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Thiamylal and Thiopental Attenuate Beta-adrenergic Signaling Pathway by Suppressing Adenylyl Cyclase in Rat Ventricular Myocytes

Ikuhiro HIDAKA*), Hiromi KUROKAWA, Toshimichi YASUDA, Hiroshi HAMADA, Masashi KAWAMOTO and Osafumi YUGE

Department of Anesthesiology and Critical Care, Division of Clinical Medical Science, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

ABSTRACT

The effects of intravenous anesthetics on myocytes have not been fully elucidated. To investigate the effects of various intravenous anesthetics such as fentanyl, morphine, ketamine, diazepam, midazolam, thiamylal, and thiopental on the beta-adrenergic signaling pathway, we measured isoproterenol-stimulated cyclic adenosine monophosphate (cAMP) production in freshly isolated rat ventricular myocytes. Fentanyl, morphine, ketamine, diazepam, and midazolam did not significantly affect isoproterenol-stimulated cAMP production. However, thiamylal and thiopental dose-dependently decreased cAMP production stimulated by isoproterenol or by forskolin, a direct adenylyl cyclase stimulator. In addition, we examined the role of protein kinase C (PKC) as a potential mediator of the thiamylal- or thiopental-induced effects on cAMP production using bisindolylmaleimide I, a non-specific PKC inhibitor. Bisindolylmaleimide I did not alter the inhibitory effects of thiamylal or thiopental. Thiamylal and thiopental significantly decreased isoproterenol-stimulated cAMP production by suppressing the adenylyl cyclase. We conclude that barbiturates such as thiamylal and thiopental decrease isoproterenol-stimulated cAMP production by suppressing the adenylyl cyclase through PKC-independent mechanisms.

Key words: *Ventricular myocytes, Beta-adrenergic signaling pathway, Adenylyl cyclase, Barbiturates*

The use of intravenous anesthetics during perioperative periods and in intensive care units has been increasing. In the presence of intravenous anesthetics, patients with depressed cardiac function, which depends on the sympathetic tone[21,22], often require catecholamines to maintain their hemodynamic state. However, the interactions between these anesthetics and catecholamines on the hemodynamic state have not been fully elucidated. Isolated cardiomyocytes are a useful model to examine the direct effects of drugs on the heart. In fact, the effects of propofol[8], fentanyl[11], morphine[11], ketamine[8], diazepam[9], midazolam[9], and thiopental[10] on the contractility of myocytes have been examined by this system. Propofol attenuates the beta-adrenergic signaling pathway via protein kinase C (PKC) in cardiomyocytes[18]. However, the effects of other intravenous anesthetics on the beta-adrenergic signaling pathway in ventricular myocytes have not been reported. The aim of this study was to investigate the effects of intravenous anesthetics on the beta-adrenergic signaling pathway in ventricular myocytes.

MATERIALS AND METHODS

This study was approved by the Animal Care and Use Committee of Hiroshima University.

Ventricular Myocyte Preparation

Ventricular myocytes were freshly isolated from adult male Sprague-Dawley rats (370-410 g), as previously described[17,18]. Immediately after sacrifice, the hearts were rapidly removed and cannulated via the aorta. The hearts were perfused using a modified Langendorff perfusion apparatus

*Ikuhiro Hidaka, M.D.
Department of Anesthesiology and Critical Care, Division of Clinical Medical Science, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan
Tel: +81-82-257-5267, Fax: +81-82-257-5269 E-mail: hikuhiro@hiroshima-u.ac.jp

in a retrograde manner at a constant flow rate (8 ml/min) with an oxygenated (95% O_2 in 5% CO_2) Krebs-Henseleit buffer (KHB; 37°C), containing 118 mM NaCl, 4.8 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $CaCl_2$, 37.5 mM $NaHCO_3$, and 16.5 mM dextrose at pH 7.35. After a 5-min equilibration period, the perfusion buffer was changed to Ca^{2+}-free KHB containing collagenase type II (1.2-1.3 mg/ml). After the collagenase digestion (30-50 min), the ventricles were minced and shaken in KHB, and the resulting cellular digest was washed, filtered, and resuspended in phosphate-free HEPES-buffered saline (HBS; 25°C), containing 118 mM NaCl, 4.8 mM KCl, 1.2 mM $MgCl_2$, 1.25 mM $CaCl_2$, 11.0 mM dextrose, 25.0 mM HEPES, and 5.0 mM pyruvate at pH 7.35. Typically, 6-9 × 10^6 cells with 80-90% viability were obtained from each heart by this procedure. The isolated myocytes were suspended in HBS until further use.

Experimental protocols

Experiments were performed in HBS, the same buffer as was used in the myocyte preparation. Rat ventricular myocytes (5.5-5.8 × 10^5 cells/ml) suspended in HBS (37°C) were used to measure cyclic adenosine monophosphate (cAMP) production. Cardiomyocytes were pre-treated with 3-isobutyl-1-methylxanthine (IBMX) (5 × 10^{-7} M; phosphodiesterase inhibitor) for 5 min at the beginning of each experiment to accumulate intracellular cAMP. As a positive control, cells were incubated with isoproterenol (1 × 10^{-7} M); experiments in which the positive control failed were excluded from the analysis. We then conducted the study by performing the following 5 assays.

Assay 1: To investigate the effects of intravenous anesthetics on isoproterenol-stimulated cAMP production, the myocytes were incubated with fentanyl (3 × 10^{-8}, 1 × 10^{-6} M), morphine (3 × 10^{-6}, 1 × 10^{-4} M), ketamine (3 × 10^{-5}, 1 × 10^{-3} M), diazepam (3 × 10^{-7}, 1 × 10^{-5} M), midazolam (3 × 10^{-7}, 1 × 10^{-5} M), thiamylal (3 × 10^{-5}, 1 × 10^{-3} M), thiopental (3 × 10^{-5}, 1 × 10^{-3} M), or an appropriate solvent for 10 min, followed by a 10-min incubation with isoproterenol (1 × 10^{-7} M). The isoproterenol concentration used in this study was the same as that reported in a previous study[18], and we confirmed that this concentration was similar to the half maximal effective concentration (EC_{50}) in preliminary experiments. The concentrations of each anesthetic used in this study were also the same as used to treat cardiomyocytes in previous reports[8-11]. We used two concentrations of each anesthetic; the lower concentration of each anesthetic approximated to the clinical concentration.

Assay 2: Because only thiamylal and thiopental decreased isoproterenol-stimulated cAMP production in assay 1, thiamylal (1 × 10^{-5}-1 × 10^{-3} M) or thiopental (1 × 10^{-5}-1 × 10^{-3} M) was added to the cells for 10 min to investigate the effects of thiamylal and thiopental on cAMP production without isoproterenol.

Assay 3: To investigate the effects of thiamylal and thiopental on isoproterenol-stimulated cAMP production, thiamylal (3 × 10^{-6}-1 × 10^{-3} M) or thiopental (3 × 10^{-6} -1 × 10^{-3} M) was added to the cells for 10 min, after which isoproterenol (1 × 10^{-7} M) was added for an additional 10 min.

Assay 4: To investigate the effects of thiamylal and thiopental on the adenylyl cyclase, myocytes were incubated with thiamylal (3 × 10^{-5}-1 × 10^{-3} M) or thiopental (3 × 10^{-5}-1 × 10^{-3} M) for 10 min, followed by forskolin (1 × 10^{-6} M), a direct adenylyl cyclase stimulator, for an additional 10 min. We used forskolin at a concentration that increased cAMP production to similar levels as isoproterenol (1 × 10^{-7} M) in a preliminary study.

Assay 5: To investigate the role of PKC as a possible mediator of the inhibitory effects of thiamylal and thiopental, cardiomyocytes were pretreated with bisindolylmaleimide I (1 × 10^{-6} M), a non-specific PKC inhibitor, for 10 min, and then treated with thiamylal (3 × 10^{-5}-1 × 10^{-3} M) or thiopental (3 × 10^{-5}-1 × 10^{-3} M) for 10 min, followed by isoproterenol (1 × 10^{-7} M) for 10 min.

Controls: The controls in assays 1, 3, 4, and 5 were cells that were stimulated with isoproterenol or forskolin without anesthetics. The control for assay 2 was cells that were incubated without barbiturates.

Measurement of cAMP

At the end of the above protocols, the cells were quickly pelleted in a microfuge (500 *g*, 5 s). After aspirating the buffer, the pellet was homogenised in 0.1 M HCl to extract cAMP. Homogenates were centrifuged (12,000 *g*, 5 min) and supernatants were collected. Samples were stored at -80°C until further analysis. cAMP was assessed using an enzyme-linked immunoassay kit (Cayman Chemical, Ann Arbor, USA) and normalized for protein content using a bicinchoninic acid (BCA) Protein Assay kit (PIERCE Biotechnology, Rockford, USA).

Statistical Analysis and Data Presentation

Each experiment was performed on multiple myocytes from the same heart and repeated with at least four different hearts. Values were calculated as a percentage of the control response, and are expressed as the means ± SEM. Statistical comparisons within the groups were performed using the Kruskal-Wallis test followed by Dunn's test. Statistical analyses were performed using GraphPad Prism version 4.03 for Windows (GraphPad Software, California, USA). A p value of <0.05 was considered statistically significant.

Materials

Collagenase type II was purchased from Worthington Biochemical (Freehold, USA). Isoproterenol, forskolin, 3-isobutyl-1-methylxanthine, bisindolylmaleimide I, and ketamine were purchased from Sigma Chemical Co. (St. Louis, USA). Fentanyl and morphine were from DAIICHI SANKYO Co. (Tokyo, Japan). Diazepam was from Takeda Pharmaceutical Co. (Osaka, Japan). Midazolam was from Astellas Pharma Inc. (Tokyo, Japan). Thiamylal was from Nichi-Iko Pharmaceutical Co. (Toyama, Japan), and thiopental was from Tanabe Seiyaku Co. (Osaka, Japan).

RESULTS

Isoproterenol (1×10^{-7} M) and forskolin (1×10^{-6} M) increased cAMP production in myocytes by 7.94 ± 0.85 fold and 7.49 ± 0.31 fold, respectively. In comparison, both thiamylal (1×10^{-3} M) and thiopental (1×10^{-3} M) significantly decreased isoproterenol-stimulated cAMP production (Fig. 1), while fentanyl, morphine, ketamine, diazepam, and midazolam had no significant effects on isoproterenol-stimulated cAMP production. Although thiamylal and thiopental did not alter cAMP production in the absence of isoproterenol (Fig. 2), thiamylal and thiopental dose-dependently decreased isoproterenol-stimulated cAMP production (Fig. 3) as well as the forskolin-stimulated cAMP production (Fig. 4). Pretreatment with bisindolylmaleimide I did not alter the inhibitory effects of thiamylal or thiopental (Fig. 5).

DISCUSSION

We demonstrated that thiamylal and thiopental decreased beta-adrenoreceptor-mediated cAMP production in rat ventricular myocytes. These findings suggest that barbiturates may lead to hemodynamic instability in clinical patients supported by catecholamines. Previous studies showed that the hemodynamic state and sympathetic nerve activity were slightly depressed at the induction of thiopental anesthesia in patients in a normal physical condition[7,20,29]. However, Thurston[28] reported that thiopental markedly decreased the contractility of rat arterial muscles when a beta-adrenergic stimulator was used. Our results are consistent with this report, which suggests that barbiturates should be carefully used in patients receiving catecholamines to support hemodynamics or cardiac dysfunction with an augmented sympathetic tone. In clinical settings, barbiturates are also administered at high concentrations for an extended time period for the purpose of cerebral protection. The blood concentration of free barbiturates in the neuroprotective therapy is 3-4 $\times 10^{-4}$ M[1,25,30]. In patients who require this therapy, the blood pressure is elevated because of high

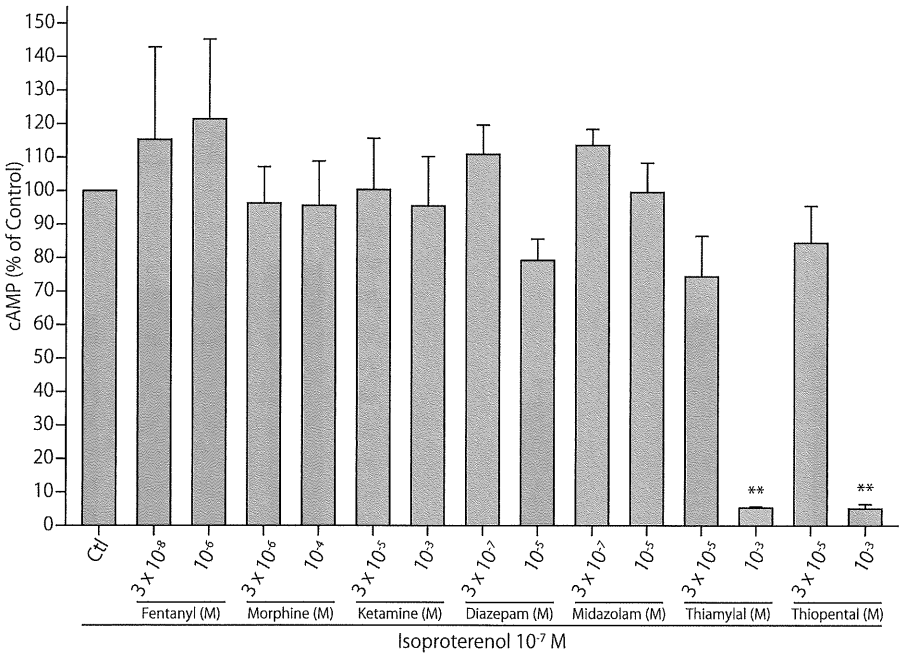

Fig. 1. The effects of anesthetics on isoproterenol (Iso)-stimulated cAMP production.

Results are expressed as a percentage of the control. ** $p<0.01$ compared with the control. n=4 (fentanyl and midazolam) or n=5 (ketamine, diazepam, thiamylal, and thiopental) hearts. Ctl = control

sympathetic tone with an increase in the intracranial pressure at the early stage, and hemodynamic supports with catecholamines are necessary for circulatory collapse following elevated blood pressure at the later stage. Our results suggest that a hemodynamic state should be carefully monitored in these cases after the administration of barbiturates.

Both thiamylal and thiopental dose-dependently decreased cAMP production in the presence of forskolin, a direct stimulator of adenylyl cyclase. Thus, it is likely that the target of these barbiturates is an adenylyl cyclase. This result is consistent with the report by Thurston[27] in which thiopental suppressed the contractility of isoproterenol-stimulated rat arterial muscles, while this suppression was reversed by dibutyryl cAMP. We attempted to locate other interaction sites of barbiturates upstream of adenylyl cyclase. Dahmani[2] reported that thiopental activated phosphorylation of non-receptor tyrosine kinase via PKC in rat hippocampal tissues. Phosphorylation of the beta-adrenergic receptor by PKC depressed the beta-adrenergic signaling pathway[4,13]. Propofol has been shown to depress the beta-adrenergic signaling pathway via PKC activation upstream of adenylyl cyclase[18]. Based on these reports, we speculated that PKC could be involved in the inhibitory effects of barbiturates on isoproterenol-stimulated cAMP production. However, pretreatment of the preparations with bisindolylmaleimide I (a non-specific PKC inhibitor) did not alter the inhibitory effects of barbiturates on cAMP production. Therefore, the mechanisms by which propofol and barbiturates inhibit isoproterenol-stimulated cAMP production may be different.

We measured cAMP to investigate the direct effects of barbiturates on the beta-adrenergic

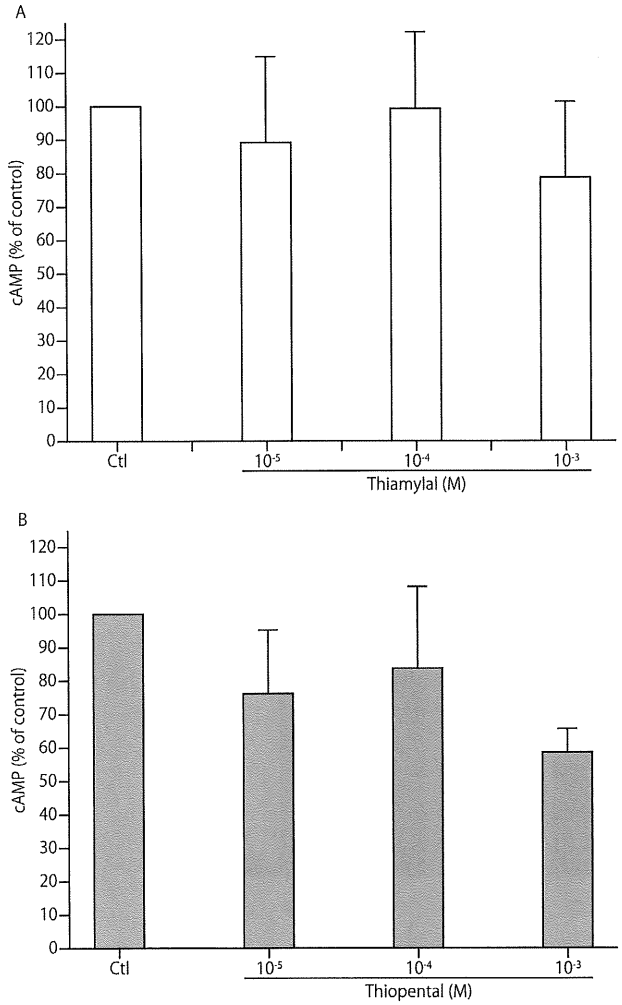

Fig. 2. The effects of thiamylal (A) and thiopental (B) on cAMP production without isoproterenol.
Results are expressed as a percentage of the control. n=5 hearts. Ctl=control.

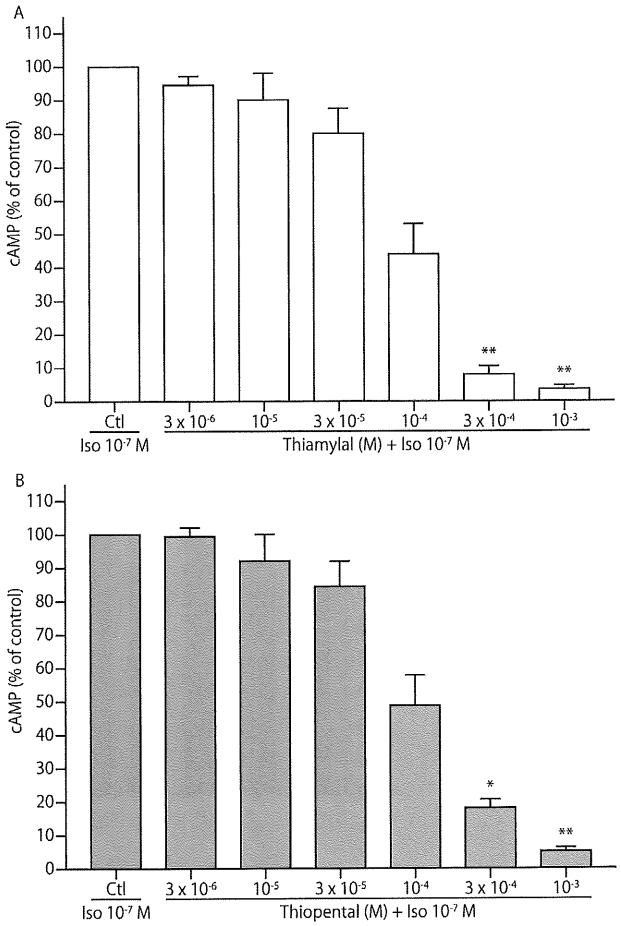

Fig. 3. The effects of thiamylal (A) and thiopental (B) on isoproterenol (Iso)-stimulated cAMP production.
Results are expressed as a percentage of the control.
* $p<0.05$ and ** $p<0.01$ compared with the control. n=5 hearts. Ctl=control.

signaling pathway. In a preliminary study, we investigated the effects of thiopental on the isoproterenol-enhanced contractility of rat ventricular myocytes by measuring the cell shortening. Thiopental (1×10^{-3} M) decreased the isoproterenol-enhanced contractility (data not shown). Changes in contractility or intracellular Ca^{2+} during the beta-adrenergic-stimulated state occur due to phosphorylation downstream of the beta-adrenergic signaling pathway. In addition, barbiturates are known to directly affect the sites downstream of the beta-adrenergic signaling pathway[3,5,6,12,14-16,19,23,24]. From these consensuses we concluded that it would be difficult to identify the specific site of action of barbiturates on the beta-adrenergic signaling pathway by measuring contractility, such as cell shortening or intracellular Ca^{2+}. Previous studies reported that thiopental decreased the steady state contractility in isolated hearts[26], papillary muscles[3,5,14,24], and cardiomyocytes[10,16]. The cellular mechanisms of thiopental could be due to inhibitions of the transsarcolemmal Ca^{2+} influx[3,5,6,12,14,16,23,24] and Ca^{2+} uptake by the sarcoplasmic reticulum (SR)[19,24], or the release of Ca^{2+} from SR[15,24]. Further studies will be needed to examine intracellular Ca^{2+}, SR, and cytoplasmic ion channels to identify the sites of action of barbiturates downstream of the beta-adrenergic signaling pathway during a beta-adrenergic-stimulated state.

In conclusion, fentanyl, morphine, ketamine, diazepam, and midazolam had no significant effect on isoproterenol-stimulated cAMP production in rat ventricular myocytes, at the concentrations used in this study. On the other hand, thiamylal and thiopental decreased isoproterenol-stimulated cAMP production by suppressing adenylyl cyclase through PKC-independent mechanisms.

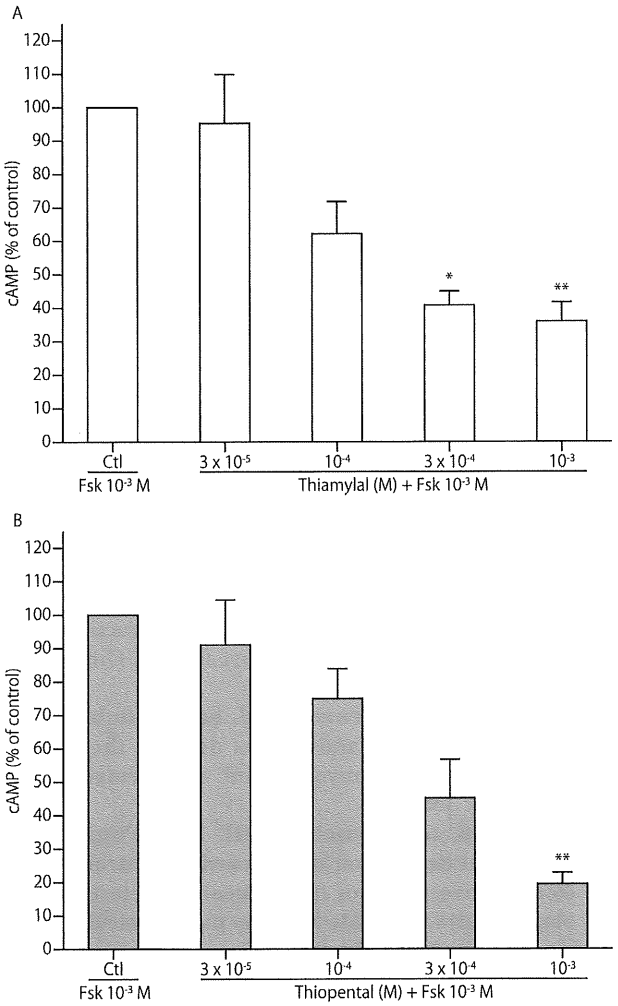

Fig. 4. The effects of thiamylal (A) and thiopental (B) on forskolin (Fsk)-stimulated cAMP production.
Results are expressed as a percentage of the control.
* $p<0.05$ and ** $p<0.01$ compared with the control. n=6 (A) or n=4 (B) hearts. Ctl=control.

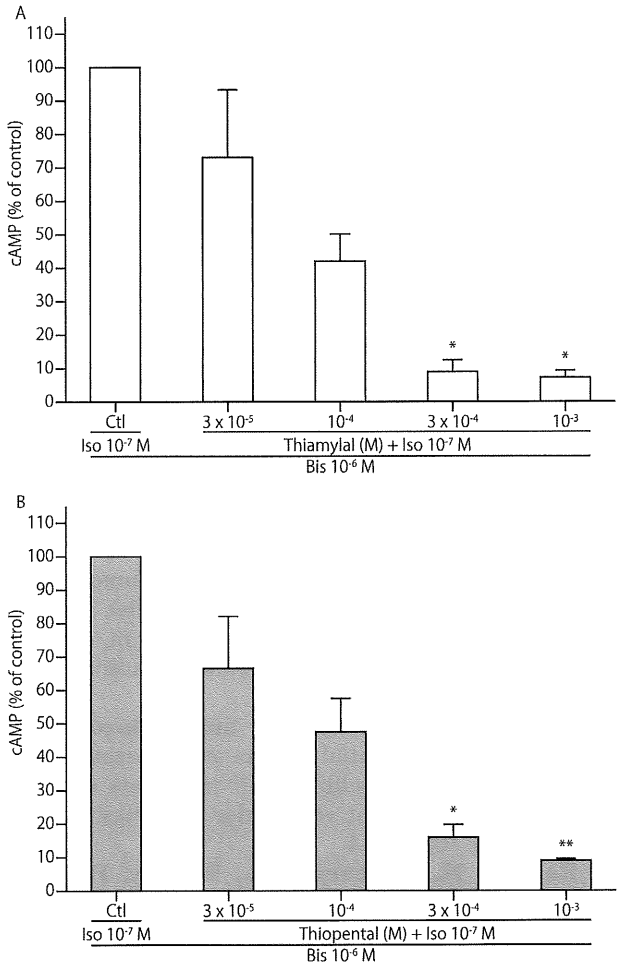

Fig. 5. The effects of bisindolylmaleimide I (Bis) with thiamylal (A) and thiopental (B) on isoproterenol (Iso)-stimulated cAMP.
Results are expressed as a percentage of the control.
* $p<0.05$ and ** $p<0.01$ compared with the control. n=4 hearts. Ctl=control.

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