Ketamine inhibits agonist-induced cAMP accumulation increase in human airway smooth muscle cells

Gary E. Hill MD,
Jodi L. Anderson BS,
Charles W. Whitten MD

Purpose: Cyclic 3',5' adenosine monophosphate (cAMP) is a second messenger of the beta adrenergic receptor (BAR). Ketamine causes an increase in the intracellular accumulation of cAMP in several non-human tissue preparations. A "species effect" may explain the differing results of ketamine on BAR mediated responses, thus reports of a ketamine-induced increase in cAMP in other species may not be applicable to humans.

Methods: The effect of ketamine (10^{-3} , 10^{-4} , or 10^{-5} M) pretreatment (60 and 120 min) on isoproterenol [ISO, a beta adrenergic receptor (AR) agonist] or forskolin [FSK, an activator of adenylylcyclase (AC)]-induced intracellular accumulation of cAMP in a human airway smooth muscle (tracheal) cell line (HASM) was evaluated. In an *in vitro* HASM culture, cells with or without pretreatment were labeled with [3 H]adenine to produce [3 H]ATP, and following stimulation with ISO or FSK to convert the [3 H]ATP to [3 H]cAMP, the intracellular accumulation of [3 H]cAMP was measured by sequential chromatography over Dowex and alumina columns.

Results: Pretreatment of the HASM cells with ketamine (10^{-3} and 10^{-4} M) caused a reduction ($P < 0.05$, when compared to untreated cells) in ISO-induced cAMP accumulation, but did not effect cAMP accumulation following FSK stimulation. This effect of ketamine was greater at 120 min of pretreatment than at 60 min (10^{-3} M ketamine only) ($P < 0.05$). No effect was found at either time period following pretreatment of the HASM cells with ketamine 10^{-5} M.

Conclusions: These results demonstrate that pretreatment of the HASM cells with ketamine reduces ISO-induced cAMP accumulation. Since only ISO-induced cAMP was effected by ketamine, these data suggest that ketamine inhibits production of cAMP proximal to AC in the cAMP production pathway. These results also demonstrate that a mechanism other than that involving the BAR and intracellular cAMP accumulation is responsible for the ketamine induced bronchodilation in humans.

Objectif : L'adénosine monophosphate-3', 5' cyclique (AMPc) est un second messenger du récepteur bêta-adrénergique (RBA). La kétamine augmente l'accumulation intracellulaire d'AMPc dans certaines préparations de tissus non humains. Un «effet lié à l'espèce» peut expliquer les différentes réactions de la kétamine sur les réponses à médiation RBA. L'augmentation d'AMPc induite par la kétamine pourrait donc ne pas s'appliquer aux humains.

Méthode : On a évalué l'effet du prétraitement (60 ou 120 min) de kétamine (10^{-3} , 10^{-4} , ou 10^{-5} M) sur l'accumulation intracellulaire d'AMPc induite par l'isoprotérénol [ISO, un agoniste des récepteurs bêta-adrénergiques (RBA)] ou par la forskoline [FSK, un activateur de l'adényl-cyclase (AC)] dans une lignée cellulaire de muscle lisse (trachéal) humain des voies aériennes (MLHVA). Les cellules, prétraitées ou non, d'une culture *in vitro* de MLHVA ont été marquées avec de l'adénine [3 H] afin de produire de l'ATP [3 H] et, à la suite de la stimulation avec l'ISO ou la FSK pour convertir l'ATP [3 H] en AMPc [3 H], l'accumulation intracellulaire d'AMPc [3 H] a été mesurée par chromatographie séquentielle sur des colonnes Dowex et d'alumine.

Résultats : Le prétraitement de cellules de MLHVA avec la kétamine (10^{-3} et 10^{-4} M) a réduit l'accumulation d'AMPc induite par l'ISO, mais n'a pas eu d'effet sur l'accumulation d'AMPc ($P < 0,05$ comparé aux cellules non traitées) à la suite d'une stimulation avec la FSK. Cet effet de la kétamine a été plus important à 120 min de prétraitement qu'à 60 min (kétamine 10^{-3} M seulement) ($P < 0,05$). Aucun effet n'a été enregistré aux temps repères suivant le prétraitement des cellules de MLHVA avec la kétamine 10^{-5} M.

Conclusion : Ces résultats démontrent que le prétraitement des cellules MLHVA avec la kétamine réduit l'accumulation d'AMPc induite par l'ISO. Puisque seule l'AMPc induite par l'ISO a subi l'effet de la kétamine, on peut penser que la kétamine inhibe la production d'AMPc en amont de l'AC dans la chaîne de production de l'AMPc. On peut aussi conclure qu'un mécanisme autre que celui qui implique le R A et l'accumulation intracellulaire d'AMPc est

From the Departments of Anesthesiology and Pain Management, the University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd., Dallas, Texas 75235-9068 USA.

Address correspondence to: Gary E. Hill MD, Phone: 214-590-7277; Fax: 214-648-5461; E-mail: gary.hill@email.swmed.edu.

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CYCLIC 3',5' adenosine monophosphate (cAMP) is the primary second messenger of the beta adrenergic receptor (β AR).¹ An increased intracellular accumulation of cAMP following β AR stimulation induces the physiological responses of airway smooth muscle relaxation and increased cardiac muscle contractile force.¹ Recent reports from our laboratory² and others³ demonstrate that *in vitro* exposure to the intravenous anesthetic ketamine causes an increase in the intracellular accumulation of cAMP in rat cardiac and mesangial cells, respectively. Similarly, Kohjitani *et al.*⁴ demonstrated that ketamine increases intracellular cAMP concentrations in rabbit esophageal muscle cells. In contrast, other reports demonstrate that ketamine has either no effect⁵ or causes a reduction of⁶ intracellular cAMP accumulation in dog airway smooth muscle and mice brain cells, respectively. Increased human airway smooth muscle cell cAMP accumulation caused by ketamine could explain the relaxation (bronchodilation) of airway smooth muscle following the clinical use of this intravenous anesthetic. In support of this hypothesis, Sato *et al.*⁷ found that ketamine relaxed pre-constricted airway (tracheal) smooth muscle in an *in vitro* guinea pig model, while Gateau *et al.*⁸ found similar results in human airway smooth muscle. Additional support for this hypothesis is the demonstration by Hirshman *et al.*⁹ that pharmacological β AR antagonism blunts ketamine-induced bronchodilation in an intact dog model, suggesting that ketamine may stimulate the β AR. Since the effect of ketamine on cAMP accumulation in human airway smooth muscle cells has not been previously reported, this study was undertaken to examine the hypothesis that ketamine causes human airway smooth muscle relaxation by increasing the intracellular concentration of cAMP. Therefore, experiments were conducted on a human airway smooth muscle cell line (HASM) utilizing the same experimental techniques used previously on rat cardiac muscle.² In addition, this study was also intended to demonstrate the possible presence of a "species effect" of ketamine. This "species effect", if present, could explain the divergent cAMP-mediated effects of ketamine reported between studies conducted on different species.¹⁰⁻¹³

In order to determine if ketamine alters β AR function and the subsequent cAMP accumulation in HASM cells, the agonist [isoproterenol (ISO), a direct acting β AR agonist, or forskolin (FSK), a direct activator of the enzyme adenylylcyclase (AC)]-induced increase of intracellular cAMP accumulation with or without ketamine pretreatment was evaluated. Since airway smooth muscle relaxation is known to occur within a short time period (within minutes) following ketamine administra-

tion,¹⁴ a similarly short exposure time (60 and 120 min) to ketamine of the HASM cell line *in vitro* was evaluated prior to measuring the agonist (ISO or FSK)-induced changes in cAMP accumulation.

Methods

Materials

The human airway (tracheal) smooth muscle cell line (HASM cells) was provided by Dr. Michael Kotlikoff (University of Pennsylvania);¹⁵ ketamine HCl from Research Biochemicals International (Natick, MA); isoproterenol from Sigma (St. Louis, MO); forskolin from Calbiochem (San Diego, CA); Dulbecco's modified Eagles's medium (DMEM) and fetal bovine serum from GIBCO (Grand Island, NY); and BudgetSolve scintillation cocktail from Research Products International (Mt. Prospect, IL).

Cell culture and drug treatment

The HASM cell line has preserved elements of the signaling pathways found in human tracheal smooth muscle cells and retains phenotypic characteristics identical to the native tissue and is stable over many passages in culture.¹⁵ More importantly, this cell line preserves a functional β AR-effector system, identical to that displayed by native human airway smooth muscle. Stimulation of the β_2 AR by ISO is responsible for the intracellular accumulation of cAMP in this cell line.¹⁵ The HASM cells were cultured to confluence in DMEM (glucose 4.5%) containing fetal bovine serum 10%. Cells passaged from one to 12 times were used for the experiments. For pre-treatments, cells were incubated in serum-containing medium in the presence or absence of ketamine (10^{-3} , 10^{-4} , or 10^{-5} M, or 1000, 100 or 10 μ M, respectively) for 60 or 120 min. Ketamine was dissolved in sterile preservative free water. Control cells were treated with the appropriate vehicles. The HASM cells were cultured to confluency in 75 cm² straight neck tissue culture polystyrene flasks (Corning Costar Corporation, Cambridge, MA), then treated with trypsin-EDTA (GIBCO), and transferred to 35 $\times$ 10 mm style tissue culture dishes (Becton Dickinson, Franklin Lakes, NJ) for the conduct of the experiments.

Assays of intact cell cAMP accumulation

The [³H] adenine prelabeling procedure of Shimizu *et al.*¹⁶ was used to measure cAMP accumulation by intact cells, essentially as described by others.¹⁷ Briefly, the growth medium, with or without the various pre-treatments, was aspirated and the cell sheets were washed once with 2 ml DMEM buffered to pH 7.4 with 20 mM HEPES (N-2-hydroxyethylpiperazine-

FIGURE 1 cAMP accumulation (% conversion) following stimulation with isoproterenol or forskolin in the HASM cell line. Data displayed are following a 60 min pretreatment period with ketamine (10^{-3} , 10^{-4} and 10^{-5} M). Data are expressed as means $\pm$ SEM. The data displayed are from three experiments done in triplicate. * $P < 0.05$, when compared to same group control (untreated) conditions.

FIGURE 2 cAMP accumulation (% conversion) following stimulation with isoproterenol or forskolin in the HASM cell line. Data displayed are following a 120 min pretreatment period with ketamine (10^{-3} , 10^{-4} and 10^{-5} M). Data are expressed as means $\pm$ SEM. The data displayed are from three experiments done in triplicate. * $P < 0.05$, when compared to same group control (untreated) conditions. † $P < 0.05$, when compared to the 60 min ketamine pretreatment group, same experimental conditions.

N²-ethanesulfonic acid) (DMEM-HEPES). The cells were then incubated for 60 min at 37°C following the addition of 1 ml of DMEM-HEPES containing 2 μ Ci of [³H]adenine to each 35-mm dish in order to label intracellular ATP pools. Cells were then washed and incubated for 10 min at 37°C in DMEM-HEPES with 0.1 mM 3-isobutyl-1-methylxanthine (IBMX), in the absence or presence of ISO (10^{-7} M or 0.1 μ M) or FSK (30 μ M) in order to stimulate cAMP formation. The concentrations of ISO and FSK were chosen for these experiments because these concentrations were previously demonstrated to be near the mid-point of their respective dose-response curves for cAMP accumulation in the HASM cell line.¹⁷ The stimulation medium was then aspirated and 1 ml of 5% trichloroacetic acid was added to stop further reactions and to extract cellular nucleotides. The trichloroacetic acid extracts were then subjected to sequential chromatography over Dowex and alumina columns to separate [³H]cAMP from [³H]ATP as described by Meeker and Harden.¹⁸ Radioactivity in the samples was quantitated with liquid scintillation spectroscopy in 14 ml BudgetSolve. Accumulation of

[³H]cAMP was then expressed as the percent of conversion of [³H]ATP to [³H]cAMP ([³H]cAMP / [³H]cAMP + [³H]ATP). Incorporation of ³H into the ATP fraction was typically 150,000 to 200,000 cpm/dish, and this value was not altered markedly by any of the pre-treatments. This assay does not give absolute values for cAMP accumulation, but has the advantage of intrinsically normalizing the data for changes in cell number or viability.^{17,18} Basal cAMP accumulation (% conversion) was determined in unstimulated HASM cells.

STATISTICAL ANALYSIS

Data are expressed as means $\pm$ SEM. Analysis of variance was used to compare means between dosing levels for the treatment groups (ISO or FSK) at the two (60 and 120 min) time intervals. Dunnett's two-tailed t test was used to determine if any dosing levels were significantly different from the control. Linear contrasts were used to test for a significant dose-response effect of ketamine pretreatment and to compare the response to dose levels at the two time points. P values of < 0.05 were regarded as statistically significant.

Results

Basal cAMP accumulation (% conversion) in unstimulated and untreated HASM cells was minimal to none.

Pretreatment of the HASM cells with ketamine (10^{-3} or 10^{-4} M) for 60 min caused a significant ($P < 0.05$) reduction in cAMP accumulation following ISO but not FSK stimulation when compared to control (untreated) cells (Figure 1).

Similarly, pretreatment of the HASM cells with ketamine (10^{-3} or 10^{-4} M) for 120 min caused a significant ($P < 0.05$) reduction in cAMP accumulation following ISO but not FSK stimulation when compared to control (untreated) cells (Figure 2).

Ketamine (10^{-3} M) pretreated cells demonstrated a significantly greater ($P < 0.05$) reduction following ISO stimulation at the 120 min pretreatment period when compared to the 60 min pretreatment period (Figure 2).

Pretreatment of the HASM cells with ketamine (10^{-5} M) for both 60 and 120 min did not significantly effect cAMP accumulation following ISO or FSK stimulation when compared to control (untreated) cells (Figures 1, 2).

Discussion

Pharmacologic stimulation of the beta adrenergic receptor (β AR) results in activation of the enzyme adenylcyclase (AC) resulting in the formation of the second messenger cAMP. Increased intracellular accumulation of cAMP results in airway smooth muscle relaxation and an increase in the rate and force of cardiac contraction. Adenylcyclase function and/or expression is considered the rate limiting step in cAMP accumulation.¹ Since increased intracellular accumulation of cAMP is the physiologic mechanism responsible for β AR mediated responses, reports that ketamine increases intracellular cAMP accumulation^{2,3,19} in rat cell cultures could explain the known airway smooth muscle relaxation caused by ketamine.^{14,20,21} Previous canine experiments performed following antigen-induced bronchospasm demonstrate that ketamine-induced bronchodilation is blunted by propranolol (a non-selective β_1 and β_2 AR antagonist).⁹ Those results⁹ suggest that ketamine causes bronchodilation by stimulation of the β AR.⁹ Unfortunately, this report demonstrates that contrary to the effects in rat cell tissue cultures^{2,3,19} and rabbit esophageal muscle strips,⁴ ketamine inhibits the agonist induced increases in intracellular cAMP accumulation in a human airway (tracheal) smooth muscle cell culture. Therefore, these results demonstrate that ketamine induces human airway smooth muscle relaxation

by a mechanism other than that involving the β AR and the resulting increase in the intracellular accumulation of cAMP.

The clinical use of ketamine ($2 \text{ mg} \cdot \text{kg}^{-1}$, *iv*) results in a plasma concentration of approximately $60 \mu\text{M}$.²² Therefore, the concentrations of ketamine used in this *in vitro* study (10, 100, and $1000 \mu\text{M}$) are similar to that used clinically. These results may suggest a mechanism for the reported ketamine-induced reduction of *in vitro* cardiac muscle contractile function reported with human cardiac muscle preparations,¹² since a reduction of intracellular cAMP accumulation will result in a reduced force of cardiac contraction.^{1,23} Because data demonstrating ketamine reduces *in vitro* myocardial contractile function in a human cardiac muscle preparation may have been influenced by prior β AR antagonist therapy,¹² the experiments reported in this study were completed in a HASM culture without any pretreatments other than ketamine. Therefore, these data give support to the concept that ketamine has a defined "species effect", that is, ketamine causes an increase in intracellular cAMP accumulation in rat^{2,3,19} and rabbit⁴ preparations but a reduction in cAMP accumulation in human tissue culture preparations.

In non-airway (cardiac muscle) tissue preparations, the pharmacologic effects of ketamine are controversial. For example, ketamine is reported to increase^{10,11} as well as to decrease^{12,13} *in vitro* cardiac contractile function, depending upon the species studied. These contrasting results support the concept of a "species effect" of ketamine on the accumulation of intracellular cAMP, since changes in the intracellular concentration of cAMP is a major factor in the β AR-mediated increase in cardiac muscle contractile force.¹ Therefore, these reports¹⁰⁻¹³ question the reliability of extrapolation of experiments performed on non-human tissue evaluating the pharmacologic effects of ketamine administration to a similar pharmacologic response in human tissue.

The β AR is coupled to AC via a stimulatory guanine nucleotide regulatory protein (Gs), activated AC in turn converts ATP to cAMP.²³ Since FSK is a direct activator of AC, these results indicate that ketamine inhibits cAMP accumulation proximal to AC, since ketamine has no significant effect on cAMP accumulation following FSK stimulation. In similar fashion to halothane and isoflurane,²⁴ ketamine inhibits cAMP accumulation only following ISO stimulation. This suggests that ketamine has an inhibitory effect on the β AR itself or an inhibitory effect at the level of the Gs protein. Halothane reduces calcium sensitivity in canine airway smooth muscle by inhibiting G protein function,²⁵ thereby causing airway smooth muscle

relaxation. While these data suggest a similar mechanism for ketamine, further studies will be required to determine the exact mechanism and location of the inhibitory effect of ketamine on cAMP accumulation in the HASM cell line. Furthermore, these data are consistent with results reported by Wilson *et al.*²⁶ that demonstrate ketamine causes bronchodilation by a mechanism not involving the β AR. In fact, Bevan *et al.*²⁷ reported evidence that ketamine may demonstrate properties characteristic of a β AR antagonist.

In summary, this study demonstrates that ketamine inhibits the agonist (isoproterenol)-induced increase of intracellular cAMP accumulation in a human airway (tracheal) smooth muscle cell culture. This is in direct contrast to other reports demonstrating ketamine increases cAMP accumulation in rat and rabbit preparations. Therefore, previous reports demonstrating ketamine causes an increase in intracellular cAMP accumulation in non-human tissue preparations cannot be extrapolated to the human. Since increased intracellular concentrations of cAMP cause β AR-mediated increases in airway smooth muscle relaxation, these results do not indicate a mechanism for ketamine-induced bronchodilation in humans^{14,20,21} and suggest mechanisms other than that involving the β AR and the resulting change in intracellular cAMP accumulation are responsible.

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