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Noncompetitive inhibition by camphor of nicotinic acetylcholine receptors

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Abstract

The effect of camphor, a monoterpenoid, on catecholamine secretion was investigated in bovine adrenal chromaffin cells. Camphor inhibited [^{3}H]norepinephrine ([^{3}H]NE) secretion induced by a nicotinic acetylcholine receptor (nAChR) agonist, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), with a half-maximal inhibitory concentration (IC_{50}) of 70 ± 12 μM. In addition, camphor inhibited the rise in cytosolic calcium ($[Ca^{2+}]_i$) and sodium ($[Na^+]_i$) induced by DMPP with IC_{50} values of 88 ± 32 and 19 ± 2 μM, respectively, suggesting that the activity of nAChRs is also inhibited by camphor. On the other hand, binding of [^{3}H]nicotine to nAChRs was not affected by camphor. $[Ca^{2+}]_i$ increases induced by high K^+, veratridine, and bradykinin were not affected by camphor. The data suggest that camphor specifically inhibits catecholamine secretion by blocking nAChRs without affecting agonist binding.

Keywords: Camphor; Nicotinic acetylcholine receptors; Catecholamine secretion; Chromaffin cells

1. Introduction

Camphor, one of the naturally occurring monoterpenoids, is a volatile and aromatic compound. It is a major component of *Cinnamomum camphora*, which is found in temperate regions such as Java, Sumatra, China (central provinces), Korea, Japan, Formasa, and Brazil. The essential oil is also present in *Piper angustifolium* [1], *Sassaafras albidum* [2] *Jasonia candicans*, and *J. montana* [3]. Camphor has been widely used as a fragrance in cosmetics, as a flavouring food additive, as a scenting agent in a variety of household products, as an active ingredient in some old drugs, and as an intermediate in the synthesis of perfume chemicals [4]. In addition, the monoterpenoid is present in a number of over-the-counter medications, mainly for external application, and is readily available in drugstores [5]. Camphor exhibits a broad range of biological properties. It has been used as an insect repellent [6], a bacteriostatic and fungistatic agent [1–3], and an antitussive [7]. On the other hand, the effect of camphor on cellular signal transduction has received little attention. We investigated the effects of camphor on catecholamine secretion and calcium increase induced by various stimulants and found that camphor specifically inhibits nAChRs, thereby blocking nAChR-mediated calcium and sodium increases and catecholamine secretion.

2. Materials and Methods

2.1. Materials

Camphor, DMPP, bradykinin, and veratridine were purchased from the Sigma Chemical Co. Fura-2/AM, SBFI/

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Abbreviations: nAChr, nicotinic acetylcholine receptor; DMPP, 1,1-dimethyl-4-phenylpiperazinium iodide; SBFI, sodium-binding benzofuran isophthalate; [^{3}H]NE, [^{3}H]norepinephrine; DMEM/F-12, Dulbecco's modified Eagle's medium/F-12; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; $[Na^+]_i$, cytosolic free Na^+ concentration; IC_{50}, half-maximal inhibitory concentration; VSCC, voltage-sensitive calcium channel; VSSC, voltage-sensitive sodium channel; and PLC, phospholipase C.

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AM, and Pluronic F-127 were purchased from the Molecular Probes, Inc. [^{3}H]NE and [^{3}H]nicotine were purchased from the NEN Life Science Products.

2.2. Chromaffin cell preparation

Chromaffin cells were isolated from bovine adrenal medulla by a two-step collagenase digestion as previously described [8]. For the measurement of [^{3}H]NE secretion and the [^{3}H]nicotine binding assay, cells were plated in 24-well plates at a density of 5×10^5 cells/well. Chromaffin cells transferred to 100-mm culture dishes (1×10^7 cells/dish) were used to measure cytosolic free calcium and sodium concentrations. The cells were maintained in DMEM/F-12 (Life Technologies, Inc.) containing 10% bovine calf serum (HyClone) and 1% antibiotics (Life Technologies, Inc.). Chromaffin cells were incubated in a humidified atmosphere of 5% CO_2/95% air at 37° for 3–7 days before use.

2.3. Measurement of [^{3}H]NE secretion

Catecholamine secretion from chromaffin cells was measured in 24-well plates following the method reported by Park *et al.* [9]. In brief, cells were loaded with [^{3}H]NE (1 μCi/mL; 68 pmol/mL) during an incubation in DMEM/F-12 containing 0.01% ascorbic acid for 1 h at 37° in 5% CO_2/95% air. The cells were washed with Ca^{2+}-free Locke's solution and were incubated in fresh Locke's solution for 10 min to measure basal secretion. The cells were subsequently stimulated with the test drugs for 10 min. After the incubation, the medium was removed from each well and transferred to scintillation vials. Residual catecholamine in the cells was extracted by the addition of 10% trichloroacetic acid, and the extract was transferred to a scintillation vial. The radioactivity in each vial was determined with a scintillation counter. The amount of [^{3}H]NE secreted was calculated as a percentage of total [^{3}H]NE content. Net stimulated secretion was obtained by subtracting the basal secretion from the stimulated secretion.

2.4. [Ca^{2+}]$_i$ measurement

[Ca^{2+}]$_i$ was determined with the help of the fluorescent Ca^{2+} indicator fura-2 as reported previously [10]. Briefly, the chromaffin cell suspension was incubated with fresh serum-free DMEM/F-12 medium containing fura-2/AM (3 μM) for 40 min at 37° with continuous stirring. The cells were then washed with Locke's solution and left at room temperature until used. Sulfinpyrazone (250 μM) was added to all solutions to prevent dye leakage. Fluorescence ratios were measured by an alternative wavelength time scanning method (dual excitation at 340 and 380 nm; emission at 500 nm).

2.5. [Na^+]$_i$ measurement

[Na^+]$_i$ was measured using the fluorescent Na^+ indicator SBFI as previously described in a report by Park *et al.* [11]. In brief, the chromaffin cell suspension was incubated in fresh DMEM/F-12 medium containing 15 μM SBFI/AM, 10% bovine calf serum, and 0.2% Pluronic F-127 for 2 h at 37° with continuous stirring.

The cells were then washed twice with fresh DMEM/F-12 medium and left at room temperature until used. Sulfinpyrazone (250 μM) was added to all solutions to prevent dye leakage. Fluorescence ratios were taken with alternate excitation at 340 and 380 nm and emission at 530 nm. Changes in [Na^+]$_i$ are presented as fluorescence ratios.

2.6. [^{3}H]Nicotine binding analysis

Binding of [^{3}H]nicotine to intact cells was measured as previously described by Park *et al.* [9]. Intact chromaffin cells in 24-well plates (5×10^5 cells/well) were washed twice with Locke's solution and incubated with 20 nM [^{3}H]nicotine and the indicated concentrations of camphor for 40 min at 25°. Then the cells were washed three times with 1 mL of ice-cold Ca^{2+}-free Locke's solution containing 100 μM EGTA. Finally, the cells were lysed and scraped in 0.5 mL of 5% Trichloroacetic acid, and the radioactivity was measured by liquid scintillation counting. Nonspecific binding, determined by coincubation with 1 mM nicotine, amounted to less than 20% of the total binding, and was subtracted routinely from the total binding. The binding data were analyzed and expressed as a percentage of total binding.

2.7. Statistical analysis

All experiments were performed at least three times, and the results were reproducible. Typical and representative data were chosen, and quantitative data are expressed as means ± SEM of triplicate measurements. The IC_{50} values were calculated with the Microcal Origin for Windows programs.

3. Results

3.1. Inhibition by camphor of DMPP-induced [^{3}H]NE secretion

To study the effects of camphor on catecholamine secretion, we treated [^{3}H]NE-loaded chromaffin cells with camphor. Whereas camphor (up to 300 μM) by itself did not induce [^{3}H]NE secretion (data not shown), it decreased the DMPP-induced secretion of [^{3}H]NE in a concentration-dependent manner with a half-maximal inhibitory concentration (IC_{50}) of 70 ± 12 μM (Fig. 1). Camphor at 300 μM completely blocked the DMPP-induced secretion of [^{3}H]NE.

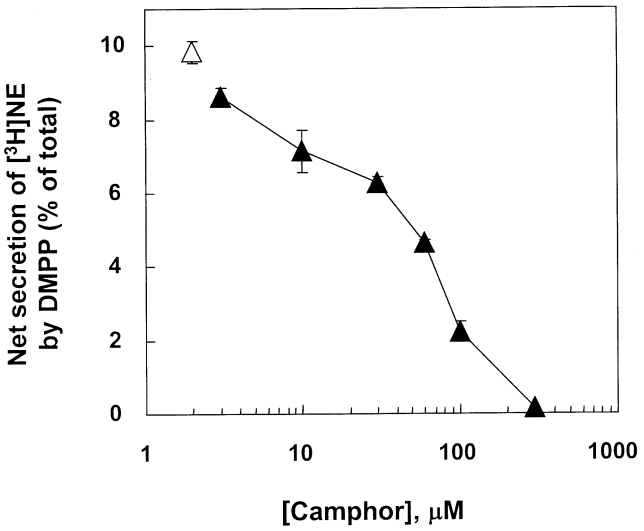

Fig. 1. Inhibitory effect of camphor on [^{3}H]NE secretion in chromaffin cells. [^{3}H]NE (1 μCi/mL; 68 pmol/mL)-loaded chromaffin cells were treated with 10 μM DMPP in the presence of the indicated concentrations of camphor (closed triangles). Secretion of [^{3}H]NE induced by DMPP in the absence of camphor is also presented (open triangle). The secreted [^{3}H]NE was measured as described under "Materials and methods" and is expressed as a percentage of total [^{3}H]NE. Three separate experiments were done, and the results were reproducible. Data are means ± SEM of triplicate measurements.

3.2. Inhibition by camphor of the DMPP-induced rise in $[Ca^{2+}]_i$

Since an increase in $[Ca^{2+}]_i$ is an essential step in catecholamine secretion, we tested the effect of camphor on the DMPP-induced rise in $[Ca^{2+}]_i$. Camphor (up to 300 μM) by itself had no effect on $[Ca^{2+}]_i$ (data not shown), whereas DMPP induced a significant increase in $[Ca^{2+}]_i$ (Fig. 2A). The DMPP-induced rise in $[Ca^{2+}]_i$ was inhibited by camphor in a concentration-dependent manner with an IC_{50} of 88 ± 32 μM (Fig. 2B). Incubation of cells with 300 μM camphor caused complete inhibition of the DMPP-induced rise in $[Ca^{2+}]_i$. The results suggest that the effect of camphor on the DMPP-evoked secretion of [^{3}H]NE resulted from its inhibition of the DMPP-induced rise in $[Ca^{2+}]_i$. In addition, the effects of camphor at differing concentrations of DMPP were also investigated to further analyze the mechanism of action of camphor. As shown in Fig. 2C, 70 μM camphor inhibited the DMPP-induced rise in $[Ca^{2+}]_i$ to an extent similar to that of all the DMPP concentrations tested, suggesting that camphor acts as a noncompetitive inhibitor.

3.3. Inhibition by camphor of the DMPP-induced rise in $[Na^+]_i$

Since VSCCs as well as nAChRs are activated upon nicotinic stimulation [9], the inhibition of the DMPP-induced rise in $[Ca^{2+}]_i$ could come from the inhibition of either nAChRs or VSCCs or both. To verify whether nAChRs were inhibited by camphor, we tested the effect of

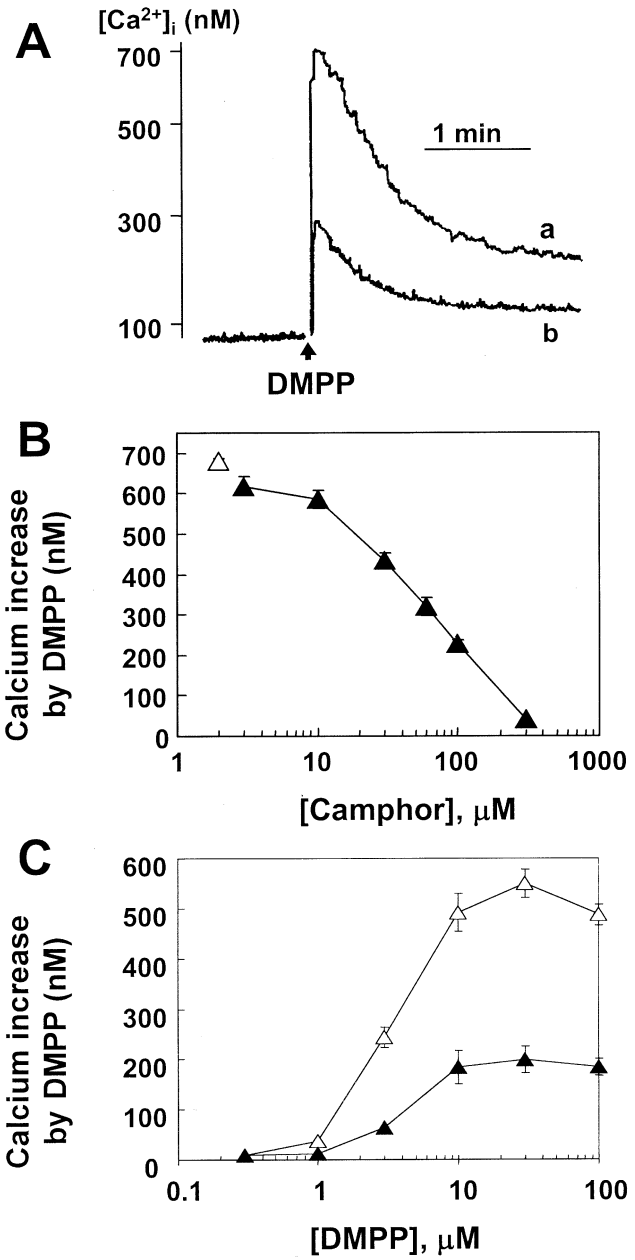

Fig. 2. Inhibitor effect of camphor on $[Ca^{2+}]_i$ evaluation in chromaffin cells. (A) The rise in intracellular $[Ca^{2+}]_i$ induced by 10 uM DMPP was measured in the absence (trace a) or the presence of (trace b) of 100 μM camphor. The experiments were performed three times independently, and the results were reproducible. Typical Ca^{2+} traces are presented. (B) The rise in $[Ca^{2+}]_i$ induced by 10 μM DMPP was measured 3 min after preincubation of the cells with the indicated concentrations of camphor (closed triangles). The peak height of each stimulation was compared to that of the control $[Ca^{2+}]_i$ increase caused by DMPP alone (open triangle). Three separate experiments were done, and the results were reproducible. Data are means ± SEM of triplicate measurements. (C) The rise in $[Ca^{2+}]_i$ induced by the indicated concentrations of DMPP was measured in the absence (open triangles) or presence (closed triangles) of 70 μM camphor. Three separate experiments were done, and the results were reproducible. Data are means ± SEM of triplicate measurements.

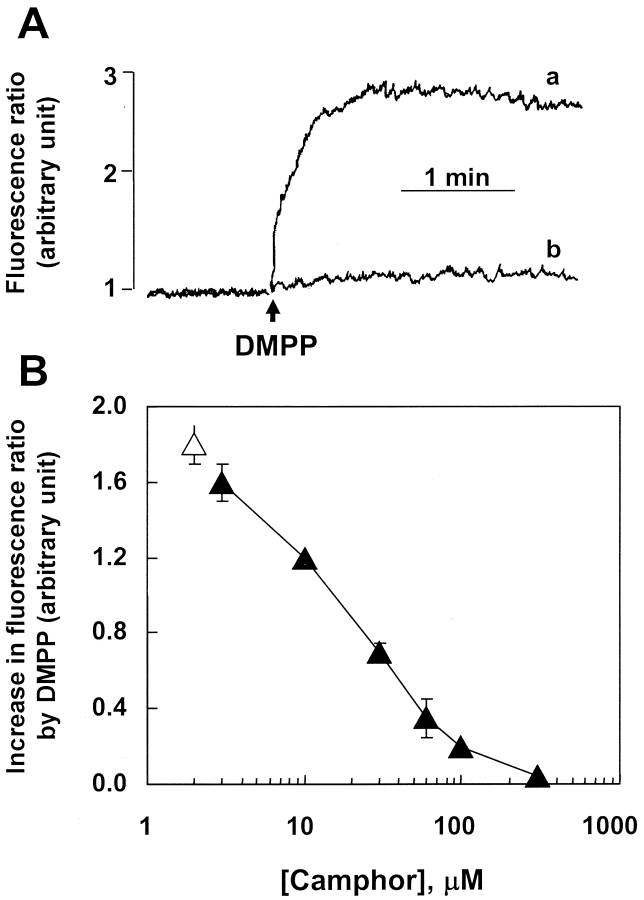

Fig. 3. Inhibitory effect of camphor on the elevation of $[Na^+]_i$ in chromaffin cells. (A) The intracellular $[Na^+]_i$ rise induced by μM DMPP was measured in the absence (trace a) or presence (trace b) of 100 μM camphor. The experiments were performed three times independently, and the results were reproducible. Typical Na^+ traces are presented. (B) The $[Na^+]_i$ rise induced by 10 μM DMPP was measured 3 min after preincubation with the indicated concentrations of camphor (closed triangles). The peak height of each stimulation was compared with that of the control $[Na^+]_i$ increase caused by DMPP alone (open triangle). Three separate experiments were done, and the results were reproducible. Data are means ± SEM of triplicate measurements.

camphor on the DMPP-induced rise in $[Na^+]_i$ that occurs only through nAChRs. As shown in Fig. 3A, DMPP induced an increase in $[Na^+]_i$. Camphor inhibited the DMPP-induced rise in $[Na^+]_i$ in a concentration-dependent manner with an IC_{50} of 19 ± 2 μM (Fig. 3B), and 300 μM camphor completely inhibited the DMPP effect. The results suggest that the inhibition by camphor of the DMPP-induced rise in $[Ca^{2+}]_i$ and $[Na^+]_i$ resulted from the inhibition of nAChRs.

3.4. Effect of camphor on [^{3}H]nicotine binding

Since the DMPP-induced rise in $[Na^+]_i$ and $[Ca^{2+}]_i$ could be inhibited by camphor, it was possible that camphor directly bound the nAChRs, thus inhibiting the activity of these receptors. We investigated whether the binding of [^{3}H]nicotine to nAChRs could be interfered with by camphor. As shown in Fig. 4, camphor at all concentrations

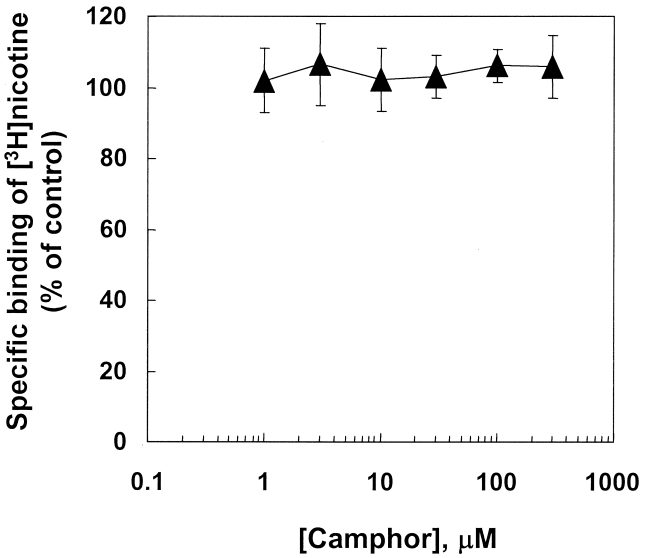

Fig. 4. Effect of camphor on [^{3}H]nicotine binding. Chromaffin cells were incubated with 20 nM [^{3}H]nicotine and various concentrations of camphor for 40 min at 25°. Specific binding of [^{3}H]nicotine is presented. Nonspecific binding was determined in the presence of 1 mM unlabeled nicotine. Four separate experiments were done, and the results were reproducible. Data are means ± SEM of triplicate measurements.

tested did not compete significantly with [^{3}H]nicotine for binding, suggesting that its binding site is distinct from that of the agonists including nicotine and acetylcholine.

3.5. Time course of camphor-induced effects

In looking for a clue towards the elucidation of the mechanism of action of camphor, we determined the time course of the camphor effect on the DMPP-induced rise in $[Ca^{2+}]_i$. When chromaffin cells were treated simultaneously with 100 μM camphor and DMPP, the initial peak height decreased by 71% (trace b in the inset of Fig. 5), in comparison to the control (trace a in the inset of Fig. 5). When the incubation time with camphor was varied, the inhibition remained similar (Fig. 5), suggesting that the effect of camphor was almost instantaneous. Interestingly, however, camphor had no inhibitory effect when it was applied 2 min after the DMPP treatment (data not shown). Therefore, it seems that the direct camphor binding occurs at the resting state and not the activated state of the nAChRs in the plasma membrane and does not involve a second messenger.

3.6. Specific action of camphor

To confirm that the inhibitory activity of camphor was specifically targeted at the nAChR, we first tested whether the DMPP-induced responses were truly mediated by nAChRs. Treatment of bovine adrenal chromaffin cells with *d*-tubocurarine, a specific blocker of nAChRs [12–13], completely inhibited the DMPP-induced rise in $[Ca^{2+}]_i$ (data not shown), suggesting that DMPP specifically activated nAChRs. We then examined the effects of camphor on

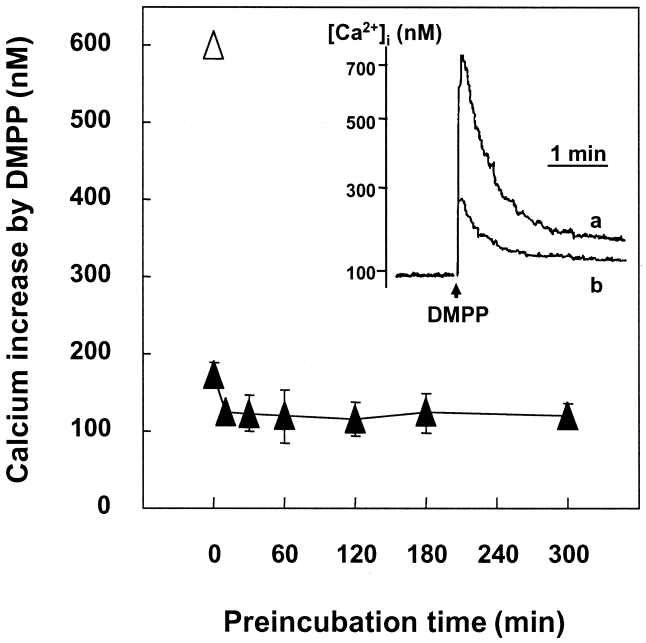

Fig. 5. Time course of the inhibitory effect of camphor. Chromaffin cells were preincubated for the indicated times with 100 μM camphor and then stimulated with 10 μM DMPP (closed triangles). Incubation labeled zero time means that camphor and DMPP were added simultaneously. The peak height of each stimulation is compared with that of the increase in the control $[Ca^{2+}]_i$ achieved by DMPP alone (open triangle). Three separate experiments were done, and the results were reproducible. Data are means ± SEM of triplicate measurements. Inset: The intracellular $[Ca^{2+}]_i$ rise induced by 10 μM DMPP was measured in the absence (trace a) or presence (trace b) of 100 μM camphor. Chromaffin cells were treated simultaneously with both camphor and DMPP (trace b). The experiments were performed three times independently, and typical Ca^{2+} traces are presented.

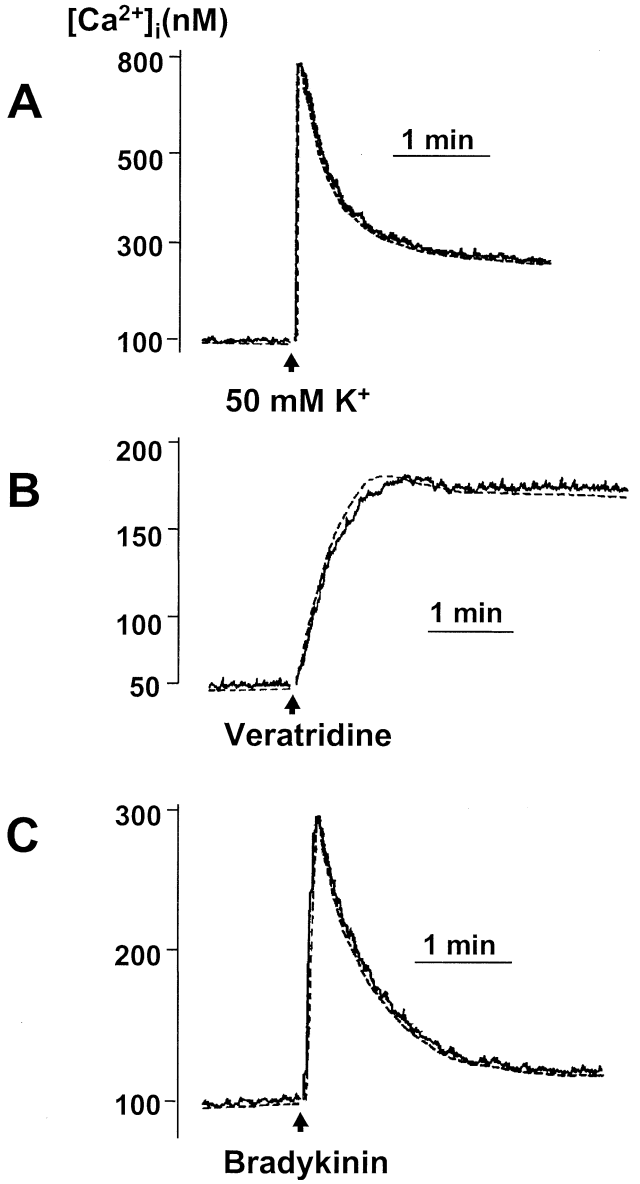

Fig. 6. Lack of a camphor effect on the rise in $[Ca^{2+}]_i$ induced by high K^+, veratridine, and bradykinine. The intracellular $[Ca^{2+}]_i$ increase was induced by 50 mM K^+ (A) 200 μM veratridine (B) or 5 μM bradykinin (C) in the absence (solid trace) or presence (dashed trace) of 100 μM camphor. The experiments were performed three times independently, and the results were reproducible. Typical Ca^{2+} traces are presented.

VSCCs, VSSCs, and PLC-linked receptor signaling. Activation of VSCCs with 50 mM K^+ evoked a rapid increase in $[Ca^{2+}]_i$ (solid trace in Fig. 6A). Pretreatment with 100 μM camphor did not affect the high K^+-evoked response (dashed trace in Fig. 6A), indicating that the VSCCs were not affected by camphor. The rise in $[Ca^{2+}]_i$ induced by veratridine, an activator of VSSCs, was also not affected by camphor (Fig. 6B). Bradykinin is known to activate B_2 bradykinin receptors in bovine adrenal chromaffin cells [14, 15]. Camphor at 100 μM had no effect on the bradykinin-evoked rise in $[Ca^{2+}]_i$ (Fig. 6C). In addition, the same concentration of camphor had no effect on muscarinic receptor- and P2Y2 purinoceptor-mediated Ca^{2+} increases in human SK-N-BE(2)C and rat pheochromocytoma (PC12) cells, respectively (data not shown). Together the data show that camphor has no effect on VSCCs, VSSCs, and PLC-linked receptors, indicating that the effect of camphor on nAChRs is highly specific.

4. Discussion

Our data clearly indicate that camphor specifically inhibits nAChRs, thereby causing inhibition of catecholamine secretion. Since all tested responses induced by DMPP including $[^3H]$NE secretion and $[Ca^{2+}]_i$ and $[Na^+]_i$ increases were inhibited by camphor, it is highly plausible that the activity of nAChRs was inhibited by the reagent. On the other hand, $[Ca^{2+}]_i$ increases induced by high K^+, veratridine, and bradykinin were not affected by camphor, which suggests that camphor has no effect on other kinds of membrane proteins such as VSCCs, VSSCs, and PLC-linked receptors. Although VSCCs are stimulated in the activation process of nAChRs, both the inhibition of the DMPP-induced rise in $[Na^+]_i$ and the absence of any inhibitory effect on the high K^+-induced increase in $[Ca^{2+}]_i$

clearly indicate that nAChRs, but not VSCCs, are the specific target of camphor. It is interesting that camphor inhibited the DMPP-induced calcium increase at all DMPP concentrations tested, suggesting that camphor does not act in a competitive manner. The lack of effect of camphor on [^{3}H]nicotine binding confirmed that camphor inhibits nAChRs in a noncompetitive manner. It seems that the monoterpenoid shares binding characteristics with many other noncompetitive inhibitors of nAChRs. It would be interesting to compare the role of a variety of physiological or pharmacological effectors such as divalent cations, neuropeptides, local and general anesthetics, membrane potential, and protein phosphorylation with the role of camphor, in modifying the properties of nAChRs, even if they do not significantly affect the binding of agonists such as acetylcholine and nicotine. A comparison study of the binding characteristics of camphor and other noncompetitive modulators on AChRs may reveal the interaction site in the nAChR molecule [16,17].

The inhibition of nAChRs by camphor may explain the toxicity caused by camphor ingestion. In several case reports, camphor caused status epilepticus [18], abortion [5], and generalized tonic-clonic seizures [19,20] when it was ingested orally in overdoses. The detailed mechanisms of the camphor-induced toxicity have not been clearly defined. Because nAChRs are known to play a major role at neuromuscular junctions, it can be expected that such toxic effects may be caused by the inhibition of nAChRs by camphor. However, it is still not clear if the toxic effects are only due to the blockage of nAChRs. Further clinical studies will be required to address this question.

Since nAChRs have been implicated in many diseases including nicotine addiction [21,22], the specific inhibitory effect of camphor on nAChRs suggests that camphor could become a promising candidate for therapeutic use. Most nicotinic antagonists, however, have inhibitory effects on VSCCs [23–25], PLC [26,27], and Na^+/Ca^{2+} exchanger [28] as well as on nAChRs. Therefore, the highly selective inhibition of nAChRs by camphor can be of great advantage in the development of medical reagents with an enhanced therapeutic effect and decreased side-effects. In addition, camphor could be a useful tool in the study of nAChR-mediated signal transduction.

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