Laboratory Investigation

Phosphatidylinositol responses are involved in the vascular effects of thiamylal and fentanyl

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Although thiobarbiturates potentiate, and fentanyl attenuates peripheral vasoconstriction, the intracellular mechanism involved in this phenomenon is not clear. Because smooth muscle contraction induced by α_1-adrenoceptor agonists is mediated by the phosphatidylinositol (PI) response, this study was carried out to clarify if thiamylal and fentanyl affect the norepinephrine-induced PI response in rat aortic slices. Rat aortic slices were incubated in Krebs-Henseleit solution containing 5 mM LiCl, [^{3}H]myo-inositol, and varying concentrations of thiamylal or fentanyl. The PI response was stimulated by 0.09 μM (ED_{50}) norepinephrine (NE). The [^{3}H]inositol monophosphate (IP_1) was separated from [^{3}H]myo-inositol by column chromatography and counted with a liquid scintillation counter. The basal IP_1 accumulation was not affected by thiamylal and fentanyl. Norepinephrine-induced IP_1 accumulation was potentiated by thiamylal at concentrations of 10 μM and 100 μM. Norepinephrine-induced IP_1 accumulation was attentuated by 1 μM and 10 μM fentanyl. The results suggest that thiamylal stimulates the NE-induced PI response, which potentiates the vasoconstriction, and fentanyl attentuates NE-induced PI response, which would attenuate the vasoconstriction.

On connaît mal le mécanisme intracellulaire qui fait que le thiamylal potentialise la vasoconstriction périphérique et que le fentanyl l'atténue. Comme la constriction du muscle lisse induite par les agonistes α_1-adrénergiques dépend de la réponse du phosphatidylinositol (PI), cette étude vise à vérifier sur des tranches d'aorte de rat si le thiamylal et le fentanyl affectent la réponse du PI induite par la norépinéphrine (NE). Celles-ci sont incubées dans une solution de Krebs-Henseleit contenant 5 mM de LiCl, du [^{3}H] myo-inositol et différentes concentrations de thiamylal ou de fentanyl. La réponse du PI est provoquée par 0,09 μM (ED_{50}) de NE. Le [^{3}H] inositol monophosphate (IP_1) est séparé du [^{3}H] myo-inositol par chromatographie sur colonne anionique et mesuré avec un compteur à scintillation liquide. L'accumulation d'IP_1 initiale n'est pas affectée par le thiamylal et le fentanyl. L'accumulation d'IP_1 induite par la NE est potentialisée par le thiamylal à des concentrations de 10 μM et de 100 μM. L'accumulation d'IP_1 induite par la NE est atténuée par 1 μM et 10 μM de fentanyl. Ces résultats suggèrent que le thiamylal stimule la réponse induite par la NE, laquelle potentialise la vasoconstriction, et que le fentanyl atténue la réponse de l'IP_1 induite par la NE, laquelle pourrait atténuer la vasoconstriction.

Key words
ANAESTHETICS, INTRAVENOUS: fentanyl, thiamylal;
ARTERIES: aorta;
PHOSPHATIDYLINOSITOL: inositol monophosphate.

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Barbiturates are used widely as agents to protect the brain from ischaemic damage by decreasing cerebral metabolism and oxygen demand or as agents for the induction of anaesthesia. Altura *et al.*[1] demonstrated, using isolated rat aortic strips, that secobarbital, amobarbital or phenobarbitone inhibited spontaneous contraction at clinically relevant concentrations, and that these barbiturates attenuated epinephrine-induced contractions in a dose dependent manner. However, they[1] and Burn *et al.*[2] observed that thiopentone could elicit contractions of aortic strips. Moriyama *et al.*[3] reported that in helical strips of dog cerebral and mesenteric arteries contracted with KCl or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), the addition of pen-

tobarbitone caused dose-related relaxation, whereas thiamylal and thiopentone caused further contraction. Terasako *et al.*[4] observed, in rat aorta, that both endothelium-dependent and -independent relaxations were considerably attenuated by thiopentone.

Fentanyl is used for balanced general anaesthesia or for cardiovascular anaesthesia. Toda *et al.*[5] reported that the contractile response of helically-cut strips of rabbit ascending aorta to transmural electrical stimulation was attenuated by fentanyl in a dose-dependent manner, and that fentanyl shifted the dose-response curve of the contractile response of the aorta to norepinephrine (NE) to the right. White *et al.*[6] reported that, in dogs with both hindlimbs isolated from the systemic circulation to allow extracorporeal perfusion at constant flow, increasing fentanyl administration caused a progressive diminution in peripheral resistance.

When α_1-adrenoceptors in the cell membrane are stimulated to activate the phospholipase C (PLC), phosphatidylinositol-4,5-bisphosphate (PIP_2) is hydrolyzed into IP_3 and diacylglycerol. Inositol 1,4,5 trisphosphate (IP_3) mobilizes Ca^{++} from sacroplasmic reticulum,[7] whereas diacylglycerol activates protein kinase C (PKC) which may also be a mechanism of modulating or controlling smooth muscle tension. Subsequently, the increase in cytoplasmic Ca^{++} concentration and activation of PKC may cause smooth muscle contraction.

Although thiopentone and thiamylal potentiate, and fentanyl attenuates the vasoconstriction, the intracellular mechanisms involved in these phenomena are not clear. Because smooth muscle contraction induced by α_1-adrenoceptor agonists is mediated by phosphatidylinositol (PI) response,[8,9] this study was carried out to clarify if thiamylal and fentanyl could affect the NE-induced PI response in rat aortic slices.

Methods

The studies were conducted under guidelines approved by the Animal Care Committee of Nagasaki University School of Medicine. The technique of Brown *et al.*[10] was used. Inositol 1,4,5 triphosphate is rapidly degraded into inositol monophosphate (IP_1) which is recycled to phosphatidylinositol (PI) via free inositol (Figure 1). Lithium inhibits the conversion of IP_1 into inositol. Thus, in the presence of lithium, the accumulation rate of IP_1 reflects the extent of PI response.[11] We measured [^{3}H]IP_1 in aortic slices incubated with [^{3}H]myo-inositol (Amersham, Tokyo Japan). Eighty-nine male Wistar rats (Charles River, Yokohama Japan) weighing 250–350 g were used for experiments. The rats were stunned by cervical dislocation, decapitated and the thoracic aorta was rapidly isolated. For tissue preparation without endothelium, endothelium was removed by rubbing with cotton gauze.

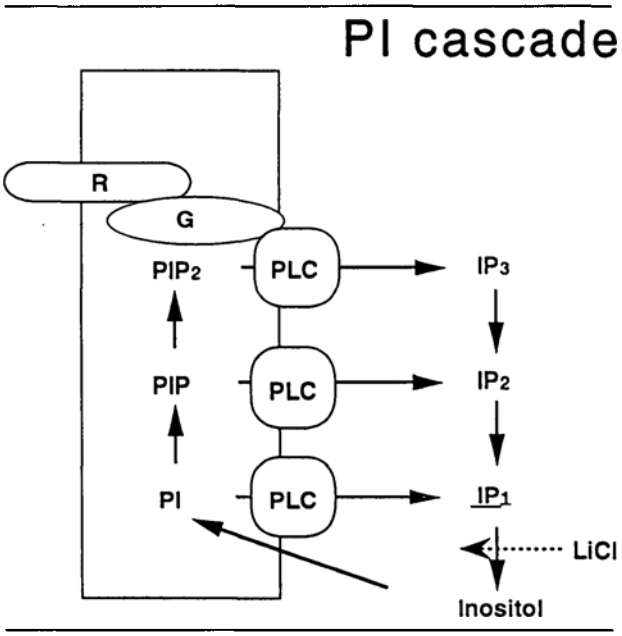

FIGURE 1 PI cascade. PI: phosphatidylinositol, PIP; phosphatidylinositol 4-phosphate, PIP_2; phosphatidylinositol 4,5-bisphosphate, IP_3; inositol 1,4,5-trisphosphate, IP_2; inositol bisphosphate, IP_1; inositol monophosphate, R; α-receptor, G; G-protein, PLC; phospholipase C.

The aorta with or without endothelium was cut longitudinally and chopped into 1-mm-wide pieces with a McIlwain tissue chopper (The Mickle Laboratory Engineering, Gomshall England). Three pieces of the aortic slice were placed in small flat-bottomed tubes and preincubated for 15 min in Krebs-Henseleit (K-H) solution (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 1.3, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 10, Na_2-EDTA 0.05) containing 5 mM LiCl. The solution was continuously aerated with 95%O_2/5%CO_2. An aliquot of 0.5 μCi [^{3}H]myo-inositol was then added to each tube (final concentration 0.1 μM in 300 μl incubation volume) and the tubes were flushed with 95%O_2/5%CO_2, capped, set in a shaking bath at 37°C and incubated for 30 min (time 0).

Firstly, we determined the ED_{50} of NE on IP_1 accumulation. After 30-min incubation with [^{3}H]myo-inositol, varying doses of NE were added to the suspension of aortic slices and the tubes were flushed with 95%O_2/5%CO_2. After an additional 60 min, the enzymatic reactions were stopped with 940 μl chloroform : methanol (1 : 2 v/v). Chloroform and water were then added (310 μl each) and the phases were separated by centrifugation with 90 g for five minutes. The [^{3}H] inositol monophosphate was separated from [^{3}H]myo-inositol in the water phase by column chromatography using Dowex AG 1-X8 resin (Bio Rad, Richmond CA)

in the formate form. The [^{3}H]IP_1 formed in the aortic slices was counted with a liquid scintillation counter and presented by disintegration per min (DPM). The counts in DPM of two samples were averaged and the average DPMs of the blank values (no slices present) were subtracted to obtain the experimental data.

Secondly, we determined the time course of the effects of NE 0.09 μM (ED_{50}) on IP_1 accumulation. The reaction was started at time 0 when NE was added. The tubes were reaerated with 95%O_2/5%CO_2, recapped and reincubated for 0, 15, 30, 45, 60 or 75 min. The reaction was stopped with 940 μl chloroform : methanol as described above.

Thirdly, we determined the effects of prazosin on NE-induced IP_1 accumulation. At time 0, varying doses of prazosin were added and 15 min later 30 μl, 0.9 μM NE were added (final NE concentration was 0.09 μM which corresponded ED_{50}). The tubes were reaerated, recapped and reincubated, for a further 60 min and the reaction was stopped.

Fourthly, we examined the effects of pentobarbitone, thiamylal and fentanyl on basal IP_1 accumulation, and on NE-induced IP_1 accumulation. After 30-min incubation of the aortic slices with [^{3}H]myo-inositol, varying doses of pentobarbitone, thiamylal or fentanyl were added to the medium and the tubes were flushed with 95%O_2/5%CO_2. After 15 min, 30 μl, 0.9 μM NE were added to the medium and the tubes were flushed with 95%O_2/5%CO_2, and reincubated for a further 60 min and then the reaction was stopped. To examine the effects on basal IP_1 accumulation, the same procedure was conducted except for the addition of NE. The reaction was stopped after a further 60 min incubation.

Lastly, we examined the effects of thiamylal and fentanyl on NE-induced IP_1 accumulation without endothelium. After 30-min incubation with [^{3}H]myo-inositol, 30 μl thiamylal (1000 μM) or fentanyl (100 μM) were added to the medium and the tubes were flushed with 95%O_2/5%CO_2. After 15 min, 30 μl, 0.9 μM NE were added to the medium and the tubes were flushed with 95%O_2/5%CO_2 and reincubated for further 60 min and then the reaction was stopped.

Data are expressed as mean ± SE. A comparison between two groups was assessed by Student's t test. A P value <0.05 was considered significant.

Results

In rat aortic slices with endothelium, IP_1 accumulation was stimulated by NE in a dose-dependent manner (Figure 2). The ED_{50} of NE on IP_1 accumulation was determined as about 0.09 μM from Figure 2. Inositol monophosphate accumulation, after adding NE (ED_{50}), continued to increase until 75 min (Figure 3). In the presence of NE, IP_1 formed was 547 ± 30 DPM after 60-min incubation. Norepinephrine-induced IP_1 accumulation was inhibited by prazosin in a dose-dependent manner (Figure 4). Basal IP_1 accumulation was not affected by pentobarbitone, thiamylal or fentanyl (Figures 5–7). Norepinephrine-induced IP_1 accumulation was not affected by pentobarbitone, but was augmented by thiamylal at concentrations of 10 μM and 100 μM ($P < 0.01$). In contrast, NE-induced IP_1 accumulation was attentuated by fentanyl at concentrations of 1 μM and 10 μM ($P < 0.01$).

Without endothelium, thiamylal (100 μM) stimulated and fentanyl (10 μM) attenuated NE-induced IP_1 accumulation (Figure 8).

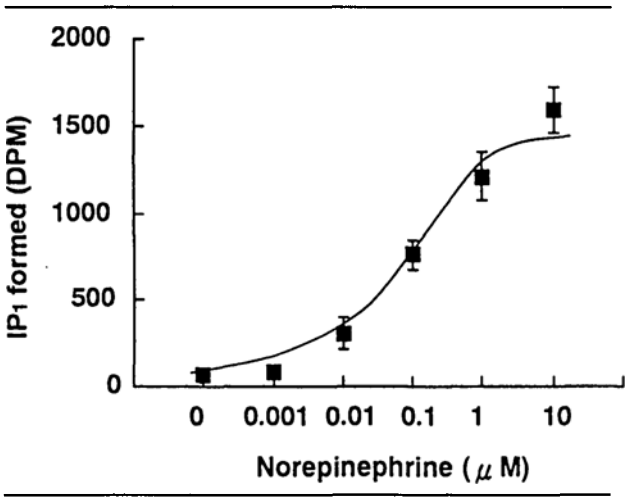

FIGURE 2 Effects of norepinephrine on IP_1 accumulation in rat aortic slices with endothelium (mean ± SE; $n = 7$ for each value).

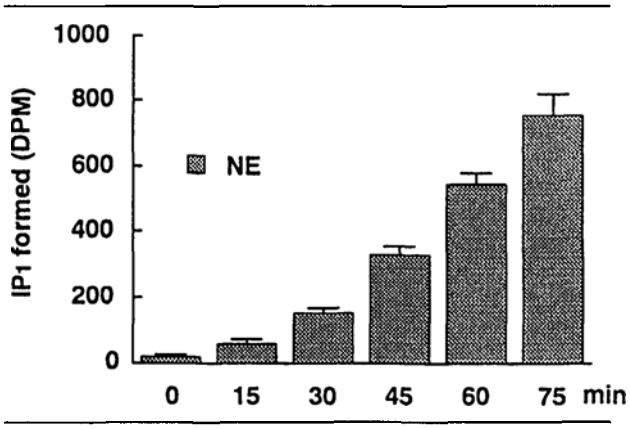

FIGURE 3 Time course of IP_1 accumulation by 0.09 μM (ED_{50}) norepinephrine (NE) in rat aortic slices with endothelium (mean ± SE; $n = 6$–8 for each value).

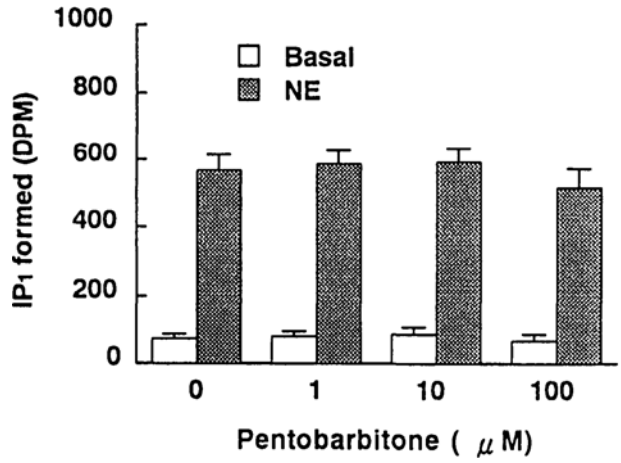

FIGURE 4 Effects of prazosin on norepinephrine (NE)-induced IP_1 accumulation in rat aorta slices with endothelium (mean ± SE; n = 5–6 for each value).

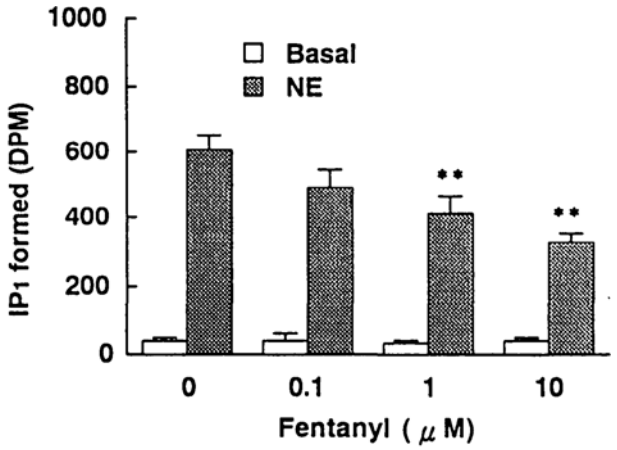

FIGURE 6 Effects of thiamylal on norepinephrine (NE)-induced IP_1 accumulation in rat aortic slices with endothelium (mean ± SE; n = 6–8 for each value). $^{**}P < 0.01$ vs thiamylal 0.

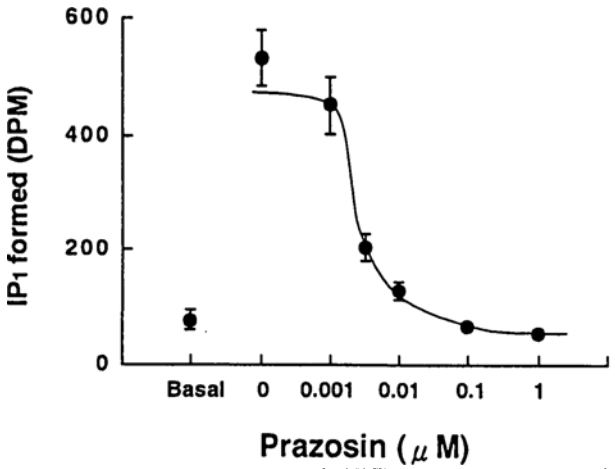

FIGURE 5 Effects of pentobarbitone on norepinephrine (NE)-induced IP_1 accumulation in rat aortic slices with endothelium (mean ± SE; n = 5–7 for each value).

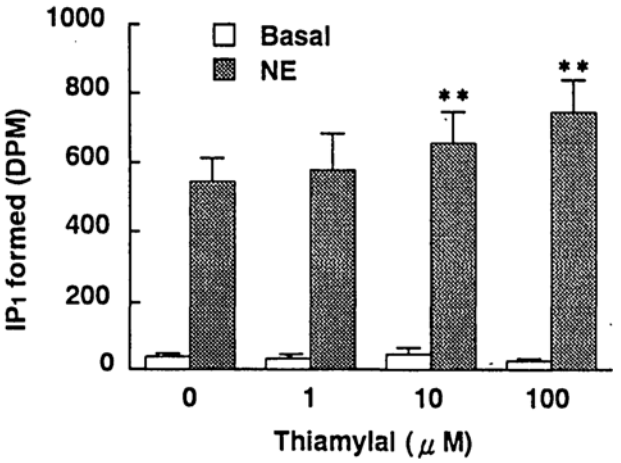

FIGURE 7 Effects of fentanyl on norepinephrine (NE)-induced IP_1 accumulation in rat aortic slices with endothelium (mean ± SE; n = 6–8 for each value). $^{**}P < 0.01$ vs fentanyl 0.

Discussion

Although the mechanisms involved in vasoconstriction are not simple, one of the important factors is Ca^{++}. Potassium chloride (KCl) depolarizes smooth muscle cells, resulting in provocation of Ca^{++} influx from extracellular fluid. On the other hand, α_1-adrenoceptor- or $PGF_{2\alpha}$-receptor-agonist activates the PI response and subsequently increases Ca^{++} release from sarcoplasmic reticulum and Ca^{++} influx from extracellular fluid.

It is known that the isolated vessel's response to NE reaches a plateau after ten minutes and then, there is a slow decrease in tension. Morgan *et al.*[12] reported that the addition of phenylephrine (α_1-adrenoceptor agonist), to isolated vessels leads to an immediate increase in intracellular Ca^{++} concentration, which peaks one to three minutes and then decreases to a value close to the basal level. In our present study NE-induced PI response continues up to 75 min. Thus, there seems to be a discrepancy between the contractive response or intracellular Ca^{++} level and the PI response. However, since Ca^{++} is cytotoxic, it is considered that Ca^{++} influx during maintenance of the PI response may be surpassed by Ca^{++} efflux to the extracellular space or uptake into sarcoplasmic reticulum, resulting in decrease in intracellular Ca^{++} level and in tension.

Different barbiturates may induce different PI re-

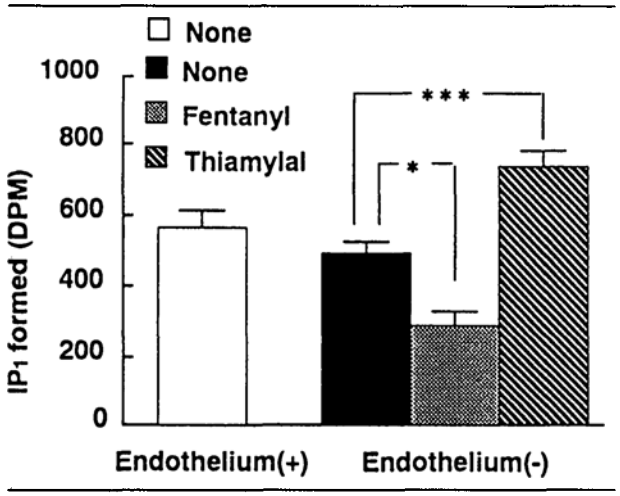

FIGURE 8 Effects of thiamylal (100 μM) and fentanyl (10 μM) on norepinephrine (NE)-induced IP_1 accumulation in rat aortic slices without endothelium (mean ± SE; $n = 6$–7 for each value). $*P < 0.05$. $***P < 0.001$ vs none.

sponses. Curry *et al.*[13] observed that thiamylal and thiopentone each produced dose-related constriction but pentobarbitone did not produce constriction in the guinea pig trachea, and concluded that thiobarbiturates, but not oxybarbiturates, produce smooth muscle contraction. In our present study, thiamylal potentiated, but pentobarbitone did not affect NE-induced PI responses. These results are consistent with those of Curry *et al.*[13]

Thiopentone and thiamylal potentiate the contractile responses in arteries.[1-3] Fukuda *et al.*[14] demonstrated that (1) potentiation by thiopentone of the responses to transmural stimulation was not affected by cocaine or hydrocortisone, which have inhibitory actions on neuronal or extraneuronal uptake of NE (2). The release of [^{3}H]NE induced by transmural stimulation was not altered by thiopentone at 100 μM (3); contraction induced by phenylephrine was potentiated by thiopentone, and (4) contractile responses to KCl were not potentiated by thiopentone. They concluded that thiopentone might specifically increase the responsiveness of the postsynaptic α_1 adrenoceptor to NE. On the other hand, Moriyama *et al.*[3] observed that, in dog cerebral and mesenteric arteries contracted with KCl or a receptor agonist, $PGF_{2\alpha}$, thiamylal at a concentration of 100 μM did not affect KCl-evoked contraction but potentiated $PGF_{2\alpha}$-evoked contraction. They also observed that, in Ca^{++} free media, the addition of $PGF_{2\alpha}$ produced only a small contractile response of arteries and this contraction was not potentiated by thiamylal, but, in the presence of Ca^{++}, $PGF_{2\alpha}$-induced contraction was potentiated by thiamylal. They concluded that thiamylal possessed a constrictor effect on vascular smooth muscle, and the mechanism involved seemed to be enhancement of Ca^{++} influx.[3] Thus, it is considered that thiobarbiturates act non-specifically on receptors and enhance Ca^{++} influx.

Intracellular Ca^{++} homeostasis is regulated by the PI response. Thus, in the present study, we measured NE-induced IP_1 accumulation, a degradation product of PI response, and found that thiamylal potentiated the NE-induced PI response in the presence of endothelium. Endothelium which releases endothelium derived constricting factor such as endothelin could also modify the response of NE in the presence of thiamylal. However, thiamylal also potentiated NE-induced PI response in the absence of endothelium. Thus, potentiation by thiamylal of the NE-induced PI response would not be mediated with endothelium.

Since barbiturates have high lipid solubility and penetrate cell membranes rapidly,[15] Altura *et al.*[1] suggested that barbiturates might act at the vascular muscle cell membrane level. Robinson-White *et al.*[16] suggested that barbiturates altered the activity of G-proteins. Therefore, thiamylal may activate G-protein, resulting in potentiation of the receptor-mediated PI response, enhancement of Ca^{++} influx and augmentation of vasoconstriction.

Barbiturates are known to be bound to plasma proteins, mainly albumin. The proportion of bound thiopentone in the plasma has been reported to be between 65 and 86%.[17] Becker[18] reported that the plasma concentration of free thiopentone necessary for anaesthesia was 6.3 $\mu g \cdot ml^{-1}$ (24 μM) in man. In the present study, thiamylal at concentrations of 10 μM and 100 μM potentiated NE-induced PI response. Thus, it seems probable that thiobarbiturates, at clinically relevant concentrations, would provoke vasoconstriction through potentiation of the PI response.

The effects of fentanyl on peripheral vascular resistance are controversial. In humans, there is one report that fentanyl reduced the peripheral vascular resistance[19-21] and another that fentanyl had no effect.[22] White *et al.*[6] found, in dogs, that high-dose fentanyl (50 $\mu g \cdot kg^{-1}$) caused a decrease in peripheral vascular resistance of 48% in the isolated hindlimb, and that pretreatment with denervation did not change the response to fentanyl. They concluded that fentanyl produced vasodilatation by direct action on the peripheral vascular smooth muscle.[6] Toda *et al.* reported that fentanyl did not alter the contractile response to histamine and serotonin but attentuated the contractile response to NE.[5] They concluded that fentanyl blocked α-adrenoceptors in vascular smooth muscle in a competitive manner.[5] Since fentanyl attenuated the NE-induced PI response in the present study, the PI response would be involved in the attenuation by fentanyl of vascular contractile response.

Fentanyl could modify the response of NE via opioid

receptors. However, Karasawa *et al.*[23] reported that pretreatment with naloxone had no effect on fentanyl-induced relaxation of rat aortic rings. White *et al.*[6] found, in dogs, that pretreatment with naloxone did not alter the vascular response to fentanyl. Makita *et al.*[24] reported that naloxone did not affect the inhibitory effect of fentanyl on the NE-induced PI response in rat cerebral cortical prisms. Thus, attenuation of the PI response by fentanyl would not be mediated by opioid receptors.

Endothelium releases autacoids such as nitric oxide or prostacyclin. These autacoids could also modify the response of NE in the presence of fentanyl. However, Karasawa *et al.*[23] reported that, in the absence of endothelium, fentanyl in concentrations >0.1 μM decreased the sensitivity of the rat aortic rings to the phenylephrine. In the present study, fentanyl attenuated the NE-induced PI response in the absence of endothelium. Therefore, fentanyl would act directly on $\alpha_1$adrenoceptors in smooth muscle cell membranes resulting in attentuation of the PI response and smooth muscle relaxation.

The mechanisms involved in the physiological action of fentanyl are inhibition of α_1-adrenergic agonist-receptor binding or G-protein including phospholipase C (PLC). In our previous study, fentanyl attentuated the carbachol- and histamine-induced PI response as well as the NE-induced PI response in rat tracheal slices.[25] Fentanyl is highly lipophilic and α_1-adrenergic, muscarinic and histamine receptors are connected with PLC through G-protein. Thus, fentanyl may non-specifically inhibit the activation of G-protein or PLC in PI cascade in vascular smooth muscle cell membrane.

Protein binding of fentanyl limits drug availability in the vascular smooth muscle cell membrane. Fentanyl is 84% bound to protein in human plasma.[26] In high-dose fentanyl anaesthesia (75–100 $\mu g \cdot kg^{-1}$), plasma fentanyl concentration is only about 100 $ng \cdot ml^{-1}$ (0.3 μM)[27] which corresponds to free drug concentrations of 0.05 μM. The concentrations used in the present study seem to be outside the clinical range. Thus, a decrease in blood pressure using fentanyl during operative hypertension is unlikely to be due to inhibition of NE-induced PI response in peripheral arteries, but mainly to the opioid effect.

In conclusion, thiamylal stimulates NE-induced PI response, which potentiates vasoconstriction. Fentanyl attenuates NE-induced PI response, which attenuates the vasoconstriction.

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