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Characterization of K_{ATP}-channels in rat basilar and middle cerebral arteries: Studies of vasomotor responses and mRNA expression

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Abstract

Changes in the activity of K^+ channels represent a major mechanism that regulates vascular tone. Cerebrovascular adenosine 5′-triphosphate-sensitive K^+(K_{ATP}) channels were characterized in studies of the molecular expression and vasomotor reactivity to different K_{ATP} channel openers in rat basilar and middle cerebral arteries. Both arteries showed strong mRNA expression of the subunits of the pore-forming inward-rectifying K^+ channel type 6.1 (Kir6.1), Kir6.2 and the connected sulfonylurea receptor (SUR) subunits, SUR1 and SUR2B, while only weak bands for SUR2A were seen. The K_{ATP} channel openers induced relaxation of prostaglalndin $F_{2\alpha}$-precontracted isolated basilar and middle cerebral arteries with the order of potency *N*-Cyano-*N*-(1,1-dimethylpropyl)-*N*″-3pyridylguanidine (P-1075)>levcromakalim>*N*-(4-Phenylsulfonylphenyl)-3,3,3-trifluoro-2-hydroxy-2-methylpropanamide (ZM226600)>pinacidil>diazoxide. The responses induced by levcromakalim, ZM226600 and diazoxide were significantly more potent in basilar arteries than in middle cerebral arteries, while pinacidil and P-1075 were equipotent. Endothelium removal decreased ($P<0.05$) the sensitivity (pIC_{50}) of basilar arteries, but not of middle cerebral arteries, to pinacidil, levcromakalim, P-1075 and ZM226600. The maximum relaxant response to P-1075 was stronger ($P<0.005$) in basilar arteries with endothelium than without endothelium. Correlation of the relaxant potency of K_{ATP} channel openers in rat basilar and middle cerebral arteries with historical measurements of affinity obtained in COS-7 cell lines expressing either SUR1, SUR2A or SUR2B showed that vasodilatation by K_{ATP} channel openers correlated with binding to either the SUR2A or the SUR2B subunit. Glibenclamide was a blocker of relaxation induced by pinacidil, levcromakalim, P-1075 and ZM226600 in basilar arteries. Only a weak antagonistic effect of glibenclamide on pinacidil-, levcromakalim- and ZM226600-induced relaxations was found in middle cerebral arteries. The subunit profile and the observed pharmacological properties suggest that the K_{ATP} channels expressed in rat basilar and middle cerebral artery are likely to be composed of SUR2B co-associated with Kir6.1 or Kir6.2. In basilar arteries, but not in middle cerebral arteries, endothelial K_{ATP} channels may be involved.

Keywords: ATP-sensitive K^+ channels (Adenosine 5′-triphosphate-sensitive K^+channels); Cerebral artery; Vasodilatation; Endothelium; Vascular smooth muscle; (Rat)

1. Introduction

Adenosine 5′-triphosphate-sensitive K^+(K_{ATP}) channels have been described in diverse cell types, including cardiomyocytes (Noma, 1983), pancreatic β cells (Ashcroft et al., 1984), neurons (Ashford et al., 1988), skeletal (Spruce et al., 1985) and vascular smooth muscle cells (Nelson et al., 1990; Standen et al., 1989), where they play important physiological and pathophysiological roles. K_{ATP} channels respond to changes in the cellular metabolic state as well as to a number of endogenous vasodilators (Nelson and Brayden, 1993). Dissociation of ATP and thus activation of K_{ATP} channels causes hyperpolarization of smooth muscle cells (Brayden, 2002) and endothelial cells (Luckhoff and Busse, 1990). In vascular smooth muscle cells, hyperpolarization prevents the opening of depolarization-activated Ca^{2+} channels, thus blocking calcium entry to the cell resulting in vasodilatation (Quast, 1996). In endothelial cells, the hyperpolarization elevates the concentration of intracellular calcium and thereby promotes the Ca^{2+}-dependent formation of nitric oxide (NO) (Luckhoff and Busse, 1990). In contrast, other reports have shown that bovine and

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Table 1
Primers designed for the detection of mRNA expression for *Kir 6.1*, *Kir 6.2*, *SUR1*, *SUR2A*, *SUR2B* and *GAPDH*

NCBI GeneBank		Primers (5′–3′)		Amplified sequence	
Gene	Acc. No	Forward	Reverse	Seq. No.	Size
Kir 6.1	NM017099	GAAGGAGAGGTGGTGCTATTCA	GTTGCTCCTCCTCATGGAGTTGT	888–1367	480
Kir 6.2	NM031358	GCCATCATCCTTCCACCTCAGTT	CCAGGCACTTCCGAAGCAAGTAT	2921–3223	303
SUR1	X97279	CTCGCCGTCGTGTGCTACTTCAT	GCCAGGTTCCTCACCATCCAGTT	3511–3908	398
SUR2A	D83598	GTTCTGCCTGGCCAGGGCC	GTCTACTTGTTGGTCATCACCAAA	4626–4910	285
SUR2B	AF019628	CTTCGGCCTGCTGAGACTGAAGA	GCATCGGTGACAGCCTTGTACCT	2242–2618	377
GAPDH	M17701	TAAAGGGCATCCTGGGCTACACT	CCTACATGGCCTCCAAGGAGTAA	833–1032	200

Each set of forward and reverse primers was designed from GeneBank extracted sequences corresponding to the shown accession numbers (Acc. No.). The primer sequences, the amplified part of the GeneBank sequence (Seq. No.) and the size of the amplified sequences are shown.

guinea pig coronary endothelial cells do not significantly hyperpolarize in response to K^+ channel openers (Gasser et al., 1998).

Several studies using synthetic K_{ATP} channel openers and sulfonylurea inhibitors suggest that K_{ATP} channels are functional in cerebral blood vessels (Kitazono et al., 1995). In situ application of direct activators of K_{ATP} channels causes dilator effects in rat basilar arteries (Faraci and Heistad, 1993; Toyoda et al., 1997a) and cerebral arterioles (Wahl et al., 1994). The functionality of K_{ATP} channels has also been shown in vitro in rat basilar arteries (Ksoll et al., 1991). In contrast to these findings, some studies failed to obtain functional evidence supporting the presence of K_{ATP} channels in middle cerebral arteries (McCarron et al., 1991; McPherson and Stork, 1992). The reason for these differences is not clear but was suggested to relate to a heterogeneous distribution of K_{ATP} channels in the rat cerebral circulation (McPherson and Stork, 1992).

The K_{ATP} channels are thought to be a heteromultimeric (tetrameric) complex of two subunits. One of them is the pore-forming inward-rectifying K^+ channel type 6.x (Kir6.x) in which 6.x can be either 6.1 or 6.2, and the other is a sulfonylurea receptor (SUR) that belongs to the ATP-binding cassette superfamily. Two different SURs have been found, SUR1 and SUR2 that are encoded by two genes containing 39 and 38 exons, respectively. In addition, the *SUR2* gene has been shown to undergo alternative splicing at exon 38, generating the two splice variants SUR2A and SUR2B (Yokoshiki et al., 1998).

Recently, Kir6.1/SUR2B was suggested to be the major K_{ATP} channel in cultured basilar arterial smooth muscle cells (Li et al., 2003; Santa et al., 2003). The presence of other Kir6.x and SUR subunits has however, not been determined in mRNA from fresh cerebral arteries.

The aims were therefore in rat cerebral arteries to: 1. Describe the subunit composition of K_{ATP} channels; 2. Pharmacologically characterize K_{ATP} channels and compare the pharmacological properties to the subunit composition; 3. Compare subunit composition and pharmacology in basilar and middle cerebral arteries.

2. Methods

2.1. Vasomotor responses

The experimental protocol was approved by the Danish committee for experiments with animals.

Young male Sprague–Dawley (Tac) rats (300–380 g, Taconic M&B, Denmark) were exsanguinated during CO_2 anaesthesia. The brains were removed and the basilar and middle cerebral arteries were carefully dissected out under an operating microscope. Each vessel was cut into 1- to 2-mm long circular segments and placed in an ice-cold buffer solution gassed with 5% CO_2 in O_2. The composition of the buffer was

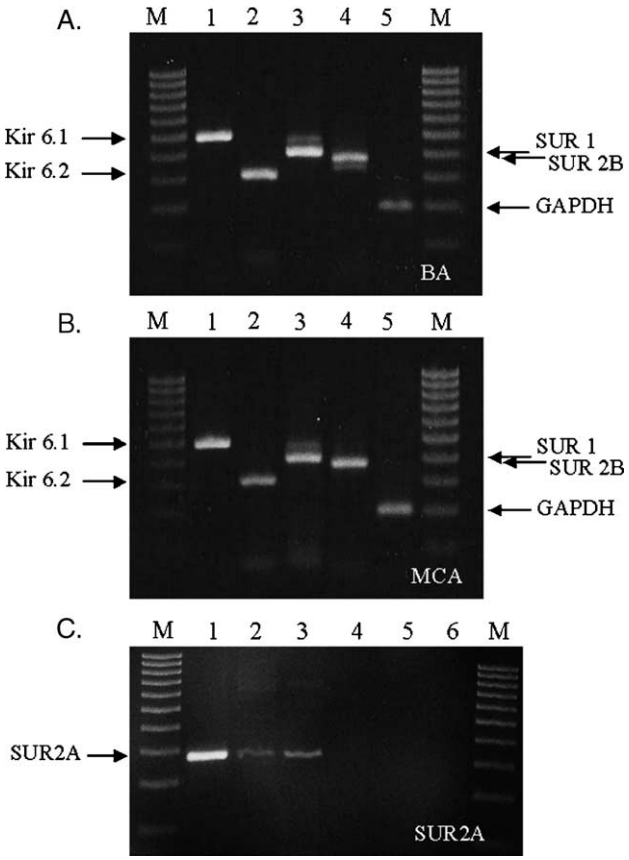

Fig. 1. Expression of mRNA for Kir6.1, Kir6.2, SUR1 and SUR2B in rat basilar (A) and middle cerebral (B) arteries as demonstrated by RT-PCR. Lanes M: Molecular weight marker showing 100 base pair ladder. Bands corresponding to the presence of mRNA encoding Kir6.1 (480 bp; lanes 1), Kir6.2 (303 bp; lanes 2), SUR1 (398 bp; lanes 3) and SUR2B (377 bp; lanes 4) are found in basilar and middle cerebral arteries. The housekeeping gene *GAPDH* is shown in lanes 5. No bands are seen in the negative controls where mRNA was not reverse transcribed to cDNA prior to amplification (not shown). In (C) the expression of mRNA for SUR2A is shown in rat heart (lane 1), rat basilar artery (lane 2) and middle cerebral artery (lane 3). No bands are seen in the negative controls for heart (lane 4), basilar artery (lane 5) and middle cerebral artery (lane 6).

(mM): NaCl 119, $NaHCO_3$ 15, KCl 4.6, $CaCl_2$ 1.5, NaH_2PO_4 1.2, $MgCl_2$ 1.2, and glucose 5.5. The buffer was continuously aerated with oxygen enriched with 5% CO_2, resulting in a pH of 7.4. Some experiments were performed in the absence of endothelium. These vessel segments were perfused with a buffer solution containing 0.1% Triton X-100 for 15 s (Hamel et al., 1987). The absence of endothelium was always checked by the lack of a dilator response to 10^{-5} M carbachol (Furchgott and Zawadzki, 1980).

In order to determine vessel tension, each segment was mounted on two metal wires 40 μm in diameter in a myograph (Model 610M, Danish Myo Technology, Denmark). The buffer solution was continuously aerated with 5% CO_2 in O_2 to maintain a stable pH of 7.4. The artery segments were allowed to equilibrate for approximately 30 min. The vessels were stretched to the internal circumference the vessel would have if relaxed and exposed to a passive transmural pressure of 100 mmHg (13.3 kPa) for the basilar artery and 52 mmHg (7.0 kPa) for the middle cerebral artery. This was in order to achieve maximal active force development (Nyborg et al., 1987). Following a second 30-min equilibration period, the vessels were constricted twice with 63 mM KCl in a modified buffer solution in which NaCl was substituted for KCl on an equimolar basis. The contraction amounted to 1.9±0.08 mN (n=89) in basilar and 0.70±0.07 mN (n=64) in middle cerebral arteries with endothelium and to 1.5±0.1 mN (n=49) in basilar and to 0.48±0.03 mN (n=68) in middle cerebral arteries without endothelium. In order to study the relaxant effect of K_{ATP} openers or 10^{-5} M carbachol (for control of endothelial functionality), the cerebral arteries were pre-contracted with 3×10^{-6} M prostaglandin $F_{2\alpha}$. This concentration was previously shown to induce a contraction of rat basilar and middle meningeal arteries of 60% to 70% of the maximum response (Hogestatt and Uski, 1987). In our preparations, it resulted in a stable tension of 1.4±0.08 mN (n=89) in basilar and 0.69±0.07 mN (n=64) in middle cerebral arteries with endothelium and 1.5±0.1 mN (n=48) in basilar and 0.41±0.03 mN (n=68) in middle cerebral arteries without endothelium, to which the agonist was added in cumulative concentrations. The tension lasted for at least 20–30 min without a significant fall in tone. In blockade experiments, the antagonist was added to the tissue bath 15–20 min before the addition of K_{ATP}-channel opener in increasing concentrations. The addition of glibenclamide did not affect the tension of the vessels. Out of eight tissue segments two served as controls (i.e., without blocker), and the others were treated with blocker in different concentrations.

All concentration–response curves were plotted graphically. I_{max} (maximum relaxant effect obtained with a K_{ATP}-channel opener), pIC_{50} (negative logarithm of the concentration of K_{ATP}-channel opener that elicited half-maximum relaxation) were calculated arithmetically from each individual concentration–response curve. Values are given as means±S.E.M. Number of experiments=n, one or two segments from each rat. The non-parametric, Mann Whitney U-test was used to determine statistical significance between two groups of data. Kruskal–Wallis test was used to determine statistical significance between multiple groups of data with Dunn's multiple comparison test as post-test. Statistical significance was assumed when $P<0.05$.

2.2. Drugs

Stock solutions of diazoxide (10^{-1} M), glibenclamide (5×10^{-1} M) and levcromakalim (10^{-2} M) (Tocris Cookson Inc., UK) were prepared by dissolving the drugs in dimethylsulfoxide (DMSO). Stock solutions of *N*-cyano-*N*-(1,1-dimethylpropyl)-*N*″-3pyridylguanidine (P-1075) (10^{-2} M), *N*-

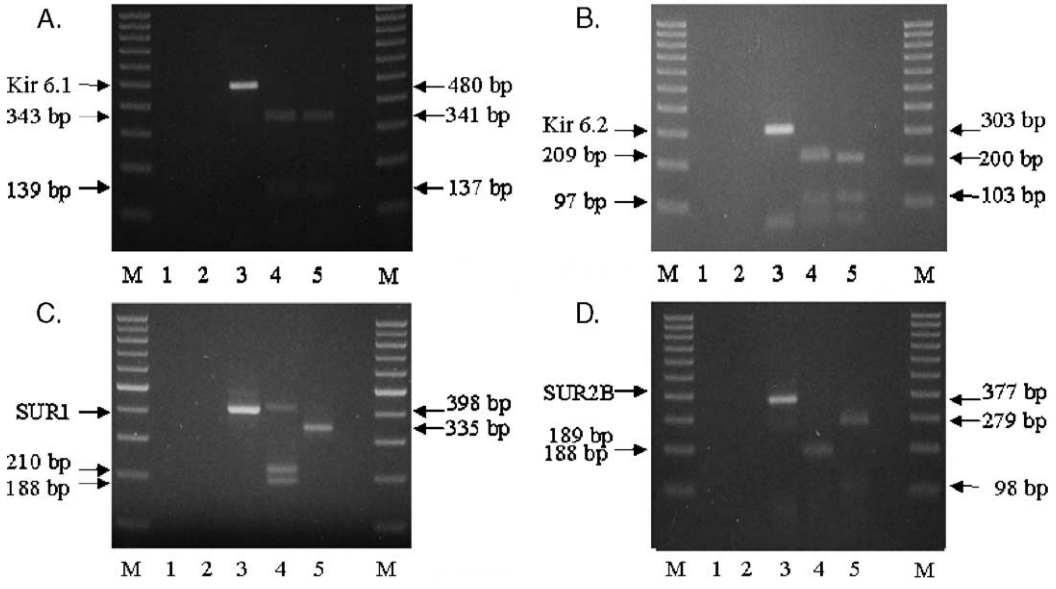

Fig. 2. Identification of mRNA for A) Kir6.1, B) Kir6.2, C) SUR 1 and D) SUR2B in rat cerebral arteries with restriction analysis. Lane M: Molecular weight marker showing 100-base pair ladder. Lane 1: Water control, Lane 2: Negative controls where mRNA was not reverse transcribed to cDNA prior to amplification (lack of RT enzyme). Lane 3: A) Kir6.1, B) Kir6.2, C) SUR1 and D) SUR2B, Lane 4: A) Kir6.1+Hae II, B) Kir6.2+EcoO109I, C) SUR1+Ear I, and D) SUR2B+AlwNI, Lane 5: A) Kir6.1+AlwNI, B) Kir6.2+Pvu II, C) SUR1+Pvu II, and D) SUR2B+EcoRI.

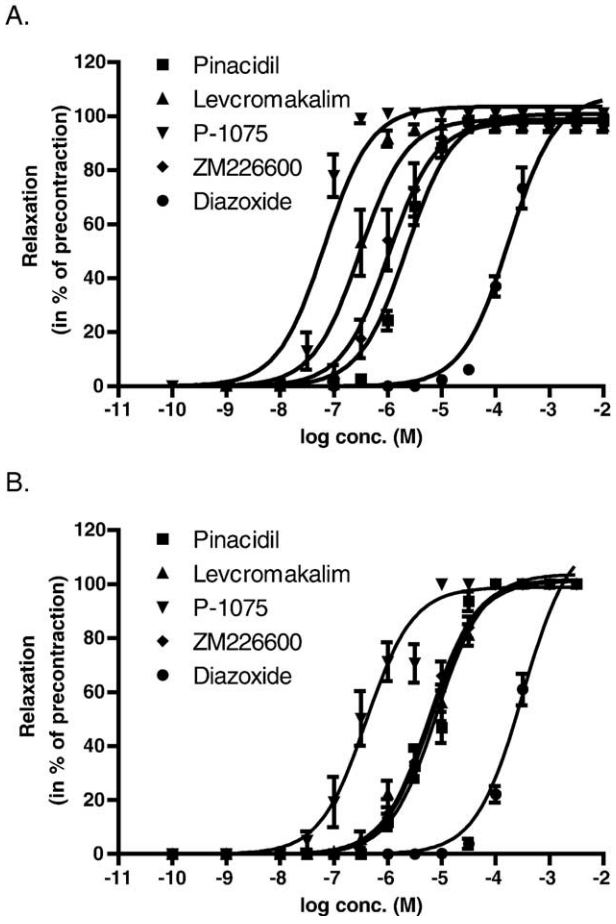

Fig. 3. Relaxation induced by cumulative concentrations of the K_{ATP} channel openers pinacidil, levcromakalim, P-1075, ZM226600 and diazoxide in rat A. basilar and B. middle cerebral arteries. The relaxation of each segment tested was calculated as a percentage of the pre-contraction induced by 3×10^{-6} M prostaglandin $F_{2\alpha}$. Each point represents the mean values with S.E.M. (vertical bars), n=16–11 from 2–8 animals.

(4-Phenylsulfonyl-phenyl)-3,3,3-trifluoro-2-hydroxy-2-methyl-propan-amide (ZM226600) (10^{-1} M) (Tocris Cookson Inc., UK) and carbachol (10^{-2} M) (Sigma, USA) were prepared by dissolving the drugs in ethanol. Pinacidil (kind gift from Leo Pharmaceutical Products, Denmark) was dissolved in 1N HCl and diluted to 10^{-2} M in 0.1 M KH_2PO_4. Prostaglandin $F_{2\alpha}$ (Sigma, USA) was dissolved in distilled water. All stock solutions were stored at −20 °C and further diluted in buffer solution just before the experiment. The concentrations are expressed as the final molar concentration in the tissue bath.

2.3. Reverse transcriptase polymerase chain reaction

Young male Sprague–Dawley (Tac) rats (300–380 g, Taconic M&B, Denmark) anaesthetized with pentobarbital were perfused transcardially with 250 ml ice-cold buffer solution (the same as used in studies of vasomotor responses). Basilar and middle cerebral arteries were carefully dissected out and placed in an autoclaved eppendorf tube containing RNAlater (Ambion (Europe) Ltd, UK) according to the manufacturer's instruction. Total RNA was extracted using Trizol (Invitrogen Life Technologies, Denmark) extraction method. Concentration and purity of RNA were determined spectrophotometrically (GeneQuant pro, Amersham Pharmacia Biotech, Denmark) by measuring absorbency at 260/280 nm. The total RNA was treated with DNAse I (Sigma-Aldrich, MO, USA) according to the manufacturer's instruction and repurified using a RNeasy Minikit (Quiagen Inc. CA, USA). cDNA was synthesized from 60 ng of purified RNA in a 20-µl reaction volume, using the GeneAmp RNA PCR kit (Perkin Elmer, USA). Reverse transcriptase-negative controls were performed for each RNA extract by substituting MuLV Revers Transcriptase enzyme in the reaction mixture with nuclease-free water. Primer sets for the subsequent PCR amplification were designed by Primer Designer 3 software (Scientific and Educational Software, NC, USA), using nucleotide sequences purchased from NCBI nucleotide query. Primers were designed to be specific for Kir6.1, Kir6.2, SUR1, SUR2A, SUR2B and GAPDH (Table 1) and did not cross-hybridize with any other known sequences. Homology with published sequences was checked using NCBI BLAST search.

PCR reactions initiated by the specific primer sets for Kir6.1, Kir6.2, SUR1, SUR2A, SUR2B and GAPDH were carried out with cDNA originating from rat basilar and middle cerebral arteries. In addition, SUR2A was investigated with cDNA originating from rat heart.

Each PCR mixture contained PCR buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3), 1.5 mM $MgCl_2$, 25 mU/µl REDTaq™ DNA Polymerase™ (Sigma-Aldrich, MO, USA) 0.2 mM dNTP mix and 0.2 µM of sense and antisense specific primers corresponding to the sequence of interest. Final volumes were 25 µl including 2 µl of cDNA solution. The reaction mixture

Table 2
I_{max} and pIC_{50} values obtained for relaxant responses to K_{ATP} channel openers in rat basilar and middle cerebral arteries with endothelium

K_{ATP} channel opener	Basilar artery			Middle cerebral artery		
	I_{max}	pIC_{50}	n	I_{max}	pIC_{50}	n
Pinacidil	98 ± 2^{ns}	5.59 ± 0.06^{ns}	15 (6)	93±6	5.30±0.25	3 (3)
Levcromakalim	$98\pm2^{ns,ns}$	$6.58\pm0.09^{a,f}$	9 (6)	88 ± 7^{ns}	5.44 ± 0.25^{ns}	3 (3)
P-1075	$102\pm1^{b,ns}$	$7.17\pm0.07^{ns,f}$	13 (7)	57 ± 10^{ns}	7.01 ± 0.11^{d}	6 (5)
ZM 226600	$97\pm3^{ns,ns}$	$5.98\pm0.09^{a,ns}$	10 (6)	84 ± 6^{ns}	5.31 ± 0.09^{ns}	4 (3)
Diazoxide	$73\pm8^{ns,d}$	$4.04\pm0.04^{b,f}$	13 (2)	71 ± 6^{ns}	3.90 ± 0.02^{d}	13 (4)

Values are given as means±S.E.M. n=number of experiments, number of animals are given in brackets. Statistical analysis (Mann Whitney U-test) was performed comparing agonist responses in basilar arteries to those found in middle cerebral arteries. $^{a}P<0.05$; $^{b}P<0.005$; $^{c}P<0.001$.
Comparison was also made of responses found for the different agonists to the response obtained with pinacidil: $^{d}P<0.05$; $^{e}P<0.005$; $^{f}P<0.001$. ns=non-significant.

Table 3
I_{max} and pIC_{50} values obtained for relaxant responses to K_{ATP} channel openers in rat basilar and middle cerebral arteries without endothelium

K_{ATP} channel opener	Basilar artery				Middle cerebral artery			
	I_{max}	pIC_{50}	n	r.p.	I_{max}	pIC_{50}	n	r.p.
Pinacidil	93 ± 3^{ns}	5.22 ± 0.11^{a}	7 (4)	2.34	96 ± 3^{ns}	5.14 ± 0.09^{ns}	10 (5)	1.45
Levcromakalim	88 ± 6^{ns}	6.32 ± 0.09^{b}	11 (2)	1.82	79 ± 5^{ns}	5.46 ± 0.17^{ns}	8 (6)	0.95
P-1075	58 ± 11^{b}	6.86 ± 0.18^{c}	8 (3)	2.04	58 ± 11^{ns}	6.78 ± 0.13^{ns}	13 (5)	1.70
ZM 226600	98 ± 2^{ns}	5.11 ± 0.08^{b}	9 (2)	7.41	84 ± 8^{ns}	5.43 ± 0.08^{ns}	8 (4)	0.76
Diazoxide	98 ± 1^{ns}	4.35 ± 0.11^{b}	4 (2)	0.49	91 ± 7^{ns}	3.77 ± 0.06^{ns}	8 (4)	1.35

Values are given as means±S.E.M. n=number of experiments, number of animals given in brackets, r.p.=relative potency comparing pIC_{50} values in arteries with endothelium to arteries without endothelium. Statistical analysis (Mann Whitney U-test) was performed comparing responses of each opener in tissue with endothelium to those found in tissue without endothelium. $^{a}P<0.05$; $^{b}P<0.005$; $^{c}P<0.001$, ns=non-significant.

was overlaid with mineral oil (Perkin-Elmer, Denmark). PCR reactions were carried out in a RoboCycler Gradient40 (Stratagene, USA) in the following manner: an initial denaturation step at 95 °C for 5 min, followed by 23 cycles (GAPDH), 34 cycles (Kir6.1) or 38 cycles (Kir6.2, SUR1, SUR2A and SUR2B) of denaturation for 1 min at 95 °C, annealing for 90 s at 63 °C and 30 s at 72 °C. After the final cycle, the temperature was maintained at 72 °C for 7 min to allow completion of synthesis of amplified products.

2.4. Electrophoretic analysis

A sample (20 μl) of each PCR amplified product was loaded on a 2% agarose gel (Gibco BRL, Invitrogen A/S, Denmark), containing 0.5 μg/ml ethidium bromide and the size of the amplified products was verified by co-electrophoresis of a 100-base pair nucleotide DNA ladder (GibcoBRL, Invitrogen A/S, Denmark).

The identity of the amplified sequences was tested by restriction analysis: GeneBank extracted sequences representing the four expected cDNAs were analyzed for specific restriction sites. The amplified DNA in the reaction mixtures derived from pial arteries was cut by a specific restriction enzyme under conditions described by the supplier (New England BioLabs, UK). For verification of the identity of the PCR products, the following specific enzymes were used: Kir6.1: AlwN I, Hae II, Kir6.2: Eco0109 I, Pvu II, SUR1: Pvu II, Ear I and SUR2B: AlwN I, EcoR I. Each of the restriction

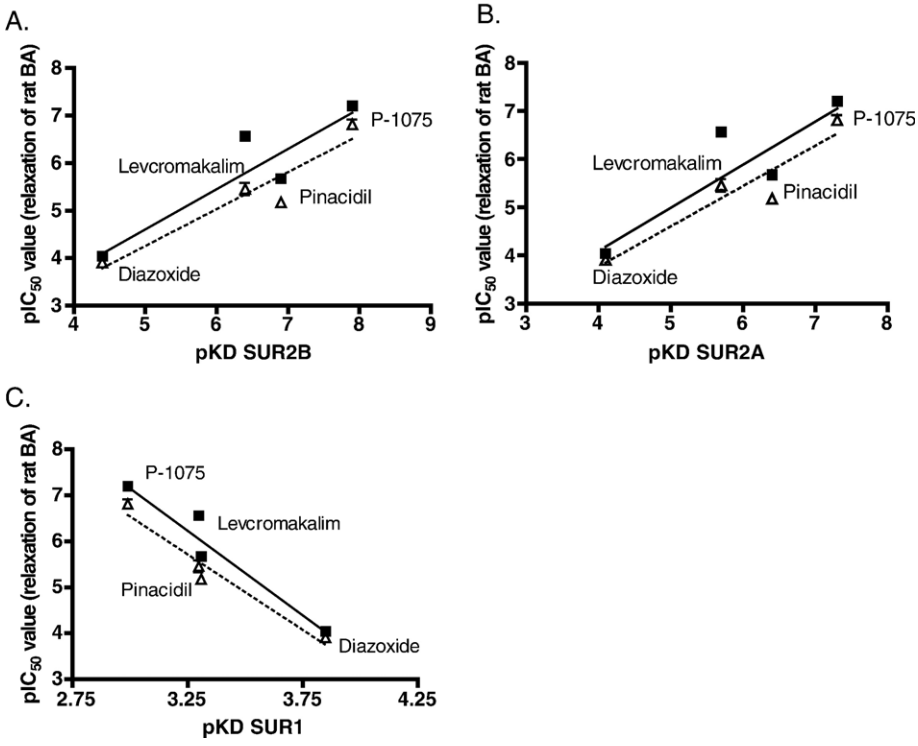

Fig. 4. Correlation between K_{ATP} channel opener-induced vasodilator potency in rat basilar (■) and middle cerebral (△) arteries (pIC_{50}) with historical measurement of affinity (pK_D) for the same four K_{ATP} channel openers at A. SUR2B, B. SUR2A and C. SUR1 transfected COS-7 cells obtained by binding studies (Schwanstecher et al., 1998). Linear regression analysis was performed using GraphPad Prism, basilar artery (full line), middle cerebral artery (broken line). Significant correlations ($P<0.05$) were found between affinity and vasodilator potency for SUR2A (basilar artery: $r=0.78$, middle cerebral artery: $r=0.81$) and SUR2B (basilar artery: $r=0.83$, middle cerebral artery: $r=0.83$). In C. a negative correlation was found between vasodilator potency and affinity for SUR1.

enzymes cut the PCR product at one (or two) restriction sites, and the restriction enzymes were chosen to obtain an even distribution of cleavage sites over the amplification sequence. The reaction mixtures were loaded on 2% agarose gels and subjected to electrophoresis to confirm the appearance of expected fragment sizes. The primers for SUR2A were characterized in a recent paper (Cao et al., 2002).

3. Results

3.1. mRNA expression by reverse transcriptase polymerase chain reaction

Agarose gel electrophoresis of the reverse transcriptase polymerase chain reaction (RT-PCR) products demonstrated bands of the expected sizes (Table 1), corresponding to the presence of mRNA encoding Kir6.1, Kir6.2, SUR1 and SUR2B in rat basilar and middle cerebral arteries (Fig. 1A and B). In contrast to the strong band found for SUR2A mRNA in rat heart, only weak SUR2A bands were detected in rat basilar and middle cerebral arteries (Fig. 1C). No bands were seen in the negative controls from which the reverse transcriptase enzyme was omitted (not shown). After treatment of the PCR products with specific restriction enzymes, bands of the expected sizes were identified by agarose gel electrophoresis (Fig. 2).

3.2. Studies of K_{ATP} openers on isolated cerebral arteries in vitro

Cumulative application of K_{ATP} channel openers to $PGF_{2\alpha}$-precontracted vessel segments resulted in dilatation of rat basilar and middle cerebral arteries with the following order of potency: P-1075 > levcromakalim > ZM226600 > pinacidil > diazoxide (Fig. 3, Table 2). The responses to P-1075 and pinacidil tended ($P > 0.05$) to be less potent in middle cerebral arteries than in basilar arteries. While the relaxations induced by levcromakalim, ZM226600 and diazoxide were significantly ($P < 0.05$) less potent in middle cerebral arteries than in basilar arteries (Table 2), P-1075 was the only K^+ channel opener that produced relaxation with a significantly higher I_{max} in basilar arteries than in middle cerebral arteries.

In rat basilar arteries, removal of the endothelium caused a significant rightward shift towards higher concentrations of pinacidil, levcromakalim, P-1075 and ZM226600. In addition, the maximum relaxant response to P-1075 was significantly ($P < 0.005$) lower in the absence of endothelium. Relaxations induced by diazoxide were significantly ($P < 0.005$) more potent in basilar arteries without an intact endothelium (Table 3). In middle cerebral arteries, no significant differences could be found in relaxant responses to K_{ATP} channel openers in arteries with endothelium as compared to arteries without endothelium (Table 3).

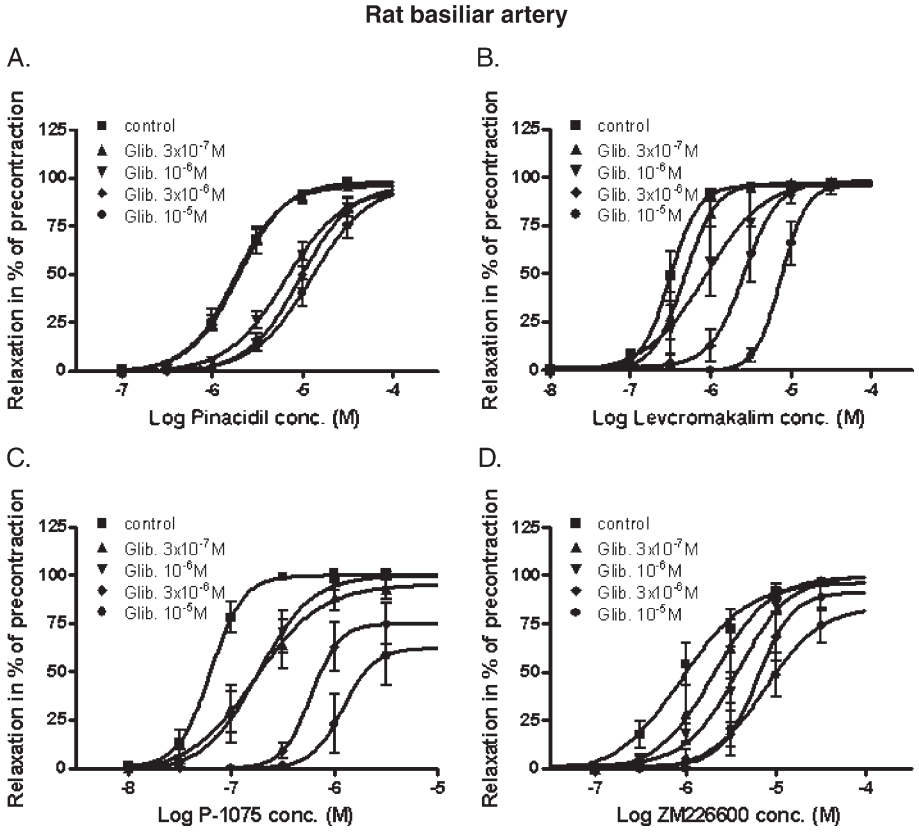

Fig. 5. Relaxation induced by increasing concentrations of the K_{ATP} channel openers A. pinacidil, B. levcromakalim, C. P-1075 and D. ZM226600 in rat basilar arteries. The experiments were performed without (■) and with glibenclamide in different concentrations (▲) 3×10^{-7} M, (▼) 10^{-6} M, (♦) 3×10^{-6} M, (●) 10^{-5} M. The relaxation of each segment tested was calculated as a percentage of the pre-contraction induced by 3×10^{-6} M prostaglandin $F_{2\alpha}$. Each point represents the mean values with S.E.M. shown by vertical bars, $n = 6–15$.

3.3. Correlation between vasodilator potency and binding affinities in SUR-transfected COS-7 cells

For the series of agonists used, there was a significant correlation between vasodilator potency in rat basilar and middle cerebral arteries and historical pK_D values obtained from COS-7 cells (Schwanstecher et al., 1998) transiently expressing rat SUR2A (basilar artery: $r^2=0.78$, $P<0.0001$; middle cerebral artery: $r^2=0.82$, $P<0.0001$) and human SUR2B (basilar artery: $r^2=0.83$, $P<0.0001$; middle cerebral artery: $r^2=0.83$, $P<0.0001$) (Fig. 4A and B). The relationsship in K_{ATP} opener affinity between COS-7 cells expressing hamster SUR1 binding sites and vasodilator potency was significantly negative ($P<0.001$) (Fig. 4C).

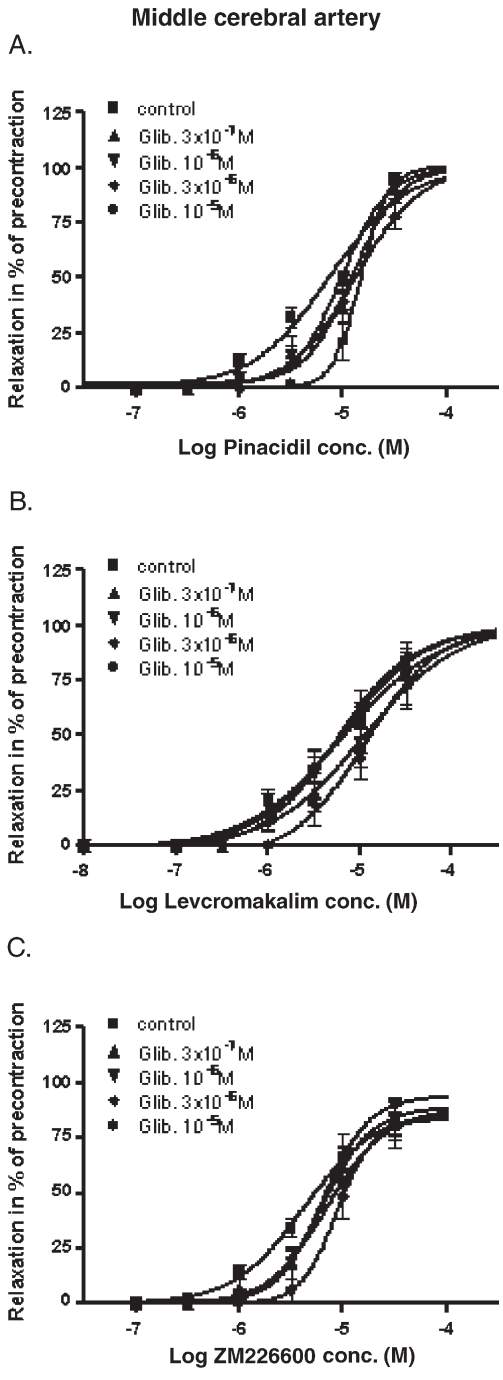

Fig. 6. Relaxation induced by increasing concentrations of the K_{ATP} channel openers A. pinacidil, B. levcromakalim and C. ZM226600 in middle cerebral arteries. The experiments were performed without (■) and with glibenclamide in different concentrations (▲) 3×10^{-7} M, (▼) 10^{-6} M, (♦) 3×10^{-6} M, (●) 10^{-5} M. The relaxation of each segment tested was calculated as a percentage of the pre-contraction induced by 3×10^{-6} M prostaglandin $F_{2\alpha}$. Each point represents the mean values with S.E.M. shown by vertical bars, $n=5$–10.

3.4. Studies of the K_{ATP} blocker glibenclamide on relaxations induced by pinacidil and levcromakalim

Glibenclamide (3×10^{-7}–10^{-5} M) acted as a blocker of relaxations induced by pinacidil, levcromakalim, P-1075 and ZM226600 in rat basilar artery (Fig. 5). In rat middle cerebral artery glibenclamide (3×10^{-7}–10^{-5} M) caused a slight block of the relaxation induced by lower concentrations of the K_{ATP} channel opener (Fig. 6).

4. Discussion

K_{ATP} channels have in previous electrophysiological and pharmacological studies been demonstrated to be present in cerebral arteries and are well known to play an important role in the regulation of vascular tone, depending on metabolic need and energy supply. However, in cerebral arteries the pharmacological properties, as compared to the molecular composition of these channels have not been examined in detail. In this study, we characterized and compared the pharmacology of K_{ATP} channels expressed in isolated rat basilar and middle cerebral arteries using a sensitive in vitro system for studying vasomotor responses.

4.1. Molecular expression of K_{ATP} channels

Molecular studies by RT-PCR showed that mRNA for Kir6.1, Kir6.2, SUR1 and SUR2B subtypes of K_{ATP} channels were present in rat basilar and middle cerebral arteries. SUR2A was found in heart but could only be detected in basilar and middle cerebral arteries as weak bands. Thus, no difference in expression between basilar arteries and middle cerebral arteries was observed. The findings indicate that at a molecular level any combination of K_{ATP} channel subunits is possible, but that SUR2A probably exists only in very small amounts. This is somewhat in contradiction to previous studies where only mRNA expression for Kir6.1 and SUR2B was found. However, in these studies the expression of SUR1 and SUR2A was not examined. Our finding of Kir6.2 expression in the two arteries may be because we used more PCR cycles (38 vs. 30) or because the molecular expression of Kir6.2 is decreased during culture of vascular smooth muscle cells. Furthermore, in accordance with the present findings, transcripts of Kir6.1, Kir6.2, SUR1 and SUR2B, but not of SUR2A, were found in endothelium-free, fresh mesenteric arteries (Cao et al., 2002).

4.2. K_{ATP} channel openers

Application of K_{ATP} openers belonging to four structurally distinct families induced relaxation of rat basilar and middle cerebral arteries. There was a tendency that the cyanoguanidines, pinacidil and P-1075, were more potent in the basilar artery than in the middle cerebral artery, but this effect was not significant. However, the responses induced by the benzopyrane, levcromakalim, the tertiary cabinol, ZM226600, and the benzothiadiazine, diazoxide, were significantly more potent in basilar arteries than in middle cerebral arteries. There are several regional and anatomical differences between the two arteries that could explain the difference in sensitivity to K_{ATP} openers. 1. The middle cerebral artery originates from the internal carotid artery while the basilar artery has its origin in the vertebral arteries. 2. The middle cerebral artery (internal diameter: ~200 μm) is smaller than the basilar artery (internal diameter: 250–300 μm). 3. The middle cerebral artery consists of three layers of smooth muscle cells while the basilar artery has four layers of smooth muscle cells (Edvinsson and Krause, 2002). The smaller size of the middle cerebral artery increases the probability of the artery becoming damaged during removal and mounting in the small-vessel myograph. However, arteries with an intact endothelium reacted with more than a 25% relaxation to carbachol, which indicates that the vessels were viable. Furthermore, we have previously shown that the even smaller rat middle meningeal artery (internal diameter ~150 μm), which also originates in the carotid artery, is more sensitive to in vitro application of pinacidil and levcromakalim than the middle cerebral artery (Gozalov et al., 2005). Thus, neither of the anatomical properties mentioned seem to be responsible for the difference in response between middle cerebral and basilar arteries. That K_{ATP} openers may display regional selectivity in their action on rat cerebral arteries is further supported by a number of in vitro and in vivo studies. The middle cerebral artery, but not the basilar artery, of Wistar rats was resistant to the effects of the K_{ATP} openers pinacidil, cromakalim and nicorandil (McCarron et al., 1991; McPherson and Stork, 1992). Moreover, the proximal arteries of the rabbit vertebrobasilar system reacted with a higher sensitivity to levcromakalim than the distal arteries (Nagao et al., 1996). The relaxant effects of pinacidil and levcromakalim were in the same potency range in the present study as in previous studies of rat basilar arteries (Ksoll et al., 1991; Toyoda et al., 1997b; Zimmermann et al., 1997) and cerebral arteries from other species (Iwamoto et al., 1993; Nagao et al., 1996; Zhang et al., 1992). To our knowledge, dose-effect studies of P-1075 and ZM226600 have previously not been performed with cerebral arteries in vitro. However, the effect of P-1075 was examined in peripheral arteries from three different species, showing a slightly higher potency (pIC_{50} 7.7–8.1) than we found in rat cerebral arteries (pIC_{50} 7.0–7.2) (Higdon et al., 1997).

4.3. Correlation between vasodilator potency and binding affinities in SUR-transfected COS-7 cells

We performed a correlation study of the relaxant potency of four K_{ATP} channel openers in rat basilar and middle cerebral arteries with measurement of affinity for the same four K_{ATP} channel openers in COS-7 cell lines expressing either human SUR2B, rat SUR2A or hamster SUR1 (Schwanstecher et al., 1998). These studies showed that there was a significant correlation between vasodilator potency and binding affinity at SUR2B and SUR2A subunits. Thus, rat basilar and middle cerebral artery vasodilatation induced by K_{ATP} channel openers is likely to be mediated via binding to either the SUR2B or SUR2A subunit. In contrast, the relationship between vasodilator potency and binding affinity at SUR1 subunits was negative. This suggests that the SUR1 protein may not be expressed at significant levels or, if it is expressed, it is barely detectable by the method used. Thus, taken together with the results from expression studies where only weak bands for SUR2A could be detected in rat basilar and middle cerebral arteries, our findings strongly suggest that K_{ATP} channels in rat cerebral arteries mainly consist of Kir6.1 or Kir6.2 in combination with SUR2B. These findings are consistent with previous reports showing the presence of Kir6.1 in combination with SUR2B in cultured and fresh smooth muscle cells from rat basilar arteries (Li et al., 2003; Santa et al., 2003).

4.4. Role of endothelium

ATP-sensitive K^+ channels have been described in aortic endothelium and in brain microvascular endothelial cells (Janigro et al., 1993). They are suggested to play a role in the regulation of endothelial cell resting potential during impaired energy supply and therefore to modulate EDRF release and cerebral blood flow (Janigro et al., 1993). In the present study, removal of the endothelium significantly reduced the sensitivity (pIC_{50}) of isolated basilar arteries to application of pinacidil, levcromakalim, P-1075 and ZM226600. Two of the K_{ATP} channel openers had a somewhat different profile of action as compared to the others. The maximum relaxant response to P-1075 was significantly lower after endothelium removal in the basilar artery. Furthermore, the maximum relaxant response in endothelium-intact vessels was significantly higher in basilar arteries than in middle cerebral arteries. The reasons for these dissimilarities remain to be established. However, it seems that endothelium-denuded basilar arteries react more like middle cerebral arteries with endothelium. Thus, the difference between the two arteries in some way seems to be related to the endothelium. In contrast, we found that the sensitivity of the basilar artery to diazoxide was increased in the absence of endothelium. It has been shown that different SUR subunits confer different sensitivity to K_{ATP} openers. For example, Kir6.2/SUR1 channels are activated strongly by diazoxide but not by pinacidil; Kir6.2/SUR2A channels are activated by pinacidil and cromakalim but only weakly by diazoxide, while Kir6.2/SUR2B channels are activated by P-1075, diazoxide, pinacidil and levcromakalim (Babenko et al., 1998; D'Hahan et al., 1999a,b; Gribble et al., 1998; Inagaki et al., 1995; Isomoto et al., 1996; Schwanstecher et al., 1998). Thus, in rat basilar artery, removal of the endothelium might reveal functional Kir6.x/SUR1 channels. In contrast, the other K_{ATP} channel openers

could act on Kir6.2/SUR2A channels on endothelial cells to cause potentiation of responses in smooth muscle cells, possibly, by increasing endothelial NO formation (Luckhoff and Busse, 1990) or via myo-endothelial communication, as described for levcromakalim in rat mesenteric artery (Murai et al., 1999). We found weak bands for SUR2A mRNA in homogenized rat basilar and middle cerebral arteries. This was not found in other studies performed with cultured smooth muscle cells and endothelium-free, fresh mesenteric arteries (Cao et al., 2002; Santa et al., 2003). One is therefore tempted to suggest that SUR2A is located on endothelial cells. However, more studies need to be performed in order to identify the specific location of SUR subunits in cerebral arteries. The lack of differences between middle cerebral arteries with and without endothelium suggests that endothelial cells do not play a role in K_{ATP} opener-induced responses in these arteries.

4.5. Blockade with glibenclamide

In rat basilar arteries, glibenclamide produced a shift towards higher concentrations of pinacidil, levcromakalim, P-1075 and ZM226600. These findings are in accordance with previous pharmacological studies performed in cerebral arteries that have shown a competitive interaction of glibenclamide with cromakalim, nicorandil and pinacidil (Kleppisch and Nelson, 1995; Ksoll et al., 1991; Masuzawa et al., 1990; Schmid-Antomarchi et al., 1987). In contrast, only a weak antagonistic effect was observed in middle cerebral arteries. The ability of glibenclamide to block K_{ATP} channel-opener induced relaxation in rat middle cerebral arteries was supported by our previous findings in which glibenclamide caused a significant inhibition of relaxation induced by a single concentration of levcromakalim and pinacidil in vitro and in vivo (Gozalov et al., 2005).

4.6. Concluding remarks

In conclusion, we have shown the presence of mRNA for Kir6.1, Kir6.2, SUR1, SUR2A and SUR2B with stronger bands for Kir6.1, SUR1 and SUR2B subunits, in rat basilar and middle cerebral arteries. The pharmacological studies showed a profile consistent with the presence of a functional Kir6.x/SUR2 subunit composition. Thus, there is a high probability that K_{ATP} channels consisting of Kir6.1/SUR2B subunits are present in rat basilar and middle cerebral arteries.

We also found a difference between basilar and middle cerebral arteries in endothelial involvement as well as in potency towards K_{ATP} openers. However, these differences were not caused by differences in mRNA expression for K_{ATP} channel subunits in the two arteries.

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