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Inhibition of Adrenal Medullary Catecholamine Secretion by Enflurane:

II. Investigations in Isolated Bovine Adrenals–Site and Mechanism of Action

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To determine the site and mechanism of action underlying the inhibition of adrenal medullary catecholamine release by enflurane, the authors measured the effects of enflurane on catecholamine secretion evoked by various secretagogues in isolated bovine adrenals perfused with Locke's solution. Catecholamine concentrations in the perfusate were measured spectrofluorometrically. Enflurane caused concentration-dependent inhibition of catecholamine release in response to activation of the nicotinic receptors in the chromaffin cells with acetylcholine or dimethylphenylpiperazinium (DMPP). An enflurane concentration of 0.88 mM caused 50 per cent inhibition of the DMPP-induced secretion. The inhibition induced by enflurane was shown to be noncompetitive. The catecholamine release evoked by activation of the muscarinic receptors with pilocarpine was only slightly decreased by 3.74 mM enflurane. At this concentration the release in response to KCl, 56 mM, was partially inhibited, whereas the output in response to tyramine (from glands perfused with calcium-free Locke's solution) was unaffected. It is concluded that the site of action of enflurane is the cell membrane. At concentrations above 1 mM, enflurane may impair calcium ion influx, but at lower concentrations it probably interacts with hydrophobic regions of the nicotinic receptor. (Key words: Anesthetics, volatile, enflurane; Sympathetic nervous system, enflurane.)

EXPERIMENTS IN CATS revealed that inhalation of enflurane decreases catecholamine secretion from the adrenal medulla.[1] In addition, it was shown that the anesthetic has a peripheral site of action on the synapses between splanchnic nerve endings and chromaffin cells, since enflurane also inhibits catecholamine secretion in response to splanchnic-nerve stimulation.[1] However, it was not possible to evaluate from these experiments whether the decrease in catecholamine output is due to an inhibition of the release of acetylcholine from the splanchnic nerve endings, to an inhibition of the effect of acetylcholine on cell membranes of the chromaffin cells, or to impairment of the exocytotic release mechanism of catecholamines.

It was the purpose of the present investigation to study whether enflurane has a postsynaptic site of action on chromaffin cells of isolated bovine adrenals. Further, we determined the effects of enflurane on the responses to drugs that increase catecholamine secretion by different mechanisms. Thus, conclusions could be drawn as to whether enflurane causes inhibition of receptor stimulation, stimulus–release coupling, or the process of exocytosis.

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Methods

The experiments were performed on adrenal glands removed from cows 10–20 min after the animals had been killed. The glands were quickly transported in ice from the slaughterhouse to the laboratory. The vein was cannulated with a polyethylene tube and the gland was scarified by making multiple incisions 0.5 to 1.0 mm deep in the cortex. The glands were perfused in a retrograde fashion with Locke's solution for at least 40 min before the first sample of perfusate was collected. The composition of the Locke's solution (saturated with oxygen) was as follows (mM): NaCl 154; KCl 5.6; $CaCl_2$ 2.2; Na_2HPO_4 2.15; NaH_2PO_4 0.86; glucose 10.

EXPERIMENTS AT 34 C

Part of the experiments were performed at 34 C and a rate of perfusion of 5 ml/min, which was maintained by means of two pumps. In detail, Locke's solution at 38 C was pumped into the perfusion cannula at a rate of 3.5 ml/min by means of a roller pump; throughout the experiments Locke's solution at 23–26 C was infused into the main stream of the perfusion fluid at a rate of 1.5 ml/min (temperature of the mixture 34 C). When the effects of enflurane and the stimulant drug were studied, Locke's solution mixed with enflurane or the secretagogue, or both, was infused. Two-minute samples of the perfusate were continuously collected in test tubes containing 1 ml 5 N perchloric acid.

The enflurane solution was prepared in a glass flask containing Locke's solution at 23–26 C equilibrated with oxygen. The volume of the gas phase in the flask was 4 per cent of that of the Locke's solution. Liquid enflurane was added directly to the solution and the bottle was closed with a glass stopper. The anesthetic was dissolved by shaking

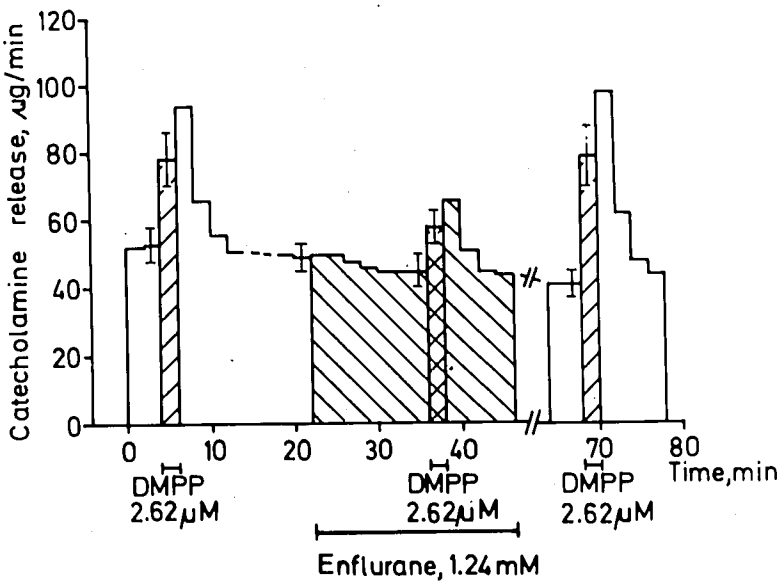

FIG. 1. Effects of enflurane on the basal catecholamine output and on the DMPP-induced catecholamine release from isolated bovine adrenals (n = 8; means ± SEM). For clarity, most of the standard errors of the means have been omitted; they varied in the same range as those indicated. DMPP and enflurane were present in the perfusion fluid during the times indicated by the horizontal bars and by the hatched areas.

the mixture for 30 min. The drops of liquid enflurane that were visible on the bottom of the flask before shaking disappeared within 15 minutes. It was calculated by use of the water–gas partition coefficient of enflurane determined at 37 C[2] that less than 5 per cent of the anesthetic would have been lost in the gas phase at this temperature. Since in aqueous media anesthetic solubility increases with decreasing temperature,[3,4] the actual loss at 23–26 C was still smaller. All-glass syringes, which were used in the infusion pump, were filled with the enflurane solution. No loss of enflurane from these syringes could occur.

Since activation of the nicotinic acetylcholine receptor is known to be the main pathway of stimulation of catecholamine release from the chromaffin cell, we investigated the effect of enflurane on the release evoked by dimethylphenylpiperazinium (DMPP). This drug, which is more stable than acetylcholine, specifically activates nicotinic receptors, leaving muscarinic receptors unaffected. In other series of experiments, pilocarpine and KCl, respectively, were used as secretagogues. Stimulation with KCl was achieved by perfusion of the glands with a modified Locke's solution in which the concentration of KCl was 56 mM and that of NaCl was decreased by a corresponding amount, as described by Douglas and Rubin.[5]

Catecholamine release was stimulated three times, twice under control conditions (S_1 and S_3) and once in the presence of enflurane (S_2). Each stimulation period lasted 2 min. Enflurane was present in the perfusion fluid 14 min before, during, and 8 min after the second stimulation period. The intervals between stimulation periods lasted 30 min (fig. 1).

EXPERIMENTS AT AMBIENT TEMPERATURE

For different reasons (see below) some of the experiments were performed with Locke's solution at room temperature (23–26 C) and at a perfusion rate of 2 ml/min. Decreases in both temperature of perfusion fluid and perfusion rate have been shown to decrease catecholamine secretion from isolated bovine adrenals.[6] Thus, exhaustion of catecholamine stores could be prevented, even when a strong secretagogue was applied for a long time. The perfusion apparatus was different from that used in the experiments at 34 C; it consisted of several pumps connected to the perfusion cannula. These pumps contained either Locke's solution (calcium-free in some of the experiments) or Locke's solution in which the secretagogue or enflurane, or both, were dissolved. By switching over from one pump to another, the adrenals could be stimulated alternately in the presence and in the absence of enflurane. Two-minute samples of the perfusates were continuously collected in test tubes containing 2 ml 1 N perchloric acid.

Acetylcholine and tyramine were used as secretagogues. The effects of acetylcholine during stimulation periods of 8 min were studied at 23–26 C, since this molecule is known to be decomposed at higher temperatures. When tyramine was used to stimulate catecholamine release, the glands were perfused with calcium-free Locke's solution throughout the experiments. Because of the slow increase in tyramine-induced catecholamine release,[7] the stimulation periods lasted 16 min. This secretagogue was investigated at room temperature, since it has been shown that at room temperature tyramine induces calcium-independent catecholamine release.[7]

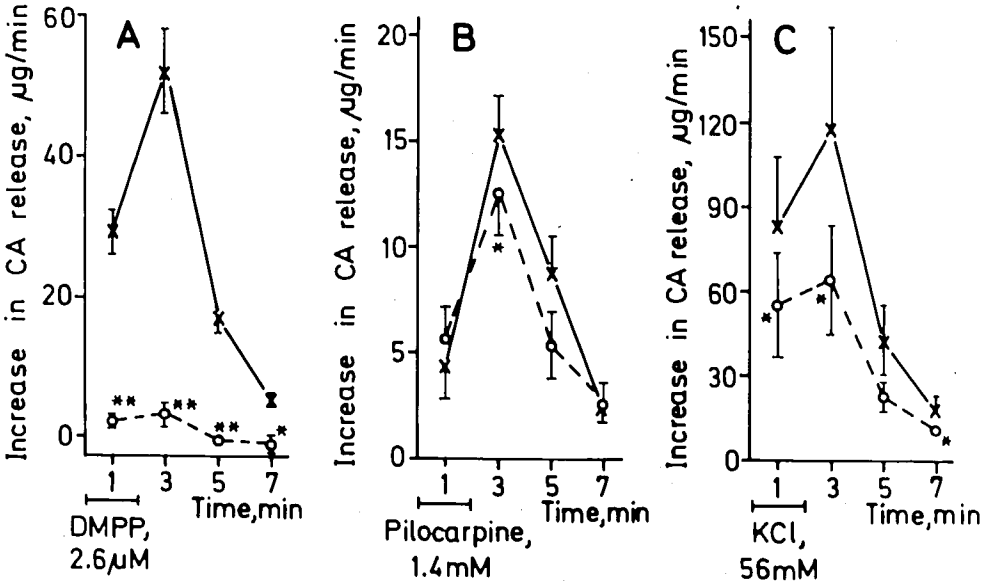

FIG. 2. Effects of 3.74 mM enflurane on catecholamine (CA) release (increase over the basal output) from isolated bovine adrenals in response to DMPP (*A*, n = 6), pilocarpine (*B*, n = 5) or KCl (*C*, n = 6). *Abscissa*: time after onset of stimulation (duration indicated by the horizontal bars). *Solid lines* (x): stimulation under control conditions. *Broken lines* (o): stimulation in the presence of enflurane (present from 14 min before until 8 min after the onset of stimulation). Means ± SEM. $*P < 0.05$; $**P < 0.001$.

In order to study the ability of enflurane to inhibit catecholamine release by these secretagogues, the glands were stimulated twice: the release evoked by stimulation in the presence of enflurane was compared with that under control conditions. In 50 per cent of the glands the first stimulation was performed under control conditions and the second in the presence of enflurane; the anesthetic was present in the perfusion fluid 10 min before and during stimulation (in the experiments with tyramine also in the subsequent 8 min). In 50 per cent of the glands the order was reversed. This sequence compensates for the gradual decrease in catecholamine release that occurs with time and is independent of enflurane. In the experiments with acetylcholine the interval between the first and second stimulation was 32 min, in those with tyramine it was 43 min because of the longer duration of the tyramine effect.[7]

Concentration–response curves of DMPP for its stimulating effect on catecholamine release were determined in a cumulative fashion (as described by Van Rossum[8]) by increasing the DMPP concentration in the Locke's solution stepwise by a factor of 10. Each concentration was present in the perfusion fluid for 4 min. For each adrenal gland we determined only one concentration–response curve: either under control conditions or in the presence of 1.24 mM enflurane (present in the perfusion fluid 10 min before and during addition of DMPP). Thirty minutes before the DMPP concentration–response curves were determined, the secretion-stimulating effect of 5.2 µM DMPP (without enflurane) was tested in both groups of adrenals in order to make sure that they did not differ in sensitivity to DMPP.

Estimation of Catecholamines

After centrifugation of the perfusate samples, catecholamine values (no differentiation between epinephrine and norepinephrine) were determined in 0.1 ml of the supernatants (dilution 1:20 with H_2O; *p*H adjusted to 6.5 with 0.4 N NaOH; no further purification) by the trihydroxyindole method.[9] Fluorescence was read at an activating wavelength of 390 nm and at 505 nm fluorescence wavelength, since at this combination of wavelengths adrenolutin and noradrenolutin did not differ in fluorescence intensity.

Calculations and Statistics

The increase in catecholamine release evoked by the secretagogues was calculated by subtration of the catecholamines released in the 2 min preceding the stimulation from the catecholamines released in the various 2-min periods of perfusate sampling during and after stimulation (until secretion had reached the prestimulation level). The inhibition by enflurane of catecholamine release was calculated from the increase in catecholamine secretion under control conditions (a) and that in the presence of enflurane (b) by the equation: per cent inhibition = 100 (1 − b/a). Since in the experiments performed at 34 C catecholamine release was stimulated twice under control conditions (S_1, S_3), the mean values of the effects evoked by S_1 and S_3 were calculated and compared with the effect in the presence of enflurane (S_2).

The pD_2 value of DMPP (negative logarithm of the concentration that produces 50 per cent of the maximum effect) and the pD_2' value of enflurane (negative logarithm of the concentration that causes depression of the effect of the agonist to 50 per

cent) were determined as described by Van Rossum.[8] Means and standard errors of the means are given. Student's t test was used to calculate the significance of the difference between two values.

Results

The spontaneous release of catecholamines from isolated bovine adrenals was not altered by 1.24 mM enflurane (fig. 1). The same results were obtained at enflurane concentrations ranging from 0.14 to 3.74 mM.

Activation of the nicotinic receptors on the chromaffin cells with 2.6 μM DMPP induced a considerable catecholamine release, which was inhibited by enflurane in a concentration-dependent fashion (figs. 1, 2A, 3). An enflurane concentration of 0.88 mM caused 50 per cent inhibition of DMPP-induced catecholamine secretion.

Before determination of concentration–response curves of DMPP (fig. 4), the catecholamine release induced by 5.2 μM DMPP was 43.9 ± 5.0 μg/min in the control adrenals and 46.5 ± 7.0 μg/min in the adrenals that were later exposed to 1.24 mM enflurane; hence, the two groups of adrenals did not differ in sensitivity to DMPP.

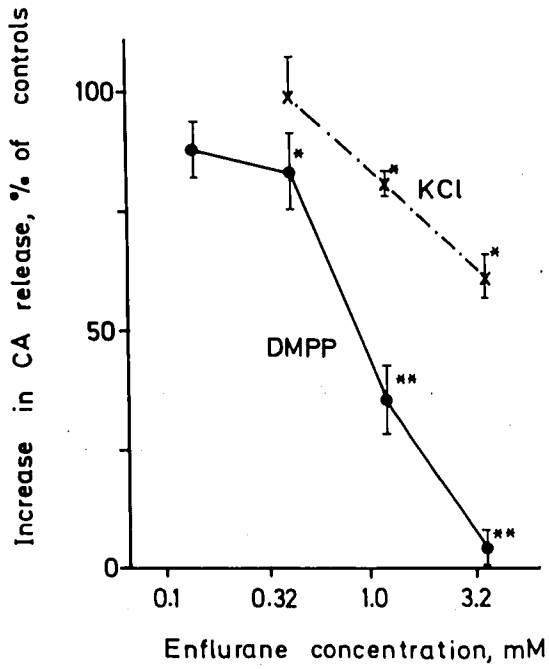

FIG. 3. Effects of various enflurane concentrations on the catecholamine (CA) release from isolated bovine adrenals in response to 2.62 μM DMPP (●) and 56 mM KCl (x). *Ordinate*: increase in catecholamine (CA) release over the basal output; results are expressed as percentage of the catecholamine release evoked by the secretagogues under control conditions. Each point: mean ± SEM of six to eight experiments. *$P < 0.05$; **$P < 0.01$.

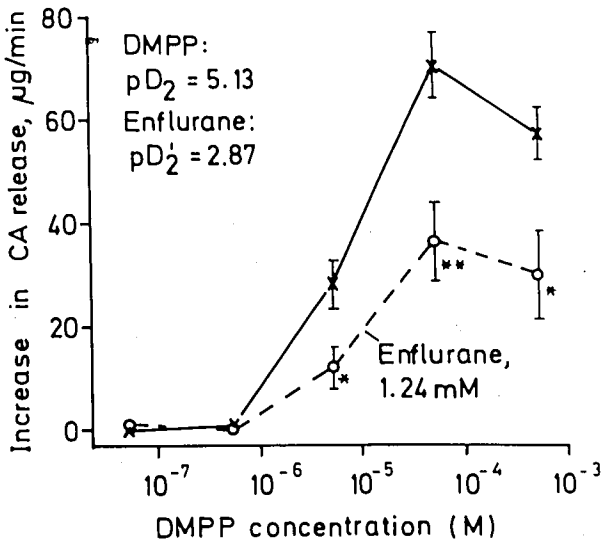

FIG. 4. Effect of enflurane on the cumulative concentration–response curve for the stimulating effect of DMPP on isolated bovine adrenals. *Ordinate*: increase in catecholamine (CA) release over the basal output. *Solid line*: stimulation under control conditions (n = 8). *Broken line*: stimulation in the presence of enflurane (n = 6). Means ± SEM. *$P < 0.05$; **$P < 0.01$. pD_2 of DMPP and pD_2' of enflurane against DMPP were determined as described by Van Rossum.[8]

Enflurane did not cause a shift of the concentration–response curve, but decreased the maximal response to DMPP (fig. 4), indicating that the anesthetic is a noncompetitive antagonist.

Enflurane, 1.24 mM, also decreased catecholamine secretion in response to acetylcholine (table 1); the extent of inhibition was the same as that caused by DMPP (fig. 3). In contrast, catecholamine release evoked by pilocarpine was only minimally decreased by 3.74 mM enflurane (fig. 2B). Catecholamine secretion in response to 56 mM KCl was only partially inhibited (by 39 per cent) by 3.74 mM enflurane, which completely inhibited catecholamine release induced by DMPP (fig. 2C). The ratio of the enflurane concentrations that caused 50 per cent inhibition of catecholamine release evoked by KCl and DMPP, respectively, was higher than 4.3 (fig. 3).

Tyramine caused a considerable release of catecholamines from glands perfused with calcium-free Locke's solution throughout the experiments (table 1); 3.74 mM enflurane did not decrease the tyramine-induced catecholamine output.

Discussion

The results of the present study of isolated adrenals provide evidence that enflurane inhibits catecholamine secretion by a direct effect on the chromaffin cell. Catecholamine secretion *in vivo* is controlled by the central nervous system via

TABLE 1. Effects of Enflurane on Catecholamine Release Evoked by Acetylcholine and Tyramine from Isolated Bovine Adrenals*

	Enflurane Concentration	Catecholamine Release (μg)		Inhibition by Enflurane
		Without Enflurane	In the Presence of Enflurane	
Acetylcholine (61 μM; n = 8)	1.24 mM	228 ± 61	74 ± 28	68 per cent ($P < 0.01$)†
Tyramine‡ (73 mM, n = 6)	3.74 mM	420 ± 145	505 ± 120	None

* Values are means ± SEM; the total amounts of catecholamines released by the secretagogues are given.

† Percentage by which secretion was decreased compared with that under control conditions.

‡ Perfusion with calcium-free Locke's solution throughout the experiments.

splanchnic nerve fibers.[10,11] Acetylcholine released from these nerve endings activates mainly nicotinic receptors on cell membranes of the chromaffin cells, thus inducing depolarization of the membrane and calcium ion influx into the cell.[12–15] Catecholamine secretion induced by stimulation of nicotinic receptors is abolished when the gland is perfused with calcium-free solution;[5,16,17] hence, calcium ion influx appears to be the vital link between receptor activation and catecholamine secretion by exocytosis (*i.e.*, extrusion of the total content of the storage vesicle after fusion with the cell membrane).[14,15] Our present study reveals that enflurane inhibits the catecholamine release induced by activation of the nicotinic receptors with either DMPP or acetylcholine. The same finding had been obtained previously with halothane, methoxyflurane, chloroform, and diethyl ether.[7,18,19]

The concentration range of enflurane in which the inhibition of catecholamine release evoked by DMPP or acetylcholine occurs corresponds to the concentrations of the anesthetic in inspired air that are necessary for induction of anesthesia in man and experimental animals. The MAC values of enflurane in man,[20] cat,[21] and dog[22] are 1.68, 1.20, and 2.20, respectively. From these values, concentrations in aqueous solutions of 0.58, 0.41, and 0.76 mM, respectively, can be calculated, using the water–gas partition coefficient of enflurane (0.82).[2] Our experiments revealed that an enflurane concentration of 0.88 mM causes 50 per cent inhibition of catecholamine release evoked by DMPP.

To evaluate the site and mechanism of action underlying the inhibition by enflurane of the secretion-stimulating effect of DMPP or acetylcholine, additional experiments were done with secretagogues that produce catecholamine release by a mechanism different from that of nicotinic agonists. Tyramine and other indirectly-acting sympathomimetic amines have been shown to induce catecholamine release independent of calcium ions in the perfusion fluid.[7,23,24] This release is not due to exocytosis. Experiments in storage vesicles isolated from bovine adrenals revealed that tyramine evokes catecholamine release by displacement of catecholamines bound to adenosinetriphosphate,[25] indicating that tyramine interacts with the storage vesicles. Since enflurane even at an extremely high concentration did not decrease the catecholamine output in response to tyramine, it appears improbable that the site of action of enflurane is the storage vesicle.

An increase in KCl concentration in the perfusion fluid evokes catecholamine release by a mechanism that is different from that of tyramine, but has several steps in common with nicotinic agonists. Thus, it has been shown that an increase in KCl concentration induces calcium influx into the cell[16,17] and that subsequently catecholamines are secreted by exocytosis.[15] In our experiments enflurane decreased the catecholamine release evoked by 56 mM KCl; however, considerably higher enflurane concentrations (>1 mM) than those that inhibit the catecholamine release in response to nicotinic agonists were necessary to produce this effect. That the inhibition of the KCl-evoked secretion by high enflurane concentrations may be due to an impairment of the process of exocytosis cannot be excluded. However, experiments performed in other models (*e.g.*, erythrocytes, nerve and muscle cells) reveal that general anesthetics predominantly affect the function of the cell membrane.[26,27] Therefore, it appears to be more probable that the decrease of the KCl-evoked catecholamine release by high enflurane concentrations is caused by an inhibition of calcium ion influx.

It is interesting that halothane even at a concentration of 14 mM failed to inhibit KCl-induced catecholamine release.[7] This concentration is 56 times higher than that which causes 50 per cent inhibition of catecholamine release evoked by acetylcholine. Hence, enflurane and halothane seem to differ in their effects on calcium ion permeability of the cell membrane of the chromaffin cell.

These suggestions are compatible with our results obtained with pilocarpine. This secretagogue induces catecholamine release by activation of muscarinic receptors on the cell membrane of the chromaffin cell, and again, calcium ion influx is the link between stimulation and secretion.[7,28] Therefore, the very slight inhibition of the pilocarpine-induced catecholamine secretion by 3.74 mM enflurane can also be explained by an inhibition of calcium ion influx into the cell. Similar to the results obtained with 56 mM KCl, the pilocarpine-induced catecholamine release was not decreased by halothane (4.3 mM).

The catecholamine release in response to nicotinic receptor stimulation is inhibited at enflurane concentrations lower than 1 mM that do not affect catecholamine release evoked by the other methods of stimulation used. Since calcium ion influx is essential for stimulus–release coupling with all methods of stimulation used, it appears improbable that the inhibition of calcium ion influx is the reason for the selective reduction of the secretion evoked by nicotinic agonists by low enflurane concentrations. However, the possibility that nicotinic agonists may open specific calcium channels, which in turn may be preferentially blocked by enflurane, cannot be excluded, but this hypothesis does not seem very probable. Hence, the possibility that the nicotinic receptor is the site of action of the anesthetic must be considered. Enflurane may induce a conformational change of the receptor, which may prevent the binding of the agonist to the receptor. The finding of noncompetitive inhibition by enflurane of DMPP-induced catecholamine release is compatible with this suggestion.

It is interesting that enflurane also decreases the norepinephrine output induced by activation of nicotinic receptors on the sympathetic nerve terminals.[29] All other inhalational anesthetics investigated also inhibit catecholamine secretion evoked by nicotinic agonists both from sympathetic nerve terminals[29,30] and from the adrenal medulla.[19] These compounds have been shown to inhibit acetylcholine-induced catecholamine secretion from the adrenal medulla in proportion to their membrane–buffer partition coefficients, *i.e.*, hydrophobic properties.[19] The coresponding data for enflurane fit the regression line calculated for this correlation. These results suggest that the interaction between the anesthetics and the membrane constituent essential to the inhibition of catecholamine release (probably the nicotinic receptor) is hydrophobic in nature. There is evidence that the nicotinic receptor is a highly hydrophobic protein[31,32] and that general anesthetics interact with hydrophobic regions of enzyme and membrane proteins, which undergo conformational change on binding the compounds.[26,33–36]

We conclude that enflurane may cause inhibition of adrenal medullary catecholamine release by hydrophobic interaction with the nicotinic receptors in the cell membrane, which may undergo conformational change on binding the compound. In this way agonist–receptor interaction may be prevented, thus inhibiting stimulus induction. At concentrations above 1 mM enflurane probably also decreases the calcium ion conductance of cell membranes of the chromaffin cells.

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