

Morphine induces preconditioning via activation of mitochondrial K_{Ca} channels

La morphine provoque le préconditionnement par l'activation des canaux mitochondriaux K_{Ca}

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Abstract

Purpose Mitochondrial calcium sensitive potassium (mK_{Ca}) channels are involved in cardioprotection induced by ischemic preconditioning. In the present study we investigated whether morphine-induced preconditioning also involves activation of mK_{Ca} channels.

Methods Isolated rat hearts (six groups; each $n = 8$) underwent global ischemia for 30 min followed by a 60-min reperfusion. Control animals were not further treated. Morphine preconditioning (MPC) was initiated by two five-minute cycles of morphine $1 \mu\text{M}$ infusion with one five-minute washout and one final ten-minute washout period before ischemia. The mK_{Ca} blocker, paxilline $1 \mu\text{M}$, was administered, with and without morphine administration (MPC + Pax and Pax). As a positive control, we added an ischemic preconditioning group (IPC) alone and combined with paxilline (IPC + Pax). At the end of reperfusion, infarct sizes were determined by triphenyltetrazoliumchloride staining.

Results Infarct size was (mean \pm SD) $45 \pm 9\%$ of the area at risk in the Control group. The infarct size was less in the morphine or ischemic preconditioning groups (MPC: $23 \pm 8\%$, IPC: $20 \pm 5\%$; each $P < 0.05$ vs Control). Infarct

size reduction was abolished by paxilline (MPC + Pax: $37 \pm 7\%$, $P < 0.05$ vs MPC and IPC + Pax: $36 \pm 6\%$, $P < 0.05$ vs IPC), whereas paxilline alone had no effect (Pax: $46 \pm 7\%$, not significantly different from Control).

Conclusion Cardioprotection by morphine-induced preconditioning is mediated by activation of mK_{Ca} channels.

Résumé

Objectif Les canaux potassiques mitochondriaux calcium-dépendants (mK_{Ca}) participent à la cardioprotection induite par le préconditionnement ischémiq. Dans la présente étude, nous avons tenté de découvrir si le préconditionnement provoqué par la morphine implique également l'activation des canaux mK_{Ca} .

Méthodes Des cœurs isolés de rats (six groupes; chacun de $n = 8$) ont été soumis à une ischémie globale pendant 30 min, suivie d'une perfusion de 60 min. Les animaux témoins n'ont reçu aucun autre traitement. Le préconditionnement provoqué par la morphine (MPC) a été initié par deux cycles de cinq minutes d'une perfusion de morphine à $1 \mu\text{M}$, avec une période de rinçage de cinq minutes et une période de rinçage finale de dix minutes avant l'ischémie. L'inhibiteur des canaux mK_{Ca} , la paxilline à $1 \mu\text{M}$, a été administré, avec ou sans administration de morphine (MPC + Pax et Pax). À titre de valeur témoin positive, nous avons ajouté un groupe soumis à un préconditionnement ischémiq (IPC) seul et combiné à la paxilline (IPC + Pax). À la fin de la reperfusion, la taille de chaque infarctus a été déterminée par la coloration du chlorure de tryphényltétrazolium.

Résultats La taille de l'infarctus couvrait $45 \pm 9\%$ (moyenne \pm écart type) de la zone à risque dans le groupe témoin. La taille de l'infarctus était moins importante dans les groupes ayant reçu de la morphine ou ayant subi un

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préconditionnement ischémique (MPC: $23 \pm 8 \%$, IPC: $20 \pm 5 \%$; chacun de $P < 0,05$ contre le groupe témoin). L'effet de réduction de la taille de l'infarctus a été éliminé par la paxilline (MPC + Pax: $37 \pm 7 \%$, $P < 0,05$ contre MPC et IPC + Pax: $36 \pm 6 \%$, $P < 0,05$ contre IPC), tandis qu'administrée seule, la paxilline n'a eu aucun effet (Pax: $46 \pm 7 \%$, aucune différence notable comparativement au groupe témoin).

Conclusion La cardioprotection par préconditionnement provoqué par la morphine est assistée par l'activation des canaux mK_{Ca} .

Keywords Morphine · Preconditioning · mK_{Ca} · Infarct size

Cardioprotection by preconditioning can be induced by various stimuli, such as brief cycles of ischemia or pharmacological agents, including volatile anesthetics and morphine^{1,2}. Both ischemic and morphine preconditioning protect the heart by sharing common cellular pathways. Opening of mitochondrial ATP-sensitive potassium (mK_{ATP}) channels is involved in regulation of mitochondrial functions, representing a key step in mediating the cardioprotective effects of ischemic- and morphine-induced preconditioning, possibly due to inhibition of mitochondrial permeability transition pore (mPTP).³⁻⁵ Besides opening of mK_{ATP} channels, activation of the mitochondrial calcium sensitive potassium (mK_{Ca}) channel is also involved in preconditioning.^{6,7} Cao *et al.* demonstrated that activation of mK_{Ca} channels plays a crucial role in ischemic preconditioning and that cardioprotection, by activation of mK_{Ca} channels, is independent of mK_{ATP} channels and vice versa.⁸ In another study, the same authors demonstrated that preconditioning by activation of κ -opioid receptors is triggered by mK_{Ca} channels.⁹ However, the opioid, morphine, is predominantly a μ -receptor agonist and has only a low affinity for κ -opioid receptors.^{10,11} Furthermore, there is no κ -opioid agonist for clinical practice available. It is not known whether activation of mK_{Ca} channels is involved in morphine-induced preconditioning. The purpose of the present study was to test the hypothesis that morphine-induced preconditioning is mediated by activation of mK_{Ca} channels.

Methods

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was performed in accordance with the requirements of the Animal Ethics Committee of the University of Duesseldorf, Duesseldorf, Germany.

Chemicals and reagents

All chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany).

Surgical preparation

Male Wistar rats were used for these studies. The rats were maintained on a 12:12 light/dark schedule (lights on at 0600 hr) with food and water provided *ad libitum*. The animals were anesthetized with pentobarbital $90 \text{ mg} \cdot \text{kg}^{-1}$ *ip*. After thoracotomy, the hearts were excised, mounted on a Langendorff system, and perfused at constant pressure (80 mmHg) with Krebs–Henseleit solution containing (in mM) 116 NaCl, 4.7 KCl, 1.1 MgSO₄, 1.17 KH₂PO₄, 24.9 NaHCO₃, 2.52 CaCl₂, 8.3 glucose, and 2.2 pyruvate at 37°C. A fluid-filled balloon was inserted into the left ventricle, and end-diastolic pressure was set at 1–4 mmHg. All hearts underwent a stabilization period of 20 min. Heart rate (HR), myocardial function (isovolumetric left ventricular pressure), coronary flow, left ventricular end-diastolic pressure, and rate of left ventricular pressure development (dp/dt_{max}) were measured continuously. The data were digitized using an analogue to digital converter (PowerLab/8SP, ADInstruments Pty Ltd, Castle Hill, Australia) at a sampling rate of 500 Hz, and they were recorded continuously on a personal computer using Chart for Windows v5.0 (ADInstruments). Maximal contracture and time to maximal contracture were noted during ischemia. Arrhythmic intervals were not used for data analysis.

Experimental protocol for infarct size determination

The hearts were assigned randomly to one of six experimental groups (Figure 1). The hearts of all groups underwent 30 min of ischemia followed by 60 min of reperfusion. In the control group (Con), the hearts were kept under baseline conditions prior to ischemia. To investigate whether morphine induces preconditioning (MPC), morphine $1 \mu\text{M}$ was given in two five-minute cycles, separated by one five-minute washout period, and ending with one final ten-minute washout period prior to ischemia. The morphine concentration was chosen because Liang *et al.* demonstrated that the preconditioning effect of morphine was maximal at $1 \mu\text{M}$.¹² Morphine was dissolved in 0.9% NaCl and separately infused into a mixing chamber placed in the perfusion system. As a positive control, the ischemic preconditioning group (IPC) underwent two similar five-minute cycles of ischemia ten minutes prior to ischemia. To test whether mK_{Ca} channels are involved in the phenomenon of preconditioning, the mK_{Ca} channel inhibitor, paxilline $1 \mu\text{M}$,^{8,9} was given over 25 min together with morphine- and

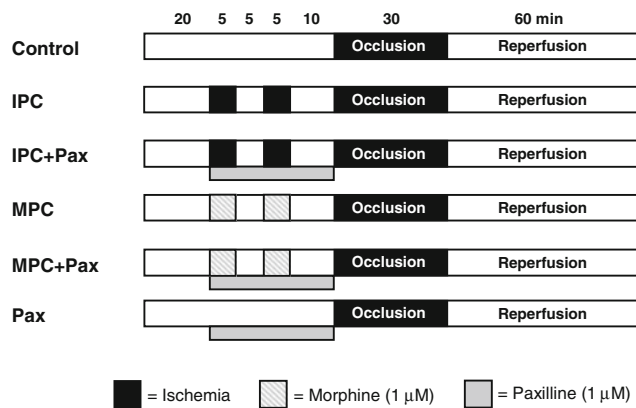


Fig. 1 Experimental protocol. IPC = ischemic preconditioning; MPC = morphine preconditioning; Pax = paxilline

ischemic-induced preconditioning (MPC + Pax and IPC + Pax). To rule out an effect of paxilline itself, we investigated the effect of paxilline alone (Pax).

After 60 min of reperfusion, the heart was cut into transverse slices, which were then stained with 0.75% triphenyltetrazoliumchloride solution. The infarcted area was determined by planimetry using SigmaScan Pro 5® computer software (SPSS Science Software, Chicago, IL, USA).

Statistical analysis

The sample size was calculated using GraphPad StatMate™ Version 1.01 (GraphPad Software, San Diego, CA, USA). Sample size analysis revealed that a group size of $n = 8$ was necessary to detect a difference in infarct size of 25% with a power of 80% and an $\alpha < 0.05$. The estimations of the mean difference of 25% and the standard deviation (SD) of 15% were based on our own data.¹³ Data are expressed as mean \pm SD. Heart rate (in min^{-1}) and mean aortic pressure (in mmHg) were measured during baseline, coronary artery occlusion, and reperfusion period. Comparisons of hemodynamics between groups or between time points in a group were performed (SPSS Science Software, version 12.0.1) using two-way analysis of variance followed by Dunnett's post hoc test. A researcher blinded to the experimental groups determined the infarct sizes. The infarcts were analyzed by Student's *t* test followed by Bonferroni's correction for multiple comparisons. Changes within and among groups were considered statistically significant if $P < 0.05$.

Results

No differences in body or heart weight were observed between the groups (Table 1). In the preconditioning groups, the level of maximal ischemic contracture was significantly

lower, and the time of maximal ischemic contracture was significantly higher compared with the Control group ($P < 0.05$ vs Con) (Table 1).

Infarct size measurement

In the Control group, infarct size was (mean \pm SD) $45 \pm 9\%$ of the area at risk (Figure 2). In the ischemic- and morphine-induced preconditioning groups, infarct size was similar and significantly less than in the Control group (IPC: $20 \pm 5\%$, MPC: $23 \pm 8\%$; each $P < 0.05$ vs Con) (Figure 2). The preconditioning effect of ischemia and morphine was attenuated significantly by the mK_{Ca}-channel inhibitor, paxilline. Infarct size was $36 \pm 6\%$ and $37 \pm 7\%$ in the IPC + Pax and MPC + Pax groups, respectively; each $P < 0.05$ vs IPC and MPC, respectively (Figure 2). Paxilline alone had no effect on infarct size (Pax: $46 \pm 7\%$; not significantly different from Con). There was no significant difference in infarct size between the preconditioning groups with paxilline compared with the Control group.

Hemodynamic variables

Hemodynamic variables are summarized in Table 2. No significant differences in left ventricular end-diastolic pressure and dP/dt_{max} were observed between the experimental groups during baseline conditions and at the beginning of ischemia (Table 2). At the end of the experiment, dP/dt_{max} was higher in the preconditioning groups (Table 2). There was no difference in HR compared with Controls at baseline and during reperfusion, with the exception of the paxilline group at time point final ten-minute washout shortly before index ischemia (Table 2).

Discussion

The main finding of our study is that the opioid, morphine, initiates preconditioning in a similar manner as ischemia, i.e., by activation of mK_{Ca} channels.

Ischemic preconditioning (IPC) describes a cardioprotective phenomenon where short periods of myocardial ischemia protect the heart against a subsequent longer period of ischemia and reduce the deleterious consequences of ischemia/reperfusion injury.¹⁴ Besides ischemic stimuli, volatile anesthetics^{15,16} and morphine can mimic the cardioprotective effect of preconditioning.¹⁷ In contrast to volatile anesthetics, morphine can be administered to patients who are subjected to organ ischemia (vascular surgery, organ transplantation, cardiac surgery) or who recently underwent regional ischemia (stroke, angina pectoris, myocardial infarction, organ transplantation) without the side effect of being "anesthetized". The mechanisms by which opioids protect the myocardium

Table 1 Weights and ischemic contracture

	Body weight (g)	Heart weight wet (g)	Heart weight dry (mg)	Maximal ischemic contracture (mmHg)	Time of maximal. ischemic contracture (min)
Control	310 ± 25	1.5 ± 0.2	171 ± 17	36 ± 3	14 ± 1
IPC	315 ± 23	1.5 ± 0.2	175 ± 18	20 ± 2*	27 ± 1*
IPC + Pax	315 ± 24	1.5 ± 0.2	181 ± 17	39 ± 3	15 ± 3
MPC	309 ± 26	1.5 ± 0.1	180 ± 20	20 ± 2*	27 ± 1*
MPC + Pax	313 ± 27	1.5 ± 0.1	185 ± 19	39 ± 3	13 ± 2
Pax	312 ± 25	1.6 ± 0.2	184 ± 19	36 ± 3	15 ± 1

Data are mean ± SD

IPC = ischemic preconditioning; MPC = morphine preconditioning; Pax = paxilline; * $P < 0.05$ vs Control

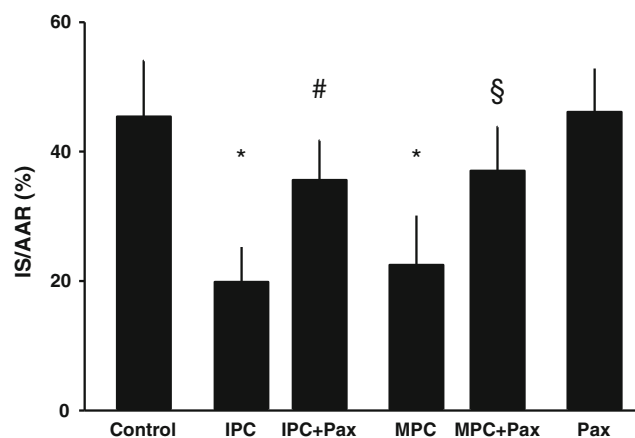


Fig. 2 Infarct size measurement. Histogram showing the infarct size (IS) as percent of area at risk (AAR) in Controls (Con, $n = 8$), morphine preconditioning (MPC, $n = 8$), ischemic preconditioning (IPC, $n = 8$), morphine preconditioning with paxilline (MPC + Pax, $n = 8$), ischemic preconditioning with paxilline (IPC + Pax, $n = 8$), and paxilline alone (Pax, $n = 8$). Data are mean ± SD; * $P < 0.05$ vs Con; # $P < 0.05$ vs IPC; § $P < 0.05$ vs MPC

share common pathways with ischemic preconditioning. Opening of mitochondrial ATP-sensitive potassium (mK_{ATP}) channels that are involved in regulating mitochondrial functions is a key step that mediates cardioprotection induced by both morphine and ischemic preconditioning, possibly through inhibition of mitochondrial permeability transition pore (mPTP) opening.^{3,5}

Mitochondrial calcium sensitive potassium (mK_{Ca}) channels seem to be another class of K^+ channels, apart from mK_{ATP} channels, that mediate cardioprotection by preconditioning.^{6,7} In 2002, Xu *et al.*⁷ reported that K_{Ca} -activated potassium channels are located on the inner mitochondrial membrane and mediate cardioprotection against ischemia and reperfusion injury in the isolated perfused guinea pig heart. Since this initial observation, mK_{Ca} -activated potassium channels have been found to be implicated in ischemic preconditioning against ischemia and reperfusion injury in isolated rat and mouse hearts, anesthetized dogs, and isolated cardiac myocytes.^{6,8,9,18,19}

The mK_{Ca} channel contains a pore forming α -subunit and a regulatory β -subunit.²⁰⁻²² The β -subunit consists of four accessory β -subunits ($\beta 1-4$). Both mK_{ATP} and mK_{Ca} channel activation triggers preconditioning that is independent one from the other and involves the mPTP.⁸ Cao *et al.* showed that ischemic preconditioning is triggered by activation of mK_{Ca} channels and is abolished by the mK_{Ca} channel inhibitor, paxilline.⁸ Paxilline is a mycotoxin produced by the fungus, *Penicillium paxilli*. It has the ability to block all subunits²³ of mK_{Ca} channels and is a selective inhibitor.²⁴ Besides paxilline, there are not many mK_{Ca} channel blockers available. Iberitoxin, for example, is a mK_{Ca} channel blocker that is also suitable for *in vivo* use,¹³ whereas paxilline is predominantly used for *in vitro* experiments. However, we did not check whether iberitoxin also abolishes morphine preconditioning. Besides ischemic preconditioning, our results demonstrate that pharmacological preconditioning with morphine also induces cardioprotection by activation of mK_{Ca} channels. Cao *et al.*⁸ administered the mK_{Ca} channel blocker, paxilline, at the onset of reperfusion after a prolonged period of ischemia. We applied paxilline during the preconditioning period, suggesting that mK_{Ca} channel inhibition blocks the infarct size reducing effect of preconditioning during the trigger phase. In a recent study, we could demonstrate that activation of mK_{Ca} channels not only reduced infarct size by preconditioning but also caused a significant reduction in the mitochondrial respiratory control index.¹³ Co-administration of the mK_{Ca} channel blocker, iberitoxin, completely abolished the reduction in the respiratory control index, and we concluded that cardioprotection is mediated by activation of mK_{Ca} channels leading to mild mitochondrial uncoupling. Mild mitochondrial uncoupling during the trigger phase of preconditioning may represent a common characteristic of mitochondria in a “conditioned” state.^{7,25-27} The involvement of mK_{Ca} channels in morphine-induced preconditioning was addressed in our study.

We demonstrated here that ischemic- and morphine-induced preconditioning reduced the infarct size to a similar extent. The mK_{Ca} channel blocker, paxilline, abolished both effects, confirming the findings of others that ischemic

Table 2 Hemodynamic variables

	Baseline	Washout 2	Time after reperfusion (min)			
			3	15	30	60
<i>Heart rate (min⁻¹)</i>						
Control	366 ± 37	359 ± 35	365 ± 29	378 ± 5	372 ± 15	373 ± 14
IPC	367 ± 11	360 ± 23	365 ± 22	377 ± 2	365 ± 20	361 ± 24
IPC + Pax	357 ± 31	355 ± 32	276 ± 73	323 ± 47	324 ± 47	357 ± 20
MPC	367 ± 30	349 ± 28	383 ± 28	383 ± 28	383 ± 28	382 ± 30
MPC + Pax	346 ± 28	333 ± 34	248 ± 215	283 ± 142	375 ± 24	372 ± 17
Pax	336 ± 25	280 ± 29*	236 ± 126	342 ± 71	343 ± 69	344 ± 68
<i>LVEDP (mmHg)</i>						
Control	12 ± 32	12 ± 5	53 ± 22 [†]	35 ± 15 [†]	26 ± 10	19 ± 7
IPC	11 ± 1	4 ± 4	52 ± 22 [†]	24 ± 13	19 ± 8	18 ± 7
IPC + Pax	14 ± 2	11 ± 11	62 ± 22 [†]	56 ± 32 [†]	45 ± 33 [†]	28 ± 18
MPC	12 ± 3	15 ± 5	51 ± 6 [†]	36 ± 7 [†]	29 ± 6	24 ± 7
MPC + Pax	13 ± 2	12 ± 4	57 ± 23 [†]	43 ± 24 [†]	38 ± 31	34 ± 34
Pax	11 ± 5	8 ± 4	43 ± 17 [†]	23 ± 10	21 ± 8	20 ± 9
<i>dP/dt_{max} (mmHg · sec⁻¹ 1,000)</i>						
Control	3.3 ± 0.6	3.3 ± 0.6	0.7 ± 0.9 [†]	0.6 ± 0.8 [†]	0.6 ± 0.7 [†]	0.4 ± 0.5 [†]
IPC	3.4 ± 1.1	3.5 ± 1.1	0.7 ± 1.0 [†]	1.4 ± 0.7 [†]	1.6 ± 0.5*, [†]	1.5 ± 0.6*, [†]
IPC + Pax	3.3 ± 0.4	2.7 ± 0.2	0.1 ± 0.1 [†]	0.3 ± 0.2 [†]	0.2 ± 0.1 [†]	0.1 ± 0.1 [†]
MPC	3.0 ± 0.4	2.7 ± 0.6	0.8 ± 0.4 [†]	1.0 ± 0.4 [†]	1.0 ± 0.3 [†]	1.0 ± 0.1*, [†]
MPC + Pax	2.6 ± 0.5	2.5 ± 0.5	0.1 ± 0.1 [†]	0.3 ± 0.2 [†]	0.4 ± 0.3 [†]	0.3 ± 0.2 [†]
Pax	3.5 ± 1.1	3.0 ± 1.4	0.2 ± 0.1 [†]	0.4 ± 0.3 [†]	0.5 ± 0.4 [†]	0.5 ± 0.3 [†]
<i>CF (mL · min⁻¹)</i>						
Control	16 ± 3	15 ± 2	9 ± 6 [†]	7 ± 6 [†]	7 ± 5 [†]	7 ± 6 [†]
IPC	14 ± 2	11 ± 5	7 ± 4 [†]	11 ± 6	8 ± 6	9 ± 6
IPC + Pax	13 ± 1	12 ± 2	11 ± 2	5 ± 2 [†]	4 ± 2 [†]	4 ± 4 [†]
MPC	16 ± 4	15 ± 4	9 ± 5 [†]	6 ± 3 [†]	5 ± 2 [†]	4 ± 2 [†]
MPC + Pax	15 ± 2	14 ± 2	13 ± 4	8 ± 3 [†]	7 ± 4 [†]	6 ± 4 [†]
Pax	14 ± 1	10 ± 2*, [†]	4 ± 2 [†]	4 ± 2 [†]	3 ± 1 [†]	2 ± 1 [†]

Data are mean ± SD

IPC = ischemic preconditioning; MPC = morphine preconditioning; Pax = paxilline

LVEDP = left ventricular end-diastolic pressure; dP/dt_{max} = rate of left ventricular pressure development; CF = coronary flow; * $P < 0.05$ vs Control; [†] $P < 0.05$ vs Baseline

preconditioning involves activation of mK_{Ca} channels. Furthermore, it supports our hypothesis that mK_{Ca} channels are involved in the trigger phase of morphine-induced preconditioning. Heinen *et al.* showed that opening of mK_{Ca} channels can cause a slight increase in mitochondrial reactive oxygen species generation.²⁸ The mK_{Ca} channel agonist, NS1619, requires superoxide radical generation during the preconditioning stimulus to induce a cardioprotective effect.²⁹ Furthermore, these authors demonstrated that cardioprotection by NS1619 reduces mitochondrial calcium overload and mitochondrial reactive oxygen species production during the subsequent period of ischemia and early reperfusion.²⁹ Such a reduction in mitochondrial calcium overload and reactive oxygen species generation has been suggested to prevent mPTP opening.^{30,31} Evidence

suggests that the mK_{Ca} channel is located upstream of the mPTP, because cardioprotection induced by activation of mK_{Ca} channels was abolished by opening of the mPTP. Vice versa, inhibition of the mK_{Ca} channel with paxilline did not block protection induced by inhibition of the mPTP with cyclosporine A (CsA).⁸ Whether cardiac preconditioning by morphine is also mediated by regulation of the mPTP due to mK_{Ca} channel activation is yet unknown.

The results of the present study have to be interpreted within the scope of some limitations. First, we did not investigate possible upstream mechanisms of mK_{Ca} channels. Opioid receptors are G-protein coupled receptors whose activation inhibits adenylyl cyclase. The post-receptor signalling following opioid receptor activation has not been well defined. We cannot rule out that morphine

confers preconditioning via intracellular pathways leading to activation of mK_{Ca} channels. However, the means by which morphine regulates mK_{Ca} channel activation to induce preconditioning is unknown. It has been shown that mK_{Ca} channel activation is involved in desflurane preconditioning and that protein kinase A (PKA) is located upstream of the mK_{Ca} channel.³² The activity of PKA as a possible upstream activator of K_{Ca} channels depends on the cellular level of cyclic adenosine monophosphate (cAMP)—PKA is known as cAMP-dependent protein kinase. Gross *et al.* demonstrated that morphine-induced cardioprotection involves glycogen synthase kinase-3 β (GSK3 β) and Akt (also called PKB).³³ However, we did not investigate in the present study whether these enzymes are related to mK_{Ca} channels.

Another limitation of our study is that we did not determine the effect of ischemic and morphine preconditioning on the mitochondria. Xi *et al.* could show that morphine prevents mPTP opening by inactivation of GSK3 β .³⁴ Our results showing that morphine confers preconditioning through activation of mK_{Ca} channels and the fact that enzymes like PKA regulate mK_{Ca} channel activation suggest future directions for investigating the underlying mechanism of morphine-induced cardioprotection. Clinically, morphine might be administered to patients who are subjected to organ ischemia (vascular surgery, organ transplantation, cardiac surgery) or who recently underwent regional ischemia (stroke, angina pectoris, myocardial infarction, organ transplantation) without the side-effect of being “anesthetized”. Furthermore, there is no κ -opioid agonist for clinical practice available. Therefore, unravelling the exact mechanisms of morphine-induced cardioprotection might have clinical consequences.

In summary, our results demonstrate that, besides ischemic preconditioning, morphine also initiates cardiac preconditioning via activation of mK_{Ca} channels.

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Conflicts of interest None declared.

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