of the GLUT4 vesicles that are in close proximity with the plasma membrane, and a second pool of GLUT4 vesicles that, under basal conditions, are tethered to the cytoskeleton, and are released from this tether upon insulin stimulation [50]. It is therefore tempting to hypothesize that there might be two populations of GLUT4 vesicles, one in close proximity with the plasma membrane ('predocked') and a reserve population that is tethered to the cytoskeleton through a synapsin like protein. Analogous with the results of Schweitzer et al. [15], the vesicles in close proximity with the plasma membrane would give an immediate response, but translocation of the tethered vesicles would be needed to give a maximal transport response in response to hypoxia/contraction or insulin. Thus inhibition of CAMKII or a similarly involved protein kinase by KN-62 would only produce a partial inhibition of transport. The known conservation of the molecular machinery for regulated secretion from neurons to insulin-sensitive cells could indicate that, analogous with neuronal cells, CAMKII-dependent and -independent components function in GLUT4-containing vesicle translocation [51,52].

In conclusion, the present study demonstrates that, in isolated rat skeletal muscle, CAMKII or a similar enzyme is involved in stimulation of glucose transport by both hypoxia/contraction and insulin, and suggests that CAMKII might be involved in the regulation of translocation of GLUT4-containing vesicles to the plasma membrane from their intracellular storage site. The exact role of CAMKII in GLUT4 vesicle trafficking, and its mechanism

of involvement, remain to be determined by further study. Nevertheless, the present data provide an insight into the possible mechanisms of CAMKII involvement in hypoxia/contraction- and insulin-stimulated glucose transport.

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