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Role of Pertussis Toxin-sensitive G-Proteins in the Analgesic and Anesthetic Actions of α_2-Adrenergic Agonists in the Rat

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Background: **α_2 Adrenoceptors are coupled to G-proteins sensitive to pertussis toxin (PTX) in the locus coeruleus. At this site, the hypnotic response to dexmedetomidine, an α_2 agonist, can be blocked by pretreatment with PTX. G-proteins sensitive to PTX may also be involved in the transduction of anesthetic and analgesic responses to α_2 agonists at supraspinal or spinal sites. To address this question the effects of pretreatment with PTX administered intracerebroventricularly, intrathecally, or a combination of the two were examined on the MAC for halothane, and the anesthetic-sparing and analgesic effects of a systemically administered α_2 agonist, dexmedetomidine.**

Methods: **Rats were cannulated intracerebroventricularly, intrathecally, and with a combination of intracerebroventricular/intrathecal and treated with PTX (0 and 2.5 μg intracerebroventricularly; 0 or 0.5 μg intrathecally; 0 + 0 or 2.5 + 0.5 intracerebroventricular-intrathecal)). After 7 days, either the analgesic (tail-flick latency) or the MAC-sparing effects of a calculated 50% effective dose of dexmedetomidine were measured. To confirm that intracerebroventricularly administered PTX was effective, ribosylation of G-proteins was assessed in periventricular brain tissue.**

Results: **The analgesic action of dexmedetomidine was blocked by PTX intrathecally but not by PTX *via* the intracerebroventricular route. The MAC-sparing action of dexmedetomidine was not blocked by PTX *via* the intrathecal or intracerebroventricular routes alone or in combination. Yet, intracerebroventricularly administered PTX effectively ribosylated the G-proteins.**

Conclusions: **Taken together with the authors' previous report, these data suggest that the hypnotic and the analgesic responses to dexmedetomidine are transduced *via* PTX-sensitive G-protein-coupled α_2 adrenoceptors but at separate sites (analgesic—spinal; hypnotic—locus coeruleus). Further studies are needed to localize the precise site(s) for the MAC-sparing effect of dexmedetomidine and to establish whether PTX-sensitive G-proteins are involved in this response. (Key words: Receptors, G-proteins: halothane; pertussis toxin. Sympathetic nervous system, α_2-adrenergic agonists: dexmedetomidine.)**

GUANINE nucleotide binding proteins (G-protein) are pivotally involved in the signal transduction of all adrenergic responses.[1] Bacterial toxins from *vibrio cholera* or *Bordetella pertussis* can covalently modify the G-proteins by the addition of an adenosine diphosphate-ribose group to the α subunit of the heterotrimeric protein. Thus, α subunits, and hence the G-proteins, can be classified into four groups according to their sensitivity to ribosylation by one, both, or neither toxin.[2]

The pertussis toxin (PTX)-sensitive G-proteins, which represent 1–2% of the total membrane protein in mammalian brain,[3] couple many inhibitory neurotransmitter receptors to their effectors.[4,5] PTX-sensitive G-proteins are known to be involved in the signal transduction of several α_2 adrenoceptor systems.[6,7] Earlier, we reported that the hypnotic response to dexmedetomidine, a highly-selective α_2 agonist, is blocked when PTX is administered intracerebroventricularly.[8] Subsequently, we demonstrated that PTX ribosylates G-proteins in the locus coeruleus to effect this blocking action.[9]

Apart from their hypnotic action, α_2 agonists have been advocated for use in the clinical practice of anesthesia because of their potent analgesic and anesthetic-sparing actions.[10] Although both supraspinal and spinal mechanisms may be involved in the analgesic re-

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sponse,[11] the dependence of the analgesic effect of α_2 agonists on PTX-sensitive G-proteins has not been rigorously tested. Likewise, for the anesthetic-sparing (MAC-reducing) action, neither the precise site(s) nor the role of PTX-sensitive G-proteins at these sites have been defined.

While the molecular mechanism of volatile anesthetic action remains obscure, there appears to be compelling evidence for the participation of a membrane-bound protein in its action (for reviews see references 12 and 13). PTX-sensitive G-proteins have also been suggested as a possible target site for the anesthetic action of volatile agents because halothane has been shown to alter the function of PTX-sensitive G-proteins.[14–16]

In the present study we examined the sites for, and the dependence on, PTX-sensitive G-proteins in the anesthetic-sparing and analgesic actions of systemically administered dexmedetomidine. Also, we sought to determine whether functional inactivation of PTX-sensitive G-proteins in the periventricular regions of the rat brain, could affect the anesthetic sensitivity to halothane in rats.

Methods

Animal Preparation

Male Sprague-Dawley rats weighing 250–300 g were used in the study after approval of the experimental protocol by the Animal Care and Use Committee at the Palo Alto Veterans Affairs Medical Center. Rat littermates were stratified into control and treatment groups to match weight distribution as closely as possible. Halothane-anesthetized rats were cannulated either in the left lateral ventricle (intracerebroventricularly), intrathecally, or by a combination of intracerebroventricular and intrathecal as previously described.[17,18] For intrathecal cannulation, an intrathecal catheter (PE-10 polyethylene tube) was passed through an incision in the dura mater overlying the cisterna magna and inserted to the level of the lumbar enlargement.[18] Two to four days after intracerebroventricular cannulation, a 30-G stainless steel needle, connected to polyethylene tubing, was inserted through the cannula and positioned 1 mm beyond its tip. After intrathecal surgery, 5–7 days were allowed for the animals to recover. Rats received either PTX (2.5 μg intracerebroventricular; 0.5 μg intrathecal; or 2.5 + 0.5 μg intracerebroventricular-intrathecal) or 10 (intracerebroventricular) to 12.5 (intrathecal) μl of the solvent vehicle (sodium phosphate buffer 6.7×10^{-2}M, pH 7.4). PTX or vehicle administration was facilitated by a microsyringe pump (CMA/100 Microinjection pump; Bioanalytic Systems Inc., West Lafayette, IN) over 2 min to minimize tissue disruption. For the intrathecal administration, an additional 12.5 μl of saline flush was needed to clear the dead space of the intrathecal catheter. Rats were used for the behavioral or biochemical experiments on day 7 after the PTX or vehicle injection as reported earlier.[9] Animals that failed to thrive after PTX administration were not tested further.

Behavioral Testing

Sensitivity to halothane was determined by measuring the MAC, which prevents a response to a supramaximal stimulus as previously described by us.[19] Briefly, halothane was vaporized in oxygen at a flow of 5 $l \cdot min^{-1}$ and introduced into a methyl methacrylate polymer exposure chamber. Anesthetic concentration was monitored continuously by an infrared spectral analyzer (Datex 222, Puritan, Bennett) that was calibrated by mass spectroscopy before every experiment. A battery-operated fan was used to distribute the gas evenly throughout the chamber. Rectal temperature was continuously monitored and maintained at 37.5 ± 1.0°C with heating blankets. The response of animals to a supramaximal noxious stimulus was assessed by applying a 6″ hemostat to the first ratchet position on the middle portion of the tail for 1 min. If the rat made a purposeful movement to the tail clamp, a positive response was recorded. The first stimulus was applied at a concentration of 0.8%, which previous studies showed would invariably result in a positive response. The halothane concentration was increased by 0.1% increments, and the next stimulation was done after allowing 30 min for equilibration. This process was repeated until no animal exhibited a positive response to tail clamping. This process allowed one to derive the particular animal's MAC, *i.e.*, the interpolated value between halothane concentrations that provided a positive and negative response. Each cohort consisted of a minimum of 12 rats. To examine the effect of pretreatment of PTX on the anesthetic-sparing effect of dexmedetomidine, rats were administered 30 $\mu g \cdot kg^{-1}$ intraperitoneal dexmedetomidine (the ≈calculated 50% effective dose for MAC-reduction)[19] and the halothane MAC was assessed again 30 min later.

Analgesic response was measured by the tail-flick latency response as previously described.[20] A high-intensity light was focused on the rat's tail and the time that it took for the rat to move its tail out of the light beam was automatically recorded (Tail-flick apparatus, Columbus Instruments, Columbus, OH) and referred as tail-flick latency. A different patch of the tail was exposed to the beam on each trial to minimize the risk of tissue damage. The animals were placed on the heating blanket to maintain body and tail temperature during the experiment. A cutoff time of 10 sec was defined at which time the trial was terminated if no response occurred. Data are expressed as maximum percent effect according to the following formula:

$$\text{MPE}(\%) = \frac{\text{Postdrug latency} - \text{basal latency}}{\text{Cutoff latency} - \text{basal latency}} \times 100.$$

Dexmedetomidine was used in the analgesic paradigm at a dose of 50 $\mu g \cdot kg^{-1}$ (an $\approx$50% maximum percent effect dose) intraperitoneally, and the tail-flick test was performed 40 min after treatment with dexmedetomidine; dose and time of testing were established by pilot experiments.

Adenosine Diphosphate Ribosylation

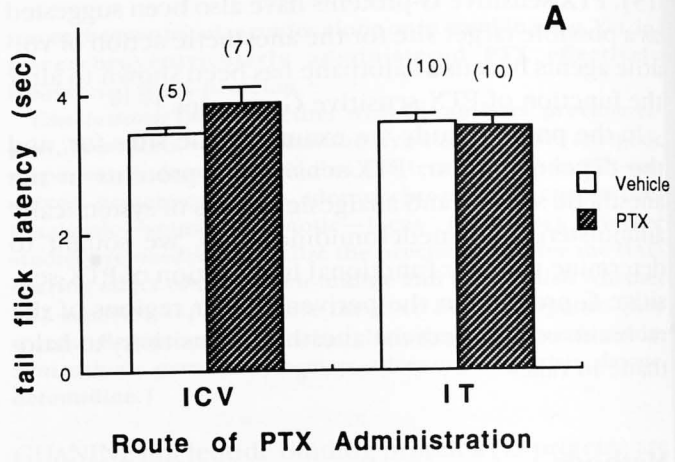
Halothane-anesthetized rats were killed on day 7 after receiving 2.5 μg PTX intracerebroventricularly. Brains were harvested and immediately frozen on dry ice and stored at −70°C. Twenty-four hours later, a 0.5 mm strip of periventricular tissue was dissected in the following manner. The brain was placed in an ice-cold brain matrix mold to cut 2-mm coronal slices. The slice(s) containing the lateral ventricle and the cannula track was isolated and placed on an ice-cold glass plate. Using a 0.5-mm bore glass pipette, punches were taken alongside the ventricle. This tissue was prepared for *in vitro* ribosylation as previously described.[9] After polyacrylamide gel electrophoresis and autoradiography, the G-protein bands were analyzed with a densitometer (Pharmacia-LKB, Ultroscan XL Enhanced Laser Densitometer).

Statistical Analysis

Data are expressed as mean ± SEM. The results of multiple groups were analyzed by repeated-measures analysis of variance followed by *post hoc* Scheffe's test. The comparison between two groups was performed by *t* test for unpaired data. A *P* value less than 0.05 was considered statistically significant.

Results

Neither intracerebroventricular nor intrathecal pretreatment of PTX changed the basal latency in the analgesia-testing paradigm (fig. 1A). While the analgesic effect of systemically administered dexmedetomidine, 50 $\mu g \cdot kg^{-1}$ intraperitoneally, was not affected by intracerebroventricular PTX treatment, it was attenuated by intrathecal PTX (fig. 1B). Neither intracerebroven-



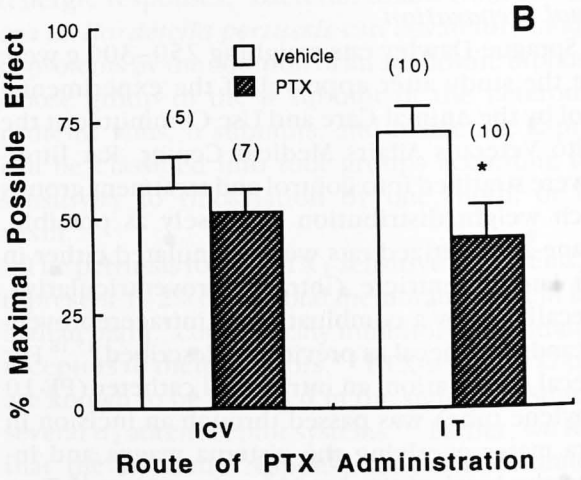


Fig. 1. Effect of intracerebroventricular and intrathecal pertussis toxin on the tail-flick latency response (*A*) and analgesic effect of dexmedetomidine (*B*). Rats were pretreated with pertussis toxin (2.5 μg intracerebroventricular or 0.5 μg intrathecal) or vehicle administered either intracerebroventricularly or intrathecally and 7 days later the tail-flick latency was measured before (*A*) or after intraperitoneal dexmedetomidine 50 $\mu g \cdot kg^{-1}$ (*B*) by tail-flick test. Data are expressed as the absolute latency (*A*) or the percent of maximal possible analgesic effect (*B*) mean ± SEM; the number of rats per group is shown in parentheses. *$P < 0.05$ compared to vehicle.

tricular, intrathecal, nor intracerebroventricular-intrathecal altered either the basal MAC for halothane or the MAC-reducing effect of systemically administered dexmedetomidine, 30 $\mu g \cdot kg^{-1}$ (fig. 2). Because of the lack of effect with intracerebroventricular-PTX we determined whether this treatment effectively ribosylated G-proteins. The intensities of the bands detected by ^{32}P-NAD ribosylation in PTX-treated and vehicle-treated groups are shown in figure 3. *In vivo* administration of PTX decreased *in vitro* ribosylation as reflected by the reduction in the incorporation of radiolabeled adenosine diphosphate-ribose in the α subunits of PTX-sensitive G-proteins.

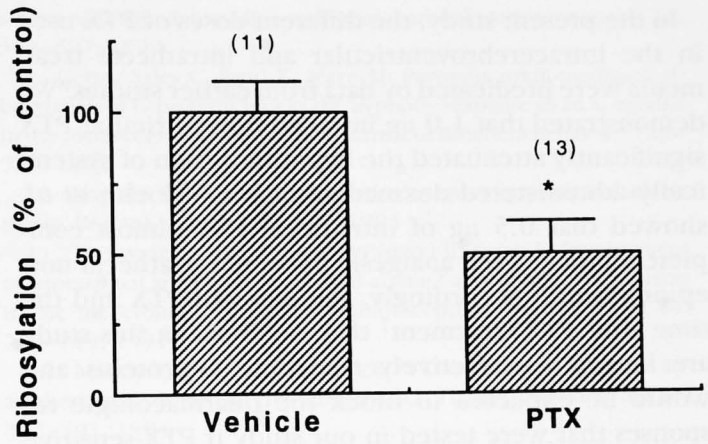


Fig. 3. Effect of *in vivo* pertussis toxin on *in vitro* adenosine diphosphate-ribosylation of G-proteins. Rats were treated with 2.5 μg pertussis toxin or vehicle intracerebroventricularly. On day 8 animals were killed and adenosine diphosphate-ribosylation was assessed by autoradiography and laser densitometry as previously described.[8] Data are normalized by expressing the densitometry as a percent of the relevant vehicle value. Data are expressed as mean ± SEM; the number of samples is shown in parentheses. *$P < 0.05$ compared to vehicle.

Discussion

The analgesic action of dexmedetomidine was blocked by PTX intrathecally but not by PTX *via* the intracerebroventricular route. The MAC-sparing action of dexmedetomidine was not blocked by PTX *via* the intrathecal or intracerebroventricular routes alone or in combination. Yet, intracerebroventricularly administered PTX effectively ribosylated the G-proteins.

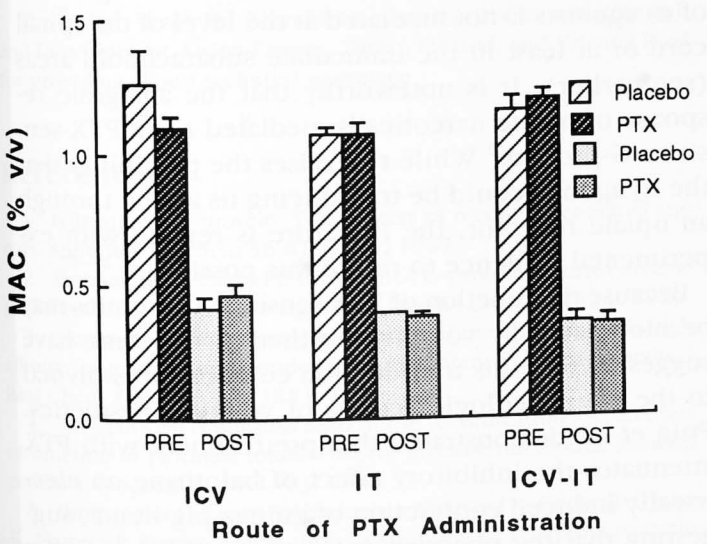


Fig. 2. Effects of pertussis toxin administered intracerebroventricularly, intrathecally, and a combination of both methods of administration on anesthetic-sparing action of dexmedetomidine. Rats were pretreated with pertussis toxin (2.5 μg intracerebroventricular; 0.5 μg intrathecal; 2.5 and 0.5 μg intracerebroventricular/intrathecal combination) or vehicle intracerebroventricularly, intrathecally, or by a combination of intracerebroventricular and intrathecal routes. Seven days later the MAC for halothane was measured before (pre) and after (post) 30 $\mu g \cdot kg^{-1}$ intraperitoneal dexmedetomidine. Data are expressed as mean ± SEM; n = 12–27.

The α_2-adrenergic agonists represent a novel class of hypnotic-anesthetic agents because of their selectivity for specific membrane receptors. Cell surface receptors use a variety of membrane signaling mechanisms to transduce the message, carried by the agonist, into cellular responses. Collectively, these mechanisms are referred to as transmembrane signaling or signal transduction. For the α_2-adrenergic responses, the transmembrane signaling system involves the coupling of the receptor protein, *via* a guanine nucleotide binding protein to an effector mechanism. PTX contains a ribosylase that catalyzes the attachment of adenosine diphosphate-ribose to a conserved cystine residue four amino acids from the carboxy terminus of the α subunit of PTX-sensitive G-proteins.[21] Once the G-protein is ribosylated by PTX, it fails to dissociate after the activation of the receptors by its agonists, resulting in the uncoupling of the α_2 adrenoceptor from its effector mechanism. Thus, pharmacologic responses to α_2 agonists are attenuated. In previous studies in which we ribosylated G-proteins with PTX and measured the behavioral response *in vivo,* we reported on the involvement of a PTX-sensitive G-protein in the transduction of the hypnotic response to dexmedetomidine.[8,9] Now we have established that the spinal analgesic response to dexmedetomidine is also transduced *via* a PTX-sensitive G-protein.

In the present study, the different doses of PTX used in the intracerebroventricular and intrathecal treatments were predicated by data from earlier studies. We demonstrated that 1.0 μg intracerebroventricular PTX significantly attenuated the hypnotic action of systemically administered dexmedetomidine.[8] Hoehn *et al.* showed that 0.5 μg of intrathecal PTX almost completely blocked the analgesic effect of intrathecal norepinephrine.[7] Accordingly, the doses of PTX and the time after PTX treatment[7] that we chose in this study are known to effectively ribosylate G-proteins and would be expected to block the pharmacologic responses that were tested in our study if PTX-sensitive G-proteins were pivotally involved at these different sites.

PTX pretreatment did not affect the MAC-reducing effect of dexmedetomidine (fig. 2), although intrathecal PTX attenuated the analgesic response (fig. 1B) and previously we have shown that intracerebroventricular PTX attenuated the hypnotic response.[8] Using a series of antagonists, we previously demonstrated that dexmedetomidine exerts its MAC-reducing effect by activating an α_2 adrenoceptor.[19] Until recently, all biologic responses that are mediated by α_2 adrenoceptors were thought to couple invariably to PTX-sensitive G-proteins; however, this tenet has been challenged by the finding that a PTX-insensitive G-protein participates in the signal transduction of an α_2 response in a recombinant system.[22] This precedent raises the possibility that the MAC-sparing effect of dexmedetomidine is transduced by a PTX-insensitive G-protein.

To further elucidate PTX's inability to reverse the MAC-sparing effect of dexmedetomidine, we demonstrated the *in vivo* ribosylation of G-proteins by PTX. While these studies clearly established the functional integrity of this toxin within our paradigm, they also revealed that more than 50% of the α subunits were *not* ribosylated by the *in vivo* treatment with PTX. The hypnotic response was recently shown to be relatively inefficient requiring the activation of more than 80% of membrane receptors while the analgesic response requires only 50% of α_2 adrenoceptors to be available.[20] Thus, a 50% reduction in ribosylation is enough to attenuate both the hypnotic,[8] and presumably the analgesic responses to dexmedetomidine. It is notable that animals rendered tolerant to the hypnotic and analgesic actions of dexmedetomidine are still capable of exhibiting this drug's MAC-sparing action (unpublished observations). Thus, it appears that the MAC-sparing action of α_2 agonists is very efficiently transduced and this response will only be blocked when considerably more than 50% of the PTX-sensitive G-proteins are inactivated. Therefore, in our study there may still be a sufficient reserve of functional G-proteins to permit the MAC-reducing effect of dexmedetomidine were this molecular component to be needed for its transmembrane signaling.

A second explanation for the inability of PTX to attenuate the MAC-sparing effect may be related to the finding that PTX penetrates tissues very poorly[23]; therefore, it is possible that the site for the MAC-sparing action of α_2 agonists is still dependent on PTX-sensitive G-proteins, which reside beyond a 1 mm radius from the ventricular system.

Similar molecular mechanisms in the spinal cord mediate the analgesic action of both opiate narcotics and the α_2 agonists. Because it was reported that morphine can exert its MAC-reducing effect at the level of the spinal cord[24] we expected that α_2 agonists would act in a similar fashion. While intrathecally administered PTX blocks the analgesic effect of dexmedetomidine it had no effect on its anesthetic-sparing property. This suggests that either a PTX- insensitive G-protein is involved (see earlier) or that the MAC-reducing action of α_2 agonists is not mediated at the level of the spinal cord or at least in the immediate subarachnoid areas (see earlier). It is noteworthy that the analgesic response of opiate narcotics is mediated *via* a PTX-sensitive G-protein.[7] While this raises the possibility that the α_2 agonist could be transducing its action through an opiate receptor, the literature is replete with experimental evidence to refute this possibility.[25–27]

Because the function of PTX-sensitive G-proteins may be modulated by volatile anesthetics,[15,16] some have suggested that this transduction component is pivotal to the pharmacologic actions of volatile anesthetics. Puig *et al.* demonstrated that pretreatment with PTX attenuates the inhibitory effect of halothane on electrically induced contraction of guinea pig ileum, suggesting that the pharmacologic effect of halothane is mediated by PTX-sensitive G-protein.[28,29] Conversely, myocardial depression exerted by volatile anesthetics was not affected by pretreatment with PTX, although the same treatment could abolish the effect of muscarinic agonists acting *via* its G-protein-coupled receptors.[30] Furthermore, PTX did not alter halothane's ability to relax airway smooth muscle.[31] Thus, the involvement of PTX-sensitive G-protein in the action of volatile

anesthetics is not universal and may depend on the individual pharmacologic response tested. The present results do not implicate periventricular PTX-sensitive G-proteins in the molecular mechanism for the anesthetic effect of the volatile agents. Based on the absence of a change in MAC after spinal cord transection, it was recently suggested that the spinal cord may be the site for anesthetic inhibition of the response to the supramaximal noxious stimulus that is used in MAC studies.[32] However, neither intrathecal nor the combination of intrathecal and intracerebroventricular PTX altered the MAC for halothane. These data do not support a mediating role for PTX-sensitive G-proteins in the spinal cord for the anesthetic inhibition of the motor response to the supramaximal noxious stimulus that is used in MAC testing.

In summary, PTX-sensitive G-proteins in the locus coeruleus and the spinal cord appear to be involved in the signal transduction for the hypnotic and analgesic effects of systemically administered dexmedetomidine, respectively. Further studies are needed to localize the site(s) and to clarify the involvement of PTX-sensitive G-proteins for the MAC-reducing effect of dexmedetomidine.

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Halothane an derived Relax Guanosine M Endothelial C Independent Cyclase Activ

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Timothy D. Le Cras, Ph.D., ‖ A

Background: Interaction of nitric oxide signaling pathw effects are controversial. The the sites and mechanism of in with the vascular nitric oxid pathway.

Methods: To specifically stud teraction with the nitric oxide- vascular smooth muscle and muscle (EC-VSM) co-culture m tures of VSM with or without on microcarrier beads were p stimulated with agonists. The on cyclic guanosine monoph ulated VSM and of VSM in w been activated by the endoth tors, sodium nitroprusside, ni termined. Experiments were of inhalational anesthetics on

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