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Vinpocetine-induced stimulation of calcium-activated potassium currents in rat pituitary GH_3 cells

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Abstract

The effects of vinpocetine, an inhibitor of cyclic GMP phosphodiesterase, on ionic currents were examined in rat pituitary GH_3 lactotrophs with the aid of the patch-clamp technique. In GH_3 cells bathed in normal Tyrode's solution, vinpocetine (10 μM) reversibly increased the amplitude of Ca^{2+}-activated K^+ current ($I_{K(Ca)}$) with an EC_{50} value of 4 μM. When the recording pipettes were filled with 10 mM EGTA, vinpocetine also stimulated $I_{K(Ca)}$. In the cell-attached configuration, application of vinpocetine to the bath increased the activity of large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels. In excised membrane patches, application of vinpocetine (10 μM) to the bath did not change the single-channel conductance of BK_{Ca} channels; however, it did increase channel activity. In the inside-out configuration, neither 8-bromo cyclic GMP nor YC-1 applied intracellularly affected BK_{Ca} channel activity. The vinpocetine-induced change in the kinetic behavior of BK_{Ca} channels was due to an increase in mean open time and a decrease in mean closed time. Vinpocetine (10 μM) caused a leftward shift in the midpoint for the voltage-dependent opening. Under the current-clamp mode, vinpocetine (10 μM) decreased the firing rate of spontaneous action potentials induced by thyrotropin-releasing hormone (10 μM) in GH_3 cells. In pheochromocytoma PC12 cells, vinpocetine (10 μM) applied intracellularly also enhanced the activity of BK_{Ca} channels without altering single-channel conductance. Thus, the present study suggests that vinpocetine-mediated stimulation of $I_{K(Ca)}$ may result from the direct activation of BK_{Ca} channels and indirectly from elevated cytosolic Ca^{2+}.

Keywords: Vinpocetine; Large-conductance Ca^{2+}-activated K^+ channels; Pituitary cells

1. Introduction

Vinpocetine, a vincamine derivative, has been known to produce neuroprotective and anticonvulsant effects [1]. Previous reports demonstrated that vinpocetine can protect against cerebrocortical cell injury [2,3] and prevent hippocampal neuronal damage [4,5] and gastric mucosal damage [6]. It was found that vinpocetine increased the neuroprotective effect of adenosine in hypoxia in cell cultures [7]. This compound was also reported to be a selective inhibitor of cyclic GMP phosphodiesterase [8] and to attenuate phenylephrine-induced pressor response in isolated rabbit aorta [9].

Several studies have demonstrated that vinpocetine can regulate receptors and/or ionic channels. For example, vinpocetine can reduce the binding of [^{3}H]2-amino-3-3-hydroxy-*s*-methylisoxasole-4-yl-propionic acid and decrease the release of dopamine and acetylcholine caused by glutamine, quisqualate, or *N*-methyl-D-aspartate in striatal slices of rat brain [10]. Vinpocetine was reported to suppress currents through ionic channels, such as voltage-dependent Na^+ and Ca^{2+} currents in rat cortical neurons and synaptosomes [1,3,11,12]. It also blocked delayed rectified K^+ current in isolated snail neurons [13].

A previous study showed that depletion of Ca^{2+} stores could facilitate the Ca^{2+} influx induced by an elevation in intracellular cyclic GMP in GH_3 lactotrophs [14]. A recent report also demonstrated that vinpocetine can suppress intracellular Ca^{2+} oscillations in GH_3 cells [15]. In addition, it is known that this cell line, in addition to the presence of voltage-dependent K^+ and Ca^{2+} currents, exhibits BK_{Ca} channels that can be regulated by arachidonic acid metabolites or intracellular cyclic GMP [16]. It is well docu-

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Abbreviations: BK_{Ca} channel, large-conductance Ca^{2+}-activated K^+ channel; $I_{K(Ca)}$, Ca^{2+}-activated BK_{Ca} channel, large-conductance Ca current; $I_{Ca,L}$, L-type Ca^{2+} current; and TRH, thyrotropin-releasing hormone.

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mented that the activity of BK_{Ca} channels plays an essential role in controlling hormonal secretion by altering the duration and frequency of action potentials [17,18]. To date, however, none of the studies have demonstrated an effect of vinpocetine on $I_{K(Ca)}$ in neurons and neuroendocrine cells.

The objective of this study was to: (a) address the question as to whether vinpocetine can affect $I_{K(Ca)}$ in GH_3 lactotrophs: (b) determine the effect of vinpocetine on the activity and gating of BK_{Ca} channels; and (c) examine whether vinpocetine can affect the activity of BK_{Ca} channels expressed in other neuroendocrine cells (i.e. rat pheochromocytoma PC12 cells). Our study demonstrated a novel and dose-dependent regulation of $I_{K(Ca)}$ by vinpocetine in GH_3 cells. The direct activation of BK_{Ca} channels by vinpocetine may also be responsible for its effect on the cellular function in these cells.

2. Materials and methods

2.1. Cell culture

The clonal strain GH_3 cell line, originally derived from a rat anterior pituitary adenoma, was obtained from the American Type Culture Collection ([CLL-82.1]) [19]. Briefly, cells were grown in monolayer culture in 50-mL plastic culture flasks in a humidified environment of 5% CO_2/95% air at 37°. Cells were maintained at a density of 10^6/mL in 5 mL Ham's F-12 nutrient medium supplemented with density of 15% heat-inactivated horse serum (v/v), 2.5% fetal bovine serum (v/v), and 2 mM L-glutamine. Experiments were performed after 5 or 6 days of subcultivation (60–80% confluence).

Stock cultures of rat pheochromocytoma PC12 cells were obtained from the Culture Collection and Research Center ([CCRC-60048]) [20]. PC12 cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum at 37° in 5% CO_2-containing humidified air.

2.2. Electrophysiological measurements

Immediately before each experiment, GH_3 or PC12 cells were dissociated, and an aliquot of the cell suspension was transferred to a recording chamber positioned on the stage of an inverted phase-contrast microscope (Diaphot-200; Nikon). Cells were bathed at room temperature (20–25°) in normal Tyrode's solution containing 1.8 mM $CaCl_2$. Patch pipettes were prepared from Kimax capillary tubes (Kimble Products) using a vertical two-step electrode puller (PB-7; Narishige), and the tips were fire-polished with a microforge (MF-83; Narishige). The resistance of the patch pipette was 3–5 MΩ when immersed in normal Tyrode's solution. A programmable stimulator (SMP-311; Bio-Logic) was used to generate rectangular or ramp pulses [21]. Experiments were performed using the whole-cell, cell-attached, and inside-out configuration of the patch-clamp technique [19, 22] by means of an RK-400 patch amplifier (Bio-Logic). All potentials were corrected for liquid junction potential, a value that would develop at the tip of the pipette when the composition of pipette solution was different from that of bath. Tested drugs were applied by perfusion or added to the bath to obtain the final concentrations indicated. In the experiments with vinpocetine plus each compound (e.g. 8-bromo cyclic GMP, YC-1, clotrimazole, and iberiotoxin), each compound was applied after the addition of vinpocetine.

2.3. Data recording and analysis

The signals consisting of voltage and current tracings were displayed on a storage oscilloscope (Model 1602; Gould) and recorded on-line using a digital audio tape recorder (Model 1204, Bio-Logic). The stored data were fed back and digitized at 5–10 kHz with a Digidata 1200 analog-to-digital device (Axon Instruments) interfaced to a Pentium III-grade computer and with the aid of Clampex subroutine in the pCLAMP 8.0 software package (Axon Instruments). Membrane currents recorded during whole-cell experiments were analyzed using Clampfit subroutine (Axon Instruments) or pCLAMP module in the Origin 6.0 software package (Microcal) to establish a current–voltage relationship for ionic currents [22].

To calculate the percentage stimulation of vinpocetine on $I_{K(Ca)}$, each cell was depolarized from 0 to +50 mV, and current amplitudes during the application of vinpocetine were compared. The increase in the amplitude of $I_{K(Ca)}$ caused by vinpocetine (100 μM) was taken to be 100%. The concentration of vinpocetine required to stimulate 50% of current amplitude was determined using a Hill function, $y = E_{max}/\{1 + (EC_{50}/[D]^{n_H})\}$, where [D] is the concentration of vinpocetine, n_H and EC_{50} are the Hill coefficient and the concentration of vinpocetine which induces a half-maximal effect, respectively, and E_{max} is the vinpocetine-induced maximal stimulation of $I_{K(Ca)}$.

Unitary currents of BK_{Ca} channels were analyzed by the pCLAMP software (Axon Instruments). Open or closed lifetime distributions were fit with logarithmically scaled bin width by using the method of McManus *et al*. [23]. To determine the effect of vinpocetine on the activation curve of BK_{Ca} channels, ramp pulses from +20 to +140 mV with a duration of 1 sec were digitally delivered [21]. This enabled us to measure single-channel conductance and channel activation more efficiently [24]. The activation curves were calculated by averaging current responses to 20 voltage ramps and dividing each point of the averaged current by the unitary amplitude of each potential after each leakage component was corrected. To obtain values for the slope factor of the voltage-dependent activation and half-maximal activation voltage, the activation curves obtained before and after the addition of vinpocetine were fitted with a Boltzmann function of the form: relative $N \cdot P_o = n/\{1 +$

exp[−(V − a)/b]}, where n is the maximal relative $N \cdot P_o$ is the slope factor of the voltage-dependent activation (i.e. change in potential required to produce an exponential (e)-fold increase in activation), and a is the voltage at which there is half-maximal activation.

To estimate all transition rates between states, the single-channel data were idealized and converted to an ASCII format (i.e. a dwell-time file format), which was readable by the maximum-interval-likelihood program (MIL) in the QUB suite. Then, the data were used to determine single-channel kinetic parameters by means of a maximum likelihood algorithm [25]. The highest log likelihood was obtained with the gating scheme: $C_1 \leftrightarrow C_2 \leftrightarrow O$. The single-channel data were modeled on the basis of this scheme. Simulated single-channel data were also obtained using the determined transition rates. K and L values represent the equilibrium dissociation constant that equals the transition rate for $C_1 \leftarrow C_2$ divided by that for $C_1 \rightarrow C_2$ and the equilibrium gating constant that equals opening rate constant divided by closing rate constant, respectively.

Values are expressed as means ± SEM. The paired or unpaired Student's *t*-test and one-way ANOVA with the least-significance difference method for multiple comparison were used for the statistical evaluation of differences among the mean values. Differences between the values were considered significant when $P < 0.05$.

2.4. Drugs and solutions

Vinpocetine ((3α, 16α)-eburnamenine-14-carboxylic acid ethyl ester) was obtained from Tocris Cookson Ltd. TRH was purchased from Sigma Chemical Co. 8-Bromo cyclic GMP (8-bromo-guanosine 3′,5′-cyclic monophosphate), clotrimazole, ionomycin, and iberiotoxin were obtained from RBI. Penitrem A was purchased from Biomol. YC-1 (3-5′-hydroxymethyl-2′-furyl)-1-benzyl indazole) was kindly provided by Professor Che-Ming Teng (Pharmacology Institute, National Taiwan University, Taiwan) [26]. Tissue culture media, penicillin–streptomycin, fungizone, and trypsin were obtained from Life Technologies, Inc. The composition of normal Tyrode's solution was as follows (in mM): NaCl 136.5, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.53, glucose 5.5, and HEPES–NaOH buffer 5.5 (pH 7.4). To record K^+ currents or membrane potentials, the patch pipette was filled with solution (in mM): KCl 140, $MgCl_2$ 1, Na_2ATP 3, Na_2GTP 0.1, EGTA 0.1, and HEPES–KOH buffer 5 (pH 7.2). To record Ca^{2+} current, KCl inside the pipette solution was replaced with equimolar CsCl, and pH was adjusted to 7.2 with CsOH. In the cell-attached configuration of single-channel recording, the high K^+-bathing solution contained (mM): KCl 145, $MgCl_2$ 0.53, $CaCl_2$ 1.8, and HEPES–KOH 5 (pH 7.4), and the pipette solution contained (mM): KCl 145, $MgCl_2$ 2, and HEPES–KOH 5 (pH 7.2). In the inside-out configuration, the high K^+-bathing solution contained (mM): KCl 145, $MgCl_2$ 0.53, and HEPES–KOH 5 (0.1 μM free Ca^{2+}; pH 7.4).

3. Results

3.1. Effect of vinpocetine on the amplitude of $I_{K(Ca)}$ in GH_3 cells

The whole-cell configuration of the patch-clamp technique was used to investigate the effect of vinpocetine on ionic currents in GH_3 cells. To examine $I_{K(Ca)}$, cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$. The pipette solution used in these experiments contained a low concentration (0.1 mM) of EGTA and 3 mM ATP. To inactivate other voltage-dependent K^+ currents, each cell was held at the level of 0 mV. As shown in Fig. 1, a family of large, noisy, outward currents that exhibited outward rectification were elicited in response to a series of voltage pulses ranging from −30 to +70 mV. These outward currents were previously identified as $I_{K(Ca)}$ [19,20]. When cells were exposed to vinpocetine (10 μM), the amplitudes of outward currents were greatly enhanced throughout the entire voltage-clamp step. For example, when depolarizing pulses from 0 to +70 mV were evoked, the presence of 10 and 30 μM vinpocetine significantly increased the current amplitude to 1336 ± 115 and 2023 ± 191 pA from a control value of 763 ± 76 pA (N = 8). This stimulatory effect was readily reversed after the removal of vinpocetine. The amplitudes of $I_{K(Ca)}$ were plotted versus membrane potentials. The averaged current–voltage relations for these currents in the absence and presence of vinpocetine are shown in Fig. 1B. The relationship between the concentration of vinpocetine and the percentage stimulation of $I_{K(Ca)}$ was also constructed. The half-maximal concentration required for the vinpocetine-induced stimulation of $I_{K(Ca)}$ was 4 μM, and 100 μM vinpocetine completely stimulated the amplitude of $I_{K(Ca)}$. These results indicated that vinpocetine caused a stimulatory effect on $I_{K(Ca)}$ in GH_3 cells.

It was further examined whether the vinpocetine-induced increase in $I_{K(Ca)}$ was related to the level of intracellular Ca^{2+}. To strongly buffer intracellular Ca^{2+}, the experiments were conducted in recording pipettes filled with 10 mM EGTA. An increase in intracellular EGTA from 0.1 to 10 mM reduced the amplitude of $I_{K(Ca)}$. However, the addition of vinpocetine (10 μM) also produced a significant increase in the amplitude of $I_{K(Ca)}$ (Fig. 2), although the vinpocetine-stimulated magnitude was reduced. For example, when cells that had been dialyzed with 10 mM EGTA were depolarized from 0 to +50 mV, vinpocetine (10 μM) significantly increased the amplitude of outward currents from 269 ± 27 pA to 346 ± 31 pA (N = 8). However, under the same voltage protocol, an approximate 80% increase in $I_{K(Ca)}$ was found when the pipette solution contained 0.1 mM EGTA and cells were exposed to vinpocetine

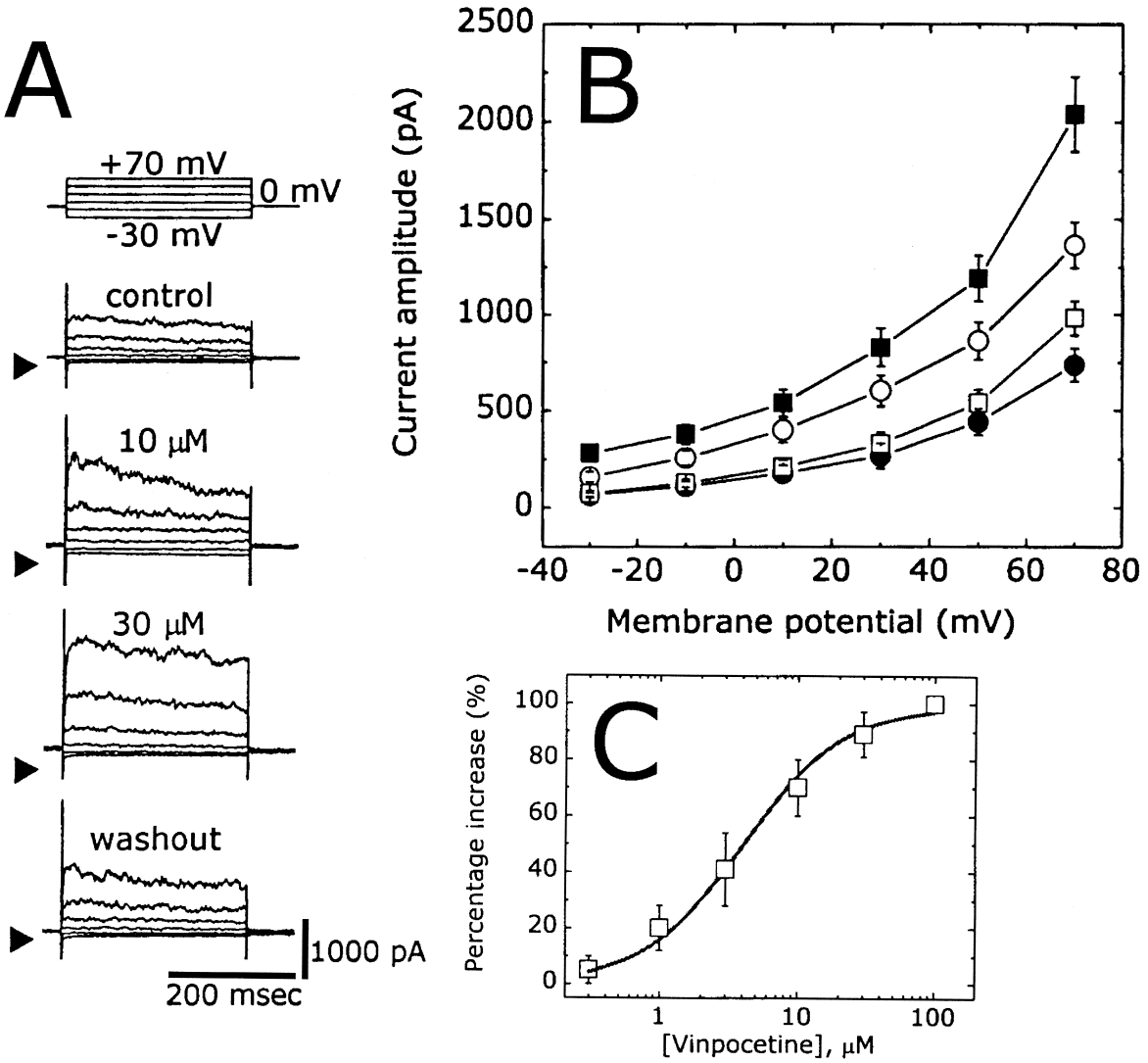


Fig. 1. Stimulatory effect of vinpocetine on $I_{K(Ca)}$ in GH_3 cells. (A) Superimposed current traces in control during exposure to 10 and 30 μM vinpocetine and after washout of vinpocetine. In these experiments, cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$. The cell was held at 0 mV and voltage pulses from −30 to +70 mV in 20-mV increments with a duration of 300 msec were delivered. The voltage protocol is shown in the upper part. Arrows indicate 0 current level. (B) Averaged current–voltage relationships of $I_{K(Ca)}$ in control (●), during exposure to 10 μM (○) and 30 μM (■) vinpocetine, and during washout of vinpocetine (□) (mean ± SEM; N = 7–9 for each point). (C) Concentration-dependent stimulation of $I_{K(Ca)}$ by vinpocetine. The relationship between the percentage stimulation of $I_{K(Ca)}$ and the concentration of vinpocetine is illustrated. $I_{K(Ca)}$ was measured at a rate of 0.1 Hz using depolarizing pulses of 300-msec duration clamped from a holding potential of 0 mV to +50 mV. Various concentrations of vinpocetine (0.3–100 μM) were applied. The values for EC_{50}, Hill coefficient, and the maximal percentage increase in $I_{K(Ca)}$ in the presence of vinpocetine were 4 μM, 1.2, and 99%, respectively.

(10 μM). The amplitude of outward currents was also increased throughout the entire voltage-clamp step (Fig. 2B).

3.2. Comparison of the effect of vinpocetine on $I_{K(Ca)}$ in the absence and presence of 8-bromo cyclic GMP or YC-1

We examined whether the stimulatory effect of vinpocetine on $I_{K(Ca)}$ was altered by the presence of 8-bromo cyclic GMP or YC-1. 8-Bromo cyclic GMP is a stable analog of cyclic GMP, while YC-1 is an activator of soluble guanylyl cyclase that can increase the level of intracellular cyclic GMP [26,27]. In these experiments, each cell was depolarized from 0 to +70 mV with a duration of 300 msec. As shown in Fig. 3, 8-bromo cyclic GMP (100 μM) and YC-1 (10 μM) alone significantly increased the amplitude of $I_{K(Ca)}$. Interestingly, the magnitude of the stimulatory effect caused by vinpocetine plus 8-bromo cyclic GMP or YC-1 on $I_{K(Ca)}$ was not different from that by vinpocetine alone (Fig. 3). However, clotrimazole (10 μM) or iberiotoxin (200 nM), which were both reported to be blockers of BK_{Ca} channels [18], significantly attenuated vinpocetine-mediated stimulation of $I_{K(Ca)}$. Thus, these data indicate that vinpocetine, 8-bromo cyclic GMP, and YC-1 increased the

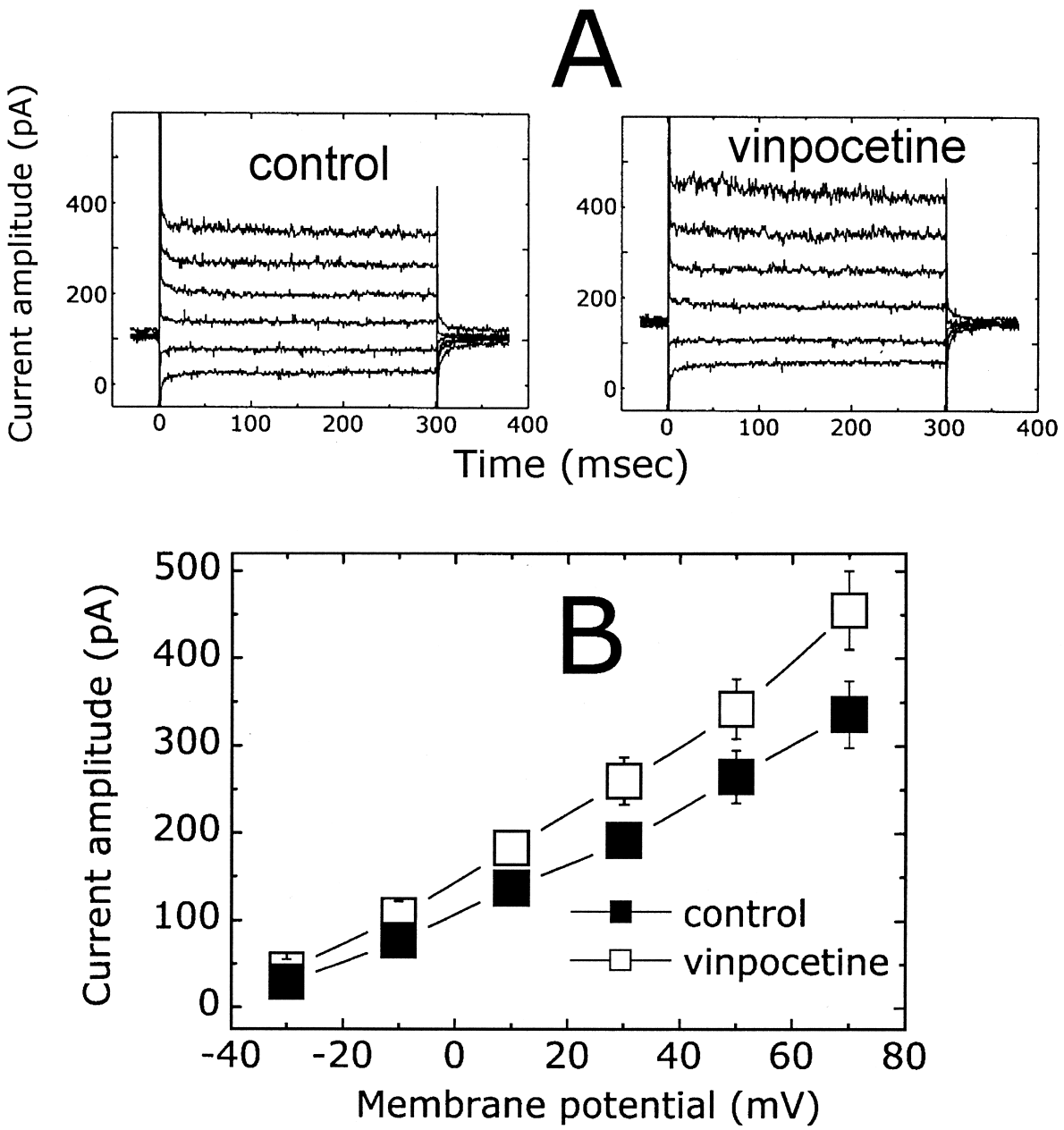


Fig. 2. Effect of vinpocetine on $I_{K(Ca)}$ in cells dialyzed with a high concentration of EGTA. The experiments were conducted in recording pipettes filled with 10 mM EGTA. (A) Superimposed current traces obtained in the absence and presence of vinpocetine (10 μM). The cell was held at 0 mV and voltage pulses from −30 to +70 mV in 20-mV increments were applied. (B) Averaged current–voltage relationships of $I_{K(Ca)}$ in the absence (■) and presence (□) of vinpocetine (10 μM). Current amplitudes were measured at the end of each voltage step. Each point represents the mean ± SEM (N = 5–8).

amplitude of $I_{K(Ca)}$ in these cells and that neither 8-bromo cyclic GMP nor YC-1 further increased the vinpocetine-stimulated $I_{K(Ca)}$.

3.3. Effects of vinpocetine on BK_{Ca} channels in cell-attached patches

In an attempt to determine how vinpocetine acts to affect $I_{K(Ca)}$, the effects of vinpocetine on BK_{Ca} channels were also assessed in the cell-attached and inside-out configurations. In the cell-attached configuration, under symmetrical K^+ (145 mM) conditions, the activity of BK_{Ca} channels can be observed as shown previously [19]. Because the potential was set constant at a certain level and no voltage pulses were applied, voltage-gated Ca^{2+} or K^+ channels would be inactivated rapidly. In these circumstances, the activity of BK_{Ca} channels could not be contaminated. The channel activity seen in these experiments is thus referred to as BK_{Ca} channels, which can be evoked by intracellular Ca^{2+} at a depolarized potential. When vinpocetine was added to

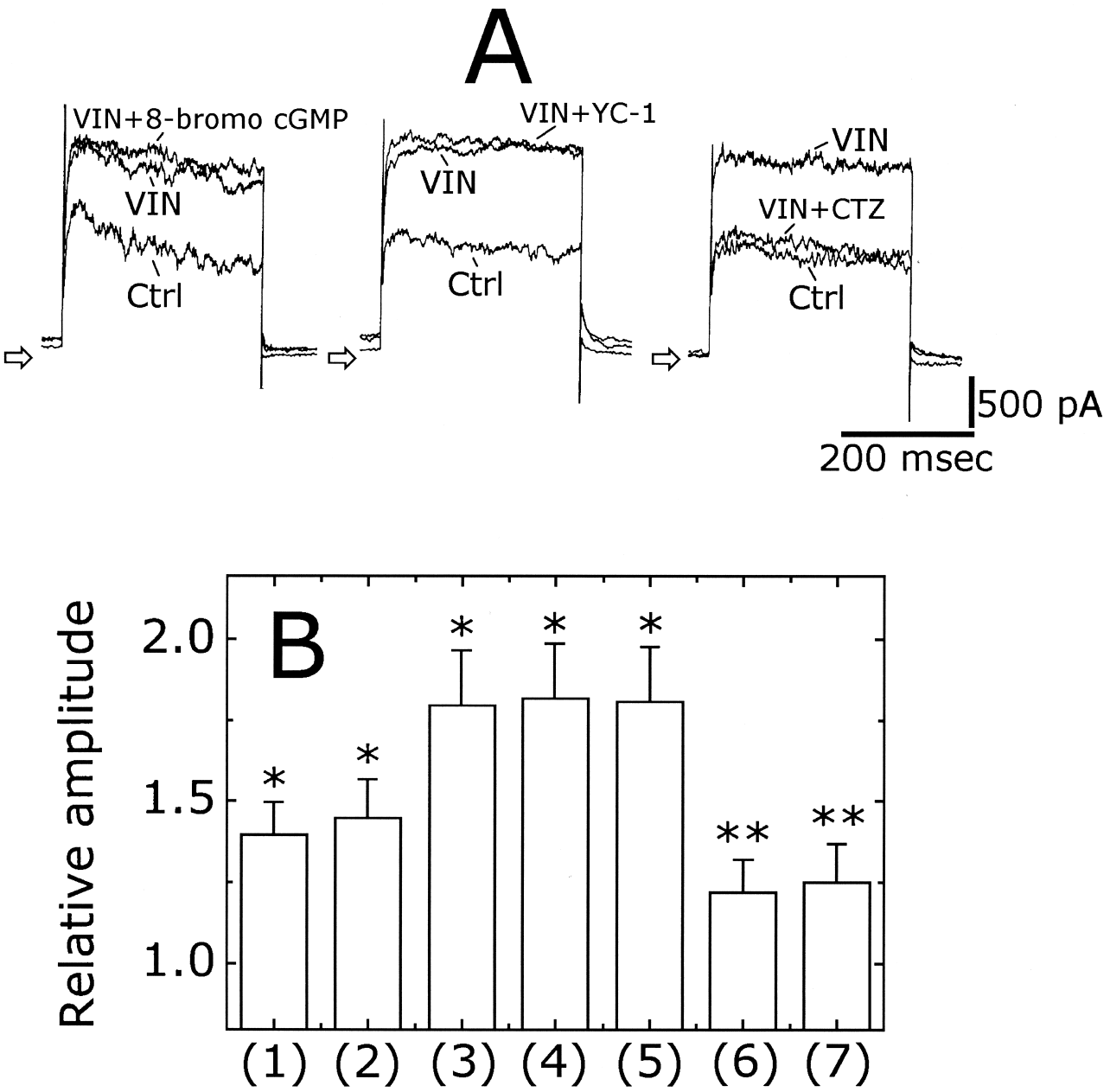
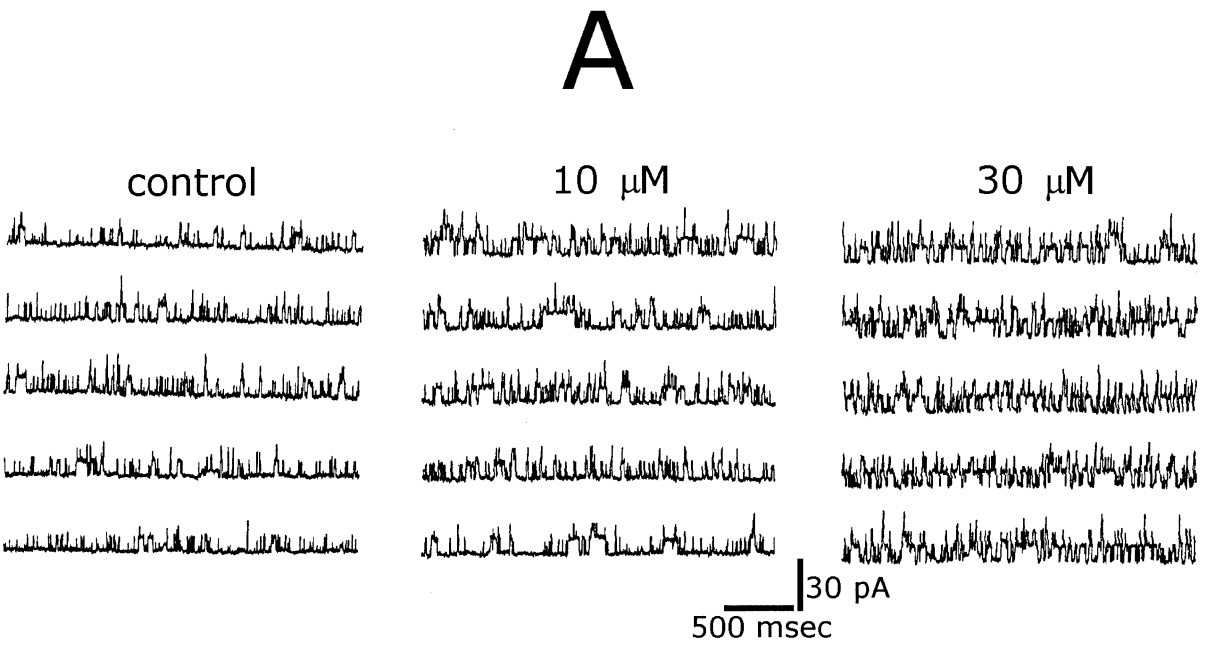


Fig. 3. Effect of vinpocetine on the amplitude of $I_{K(Ca)}$ in the absence and presence of 8-bromo cyclic GMP or YC-1. Each cell was depolarized from 0 to +70 mV with a duration of 300 msec. (A) Original current traces showing the effect of vinpocetine (VIN: 10 μM) with and without addition of 8-bromo cyclic GMP (8-bromo cGMP: 100 μM), YC-1 (10 μM), or clotrimazole (CTZ: 10 μM) on $I_{K(Ca)}$. Ctrl: control. Open arrows indicate 0 current level. (B) Summary of data depicting the effect of 8-bromo cyclic GMP or YC-1 on $I_{K(Ca)}$ and the effects of vinpocetine in the absence and presence of 8-bromo cyclic GMP (100 μM), YC-1 (10 μM), clotrimazole (10 μM), or iberiotoxin (200 nM). Current amplitudes were measured at the end of the voltage pulses. The amplitude of $I_{K(Ca)}$ in the control was considered to be 1.0, and the relative amplitude of $I_{K(Ca)}$ obtained in the presence of each agent was then plotted: (1) 8-bromo cyclic GMP (100 μM); (2) YC-1 (10 μM); (3) vinpocetine (10 μM); (4) vinpocetine (10 μM) plus 8-bromo cyclic GMP (100 μM); (5) vinpocetine (10 μM) plus YC-1 (10 μM); (6) vinpocetine (10 μM) plus clotrimazole (10 μM); and (7) vinpocetine (10 μM) plus iberiotoxin (200 nM). Each point represents the mean ± SEM (N = 7–10). *, significant difference from control group ($P < 0.05$). **, significant difference between vinpocetine-alone group and vinpocetine-plus-clotrimazole or vinpoceteine-plus-iberiotoxin group ($P < .05$).

the bath solution, the frequency of channel openings was greatly increased (Fig. 4). The opening probability of BK_{Ca} channels at the level of +80 mV in the control was 0.23 ± 0.08 (N = 7). One minute after the addition of 10 and 30 μM vinpocetine to the bath, the opening probability was significantly increased to 0.61 ± 0.12 and 0.88 ± 0.15 (N = 7), respectively. Moreover, one minute after subsequent application of EGTA (1 mM), the vinpocetine-induced channel activity was significantly reduced to 0.41 ± 0.11 ($P < 0.05$, N = 5). These results indicate that vinpocetine induced the activity of BK_{Ca} channels and that this vinpocetine-induced channel activity was atten-



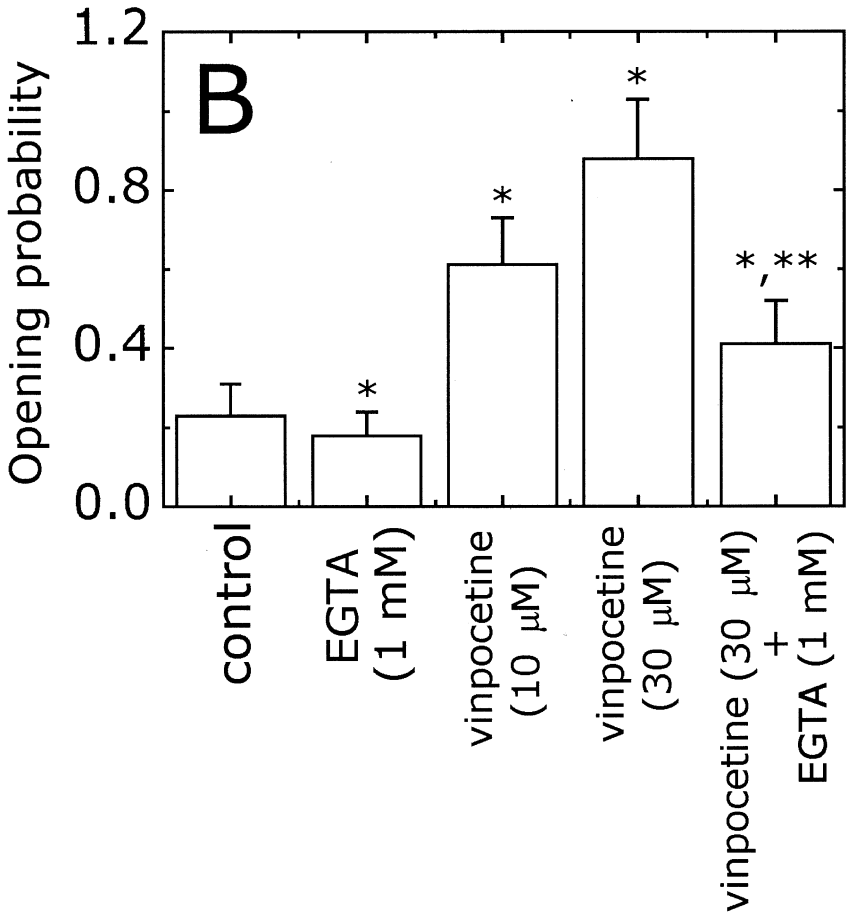


Fig. 4. Effect of vinpocetine on the activity of BK_{Ca} channels in cell-attached patches. GH_3 cells were bathed in high K^+ solution containing 1.8 mM $CaCl_2$. The cell was held at +80 mV and the original current trace was obtained in control and 1 min after application of 10 and 30 μM vinpocetine into the bath (A). Channel openings are shown as an upward deflection. (B) Summary of data showing the effect of vinpocetine on BK_{Ca} channels and the inhibitory effect of 1 mM EGTA on vinpocetine-induced channel activity. Each point represents the mean ± SEM (N = 5–7). *, significant difference from control group ($P < .05$). **, significant difference between vinpocetine-alone group and vinpocetine-plus-EGTA group ($P < .05$).

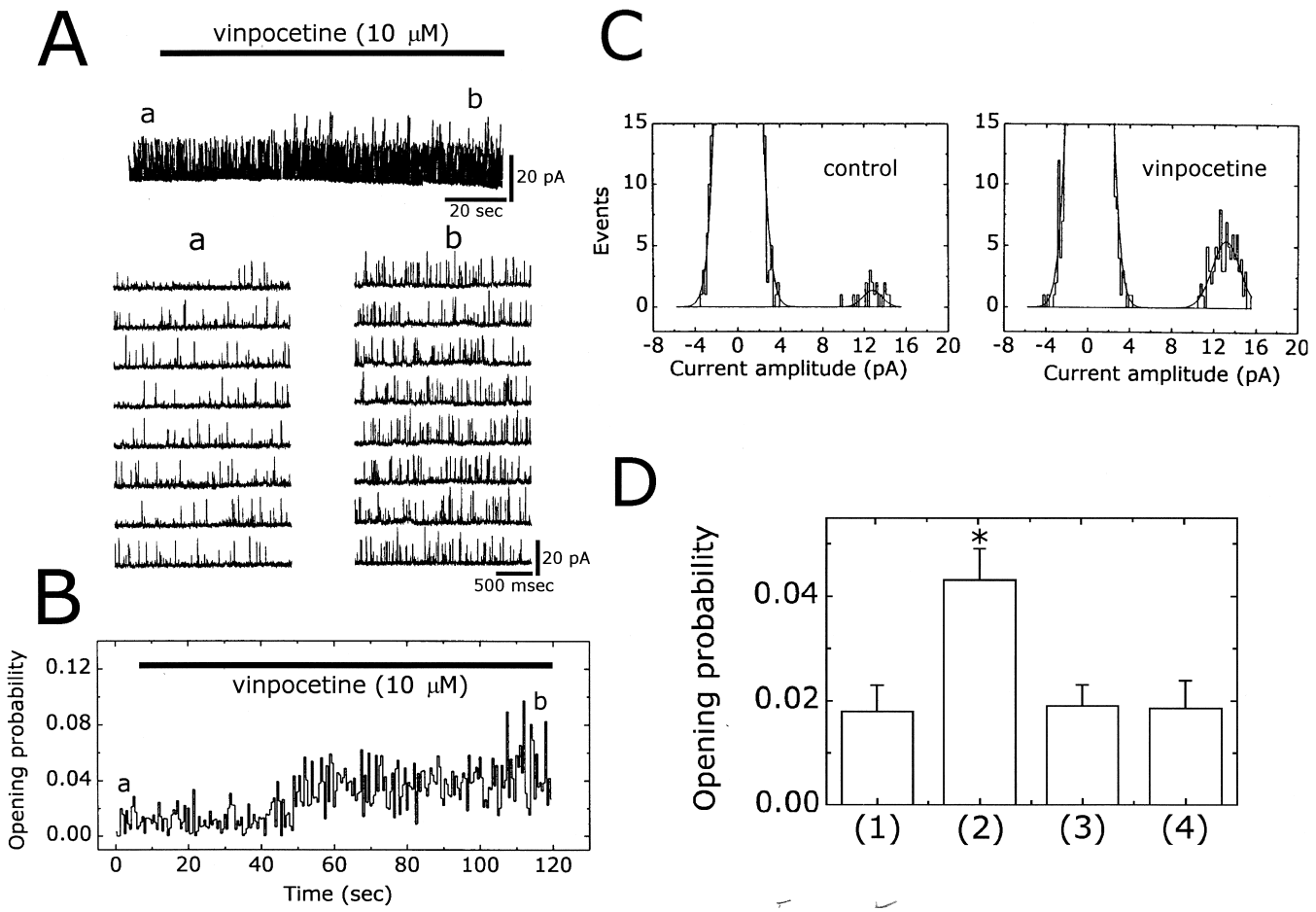


Fig. 5. The effect of vinpocetine on the activity of BK_{Ca} channels recorded from excised inside-out patches. Single-channel currents were recorded in the presence of a symmetrical K^+ concentration (145 mM). The bath solution contained 0.1 μM Ca^{2+}. Membrane potential was held at +80 mV. (A) (Top) Original current trace showing the change in the activity of BK_{Ca} channels after the addition of vinpocetine (10 μM). Upward deflections are the opening events of the channel. (Bottom) Current traces obtained in expanded time scale corresponding to those labeled a and b shown in the top and in (B). (B) Opening probability of BK_{Ca} channels shown in (A) plotted against time of recording. Bin width, 0.5 sec. Horizontal bar indicates the application of vinpocetine (10 μM) to the bath. (C) Amplitude histograms in the absence and presence of vinpocetine (10 μM). All data points shown in the amplitude histograms were fitted by one or more Gaussian distributions using the method of maximum-likelihood. The closed state corresponds to the peak at 0 pA. (D) Summary of data showing the effect of vinpocetine, 8-bromo cyclic GMP, and YC-1 on BK_{Ca} channel activity. In each experiment, the inside-out configuration was performed, holding potential was set at +80 mV, and bath medium contained 0.1 μM Ca^{2+}. Each compound was applied into bath medium. (1) Control; (2) vinpocetine (10 μM) (3) 8-bromo cyclic GMP (100 μM) (4) YC-1 (10 μM). Each point represents the mean ± SEM (N = 4–7). *, significant difference from control group ($P < .05$).

uated by the reduction of extracellular Ca^{2+} with EGTA (1 mM).

3.4. Effects of vinpocetine on BK_{Ca} channels in inside-out patches

Although the magnitude of vinpocetine-stimulated $I_{K(Ca)}$ was diminished, vinpocetine still increased the amplitude of $I_{K(Ca)}$ when the patch pipettes were filled with a high concentration (10 mM) of EGTA. In the next series of experiments, we thus tested the hypothesis that vinpocetine may directly increase the activity of BK_{Ca} channels. As shown in Fig. 5, in an inside-out configuration, when vinpocetine (10 μM) was applied to the bath containing 0.1 μM Ca^{2+}, channel activity was enhanced. The opening probability of BK_{Ca} channels in control at the level of +80 mV was 0.018 ± 0.005 (N = 10). One minute after the application of vinpocetine to the bath, the channel activity was increased significantly to 0.043 ± 0.006 (N = 10). In contrast, when detached membrane patches were intracellularly exposed to 8-bromo cyclic GMP (100 μM) or YC-1 (10 μM), no significant changes in the activity of BK_{Ca} channels were observed (Fig. 5D). Thus, vinpocetine can effectively increase the opening probability of BK_{Ca} channels in GH_3 cells.

3.5. Effect of vinpocetine on the activation curve of BK_{Ca} channels

Fig. 6A shows the activation curve of BK_{Ca} channels in the absence and presence of vinpocetine (10 μM). In these

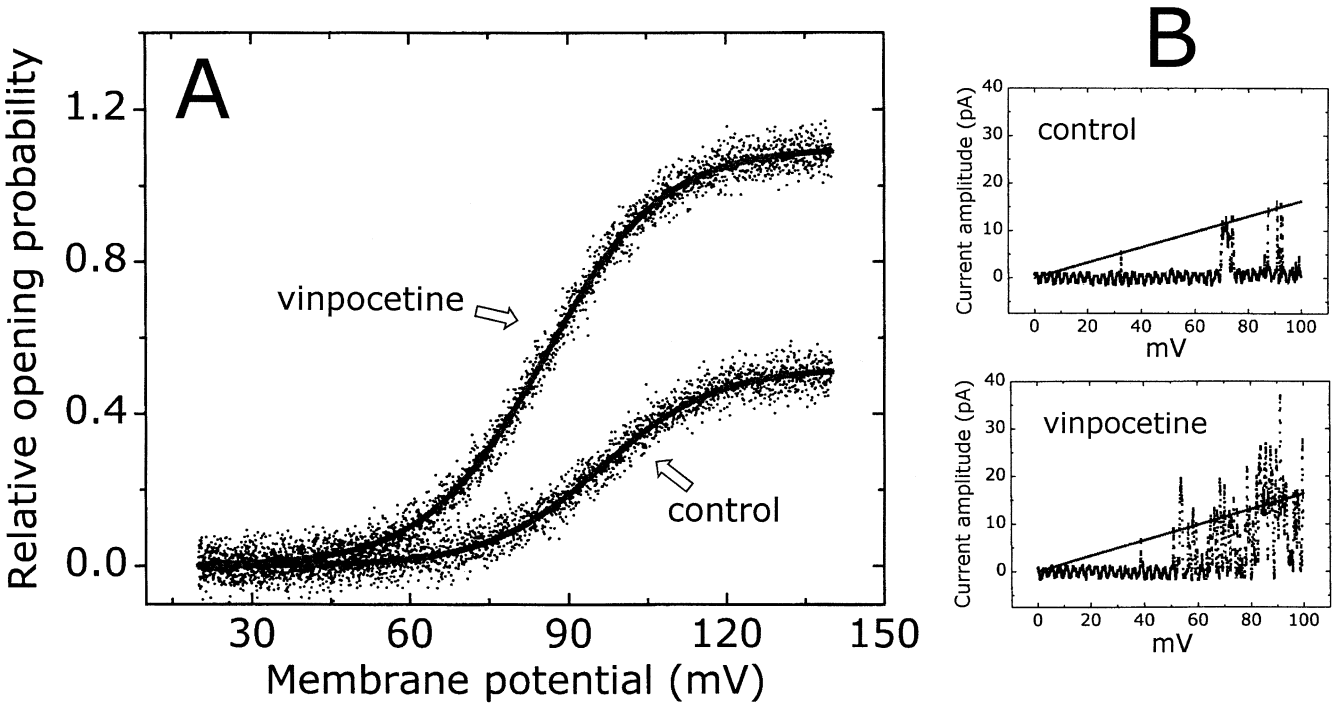


Fig. 6. The voltage-dependent effects of vinpocetine on BK_{Ca} channels in GH_3 cells. The experiments were conducted with a symmetrical K^+ concentration. Under the inside-out configuration, holding potential was +60 mV and bath medium contained 0.1 μM Ca^{2+}. (A) The effect of vinpocetine on the activation curve of BK_{Ca} channels. The activation curves were obtained by means of ramp pulses that ranged from +20 to +140 mV with a duration of 1 sec. Smooth lines show a Boltzmann fit of the data yielding a value for half-maximal activation (i.e. a) of 96 mV for control and 85 mV when the membrane patch was exposed to vinpocetine (10 μM). (B) Lack of effect of vinpocetine on the single-channel conductance of BK_{Ca} channels. Under the symmetrical K^+ condition, holding potential was +60 mV in an inside-out configuration and bath solution conatined 0.1 μM Ca^{2+}. Voltage ramp pulses from 0 to +100 mV with a duration of 1 sec were used to measure single-channel conductance. The straight lines with a reversal potential of 0 mV represent the I-V relationships of BK_{Ca} channels in the absence (upper part) and presence (lower part) of vinpoceetine (10 μM).

experiments, the activation curves of BK_{Ca} channels were obtained with the aid of voltage ramp protocols. The ramp pulses were delivered at voltages from +20 to +140 mV with a duration of 1 sec. The plots of opening probability of BK_{Ca} channels as a function of membrane potential were constructed and fitted with a Boltzmann function as described in Materials and Methods. In control, n = 0.52 ± 0.04, a = 96.2 ± 1.6 mV, and b = 10.9 ± 0.4 mV (N = 7), whereas in the presence of vinpocetine (10 μM), n = 1.12 ± 0.04, a = 85.4 ± 1.3 mV and b = 11.2 ± 0.4 mV (N = 7). Thus, the presence of vinpocetine (10 μM) not only caused a 2-fold increase in the maximal opening probability of BK_{Ca} channels, but also significantly shifted the activation curve to a less positive membrane potential by approximately 10 mV. In contrast, our results revealed no significant difference in the slope (i.e. b) of the activation curve between the absence and presence of vinpocetine. Thus, vinpocetine stimulated BK_{Ca} channel activity in a voltage-dependent fashion in GH_3 cells.

3.6. Lack of effect of vinpocetine on single-channel conductance of BK_{Ca} channels

We examined whether vinpocetine affects the single-channel conductance of BK_{Ca} channels. To construct plots of current amplitude as a function of membrane potential, voltage ramp pulses from 0 to +100 mV with a duration of 1 sec were applied at a rate of 0.1 Hz. Fig. 6B illustrates I-V (current-voltage) relationships of BK_{Ca} channels in the absence and presence of vinpocetine (10 μM). The single-channel conductance of BK_{Ca} channels calculated from the linear I-V relationship in control (i.e. in the absence of vinpocetine) was 162 ± 5 pS (N = 12) with a reversal potential of 0 ± 3 mV (N = 12). The value of unitary conductance for these channels was found to be similar to that reported previously [19,20], but not significantly different from that (163 ± 5 pS, N = 8) measured when excised membrane patches were exposed to vinpocetine (10 μM). These data showed that the presence of vinpocetine produced no significant change in the single-channel conductance of BK_{Ca} channels, although it did enhance the channel activity in these cells.

3.7. Effect of vinpocetine on the kinetic behavior of BK_{Ca} channels

The effect of vinpocetine on the kinetic behavior of BK_{Ca} channels was also examined and analyzed. As shown in Fig. 7A, in excised membrane patches of control cells (i.e. in the absence of vinpocetine), the open-time histogram

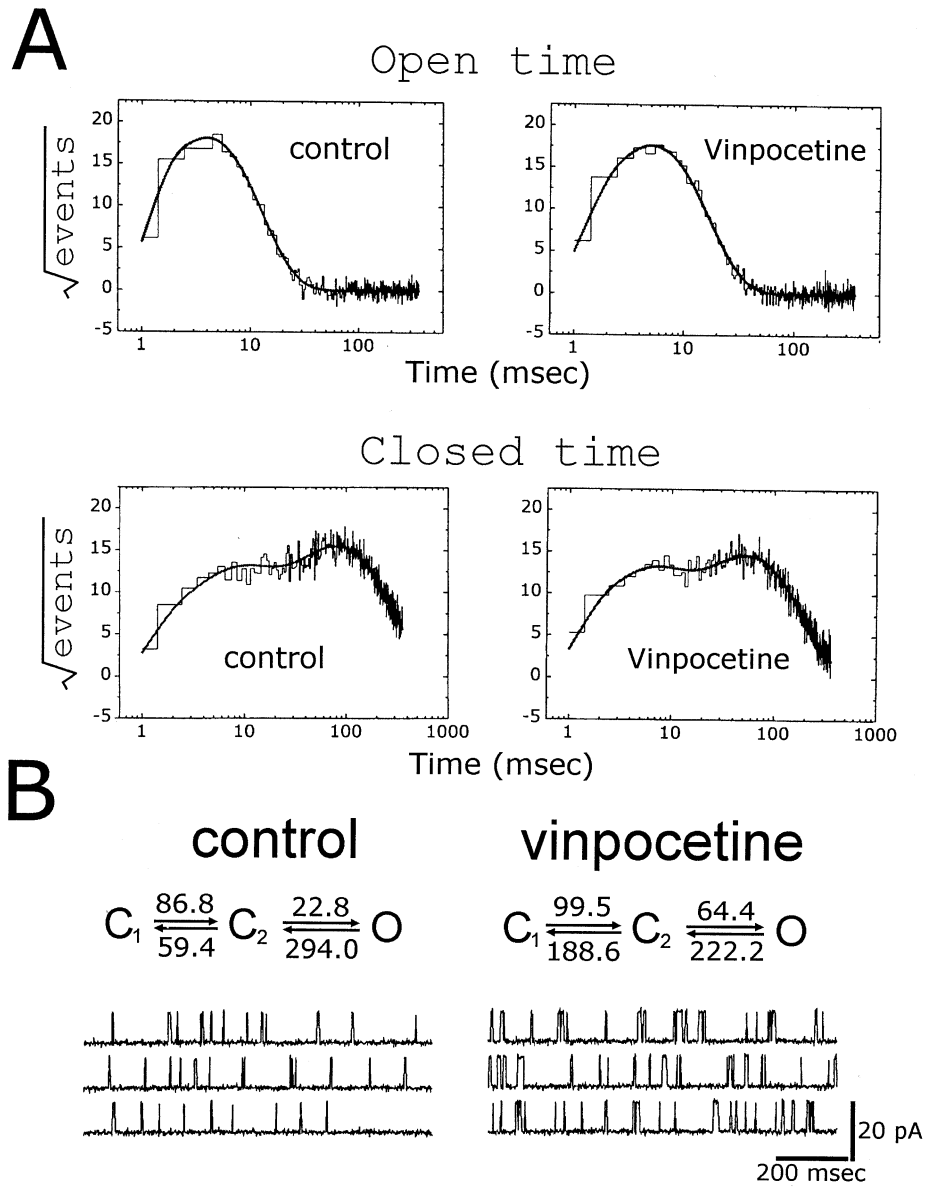


Fig. 7. Effect of vinpocetine on kinetic properties of BK_{Ca} channels. (A) The mean open- (upper panels) and closed-time (lower panels) histograms of BK_{Ca} channels in the absence (left) and presence (right) of vinpocetine in GH_3 cells. Under a symmetrical K^+ concentration in which bath medium contained 0.1 μM Ca^{2+}, holding potential was +80 mV and the inside-out configuration was performed. In control, the open-time histogram was fitted by a single-exponential function with a mean open time of 3.4 msec, whereas the closed-time histogram was fitted by a sum of two-exponential function with a mean closed time of 6.4 and 79.1 msec. The open- and closed-time histograms after the presence of vinpocetine (10 μM) are shown on the right. The mean open time was increased to 4.7 msec, whereas the mean closed time was decreased to 3.1 and 52.2 msec. Notably, abscissa and ordinate show the logarithm of open or closed time (msec) and the square root of the number of evens ($n^{1/2}$), respectively. In control, data were obtained from the measurement of 873 channel openings with a total recorded time of 2 min, whereas in the presence of vinpocetine, data were measured from 798 channel openings with a total recorded time of 30 sec. (B) Simulated unitary currents in the absence (left) and presence (right) of vinpocetine (10 μM). The kinetic models that can account for the observed data are shown in the upper part of each simulated current. Each horizontal arrow pointing to the left represents bindings of vinpocetine or closing of channel, whereas each arrow pointing to the right represents dissociation of vinpocetine or opening of channel. Units are $\mu M^{-1}sec^{-1}$ or sec^{-1}.

of BK_{Ca} channels at the level of +80 mV could be fitted by a single-exponential curve with a mean open time of 3.4 ± 0.3 msec (N = 6). The closed-time histogram in control cells was fitted by a two-exponential curve with a mean closed time of 6.4 ± 1.2 and 79.1 ± 4.5 msec (N = 6). The presence of vinpocetine (10 μM) was found to increase the lifetime of the open state to 4.7 ± 0.3 msec (N = 6), while it decreased the mean closed time to 3.1 ± 0.7 and 52.2 ± 2.2 msec (N = 6). Thus, the activation of these channels by vinpocetine could be associated with an increase in open time and a decrease in closed time.

In order to characterize the effect of vinpocetine on the mean lifetimes of open or closed states, channel currents were also idealized and modeled by using the kinetic scheme described in Materials and Methods. This gating scheme consists of one open and two closed states. Transi-

tion rates between states were derived from the maximum-likelihood estimation [25]. Fig. 7B shows examples of simulated single-channel data generated using the transition rates obtained for BK_{Ca} channels activated in the absence and presence of vinpocetine. The presence of vinpocetine (10 μM) not only produced an increase in the equilibrium dissociation constant (K value) from 0.68 ± 0.03 to 1.91 ± 0.03 (N = 5), but also increased the equilibrium gating constant (L value) from 0.08 ± 0.01 to 0.29 ± 0.02 (N = 5). These results indicate that vinpocetine can affect both the dissociation and gating rate constants.

3.8. Lack of effect of vinpocetine on $I_{Ca,L}$ in GH_3 cells

We also examined whether vinpocetine can cause any effect on $I_{Ca,L}$ in these cells. These experiments were conducted with Cs^+-containing pipette solution. As shown in Fig. 8, the cell was held at −50 mV, and depolarizing pulses (300 msec in duration) to 0 mV were delivered at 0.1 Hz. The presence of vinpocetine (10 μM) caused no significant effect on the amplitude of $I_{Ca,L}$. However, further addition of nifedipine (1 μM) significantly suppressed its amplitude, although there was no significant change in the current–voltage relationship of $I_{Ca,L}$ between the presence and absence of nifedipine (1 μM). These results indicate that vinpocetine has no effect on $I_{Ca,L}$ in GH_3 cells and that vinpocetine-stimulated BK_{Ca} channel activity is unlikely to be associated with the amplitude of $I_{Ca,L}$.

3.9. Effect of vinpocetine on membrane potential in GH_3 cells

The next series of experiments was designed to test the effect of vinpocetine on the change in membrane potential in GH_3 cells. GH_3 cells, bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$, had a resting membrane potential of −45 ± 6 mV (N = 24) under the current-clamp configuration. The typical effects of vinpocetine on membrane potentials in these cells are illustrated in Fig. 9. The presence of vinpocetine (10 μM) significantly decreased the firing frequency of spontaneous action potentials from 0.67 ± 0.08 to 0.17 ± 0.04 Hz (N = 7). The resting membrane potential was significantly hyperpolarized to −51 ± 7 mV from a control value of −46 ± 5 mV (N = 7). When vinpocetine was washed out, resting potential was returned to the control level. Furthermore, the increased firing frequency of action potentials caused by TRH (10 μM) was decreased by the subsequent application of vinpocetine. When cells were exposed to TRH (10 μM), the repetitive firing of action potentials was increased to 1.38 ± 0.14 Hz from a control value of 0.72 ± 0.13 Hz (N = 6). The application of vinpocetine (10 μM) in the continued presence of TRH significantly decreased the firing frequency to 0.62 ± 0.11 (N = 6). Thus, it is clear that vinpocetine can regulate the firing of action-potentials in GH_3 cells.

3.10. Stimulatory effect of vinpocetine on BK_{Ca} channels in pheochromocytoma PC12 cells

In the final series of experiments, we examined the effect of vinpocetine in pheochromocytoma PC12 cells to determine whether vinpocetine can interact with the BK_{Ca} channel in other types of neuroendocrine cells. As shown in Fig. 10, in the inside-out configuration with symmetrical K^+ concentration, when the potential was constantly held at +60 mV and the bath medium contained 0.1 μM Ca^{2+}, the activity of non-inactivating components of BK_{Ca} channels in these cells was observed [20,28]. The intracellular application of vinpocetine (10 μM) caused a rapid increase in the channel activity from 0.043 ± 0.012 to 0.131 ± 0.013 (N = 5). After vinpocetine was removed, the activity of BK_{Ca} channels almost returned to the control level. Vinpocetine-induced channel activity was reversed by penitrem A (1 μM), a known blocker of BK_{Ca} channels [29]. The further application of penitrem A (1 μM) into the bath significantly decreased the channel activity to 0.041 ± 0.011 (N = 4). The amplitude of unitary currents between the absence and presence of vinpocetine did not differ significantly (11.6 ± 1.2 pA [N = 5] vs 11.3 ± 1.8 pA [N = 5]). The effect of vinpocetine on BK_{Ca} channels at various membrane potentials was also examined. Similar to the results seen in GH_3 cells, there was no significant difference in single-channel conductance between the absence and presence of vinpocetine (10 μM) (193 ± 12 pS [N = 6] vs 195 ± 14 pS [N = 5]) (Fig. 10C). Thus, the results indicate that vinpocetine is capable of stimulating non-inactivating BK_{Ca} channels present in PC12 cells and that the ability of vinpocetine to activate the channels can be eliminated with the addition of penitrem A.

4. Discussion

The major findings of the study are as follows. First, in pituitary GH_3 cells, vinpocetine can increase the amplitude of $I_{K(Ca)}$ in a concentration-dependent manner. Second, vinpocetine can stimulate the activity of BK_{Ca} channels in both excised and cell-attached membrane patches. Third, vinpocetine enhances channel activity in a voltage-dependent manner, but does not change single-channel conductance. Fourth, vinpocetine-induced channel activity is related to an increase in time constant in the open state and a decrease in time constant in the closed state. Fifth, vinpocetine decreases the repetitive firing of action potentials in GH_3 cells. Sixth, vinpocetine can also enhance the activity of BK_{Ca} channels in rat pheochromocytoma PC12 cells. The stimulatory effect of vinpocetine on these channels will lead to a decrease in hormonal secretion in these cells if similar results occur in neurons or neuroendocrine cells *in vivo*.

The EC_{50} value for the vinpocetine-induced stimulation of $I_{K(Ca)}$ observed in our study was 4 μM. This value is similar to those required to reduce the veratridine-induced rise in

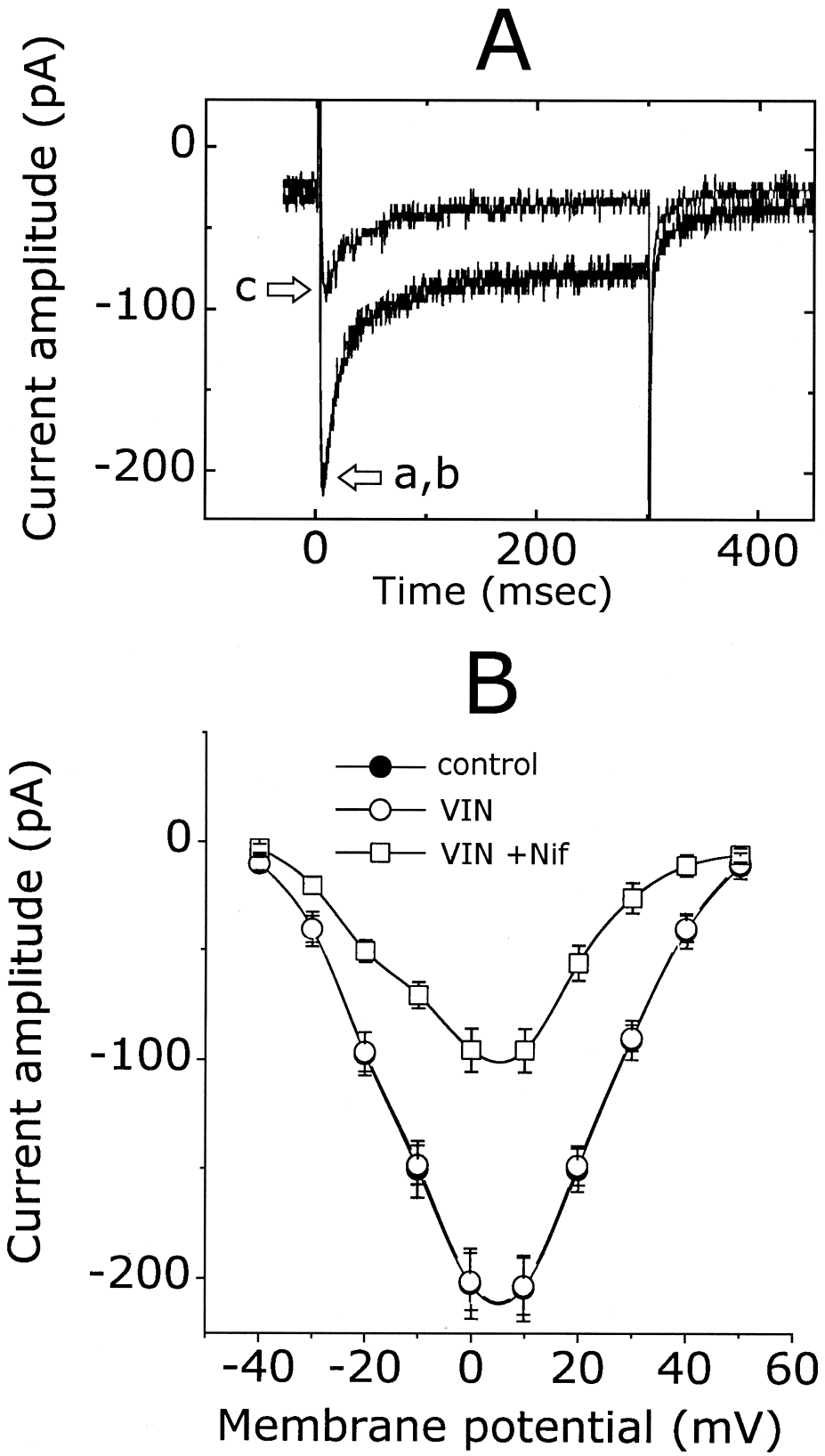


Fig. 8. Lack of effect of vinpocetine on $I_{Ca,L}$ in GH_3 cells. The patch pipette was filled with Cs^+-containing solution, and cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$, tetrodotoxin (1 μM), and tetracethylammonium chloride (10 mM). (A) Original current traces obtained when the cell was depolarized from −50 to 0 mV. Labeled a is control, labeled b was obtained in the presence of vinpocetine (10 μM), and labeled c was after the addition of nifedipine (1 μM), but still in the presence of vinpocetine (10 μM). (B) Averaged current–voltage relationships of $I_{Ca,L}$ in control (●), and during exposure to 10 μM vinpocetine (○) and 10 μM vincopetine plus 1 μM nifedipine (□) (mean ± SEM; N = 4–7 for each point).

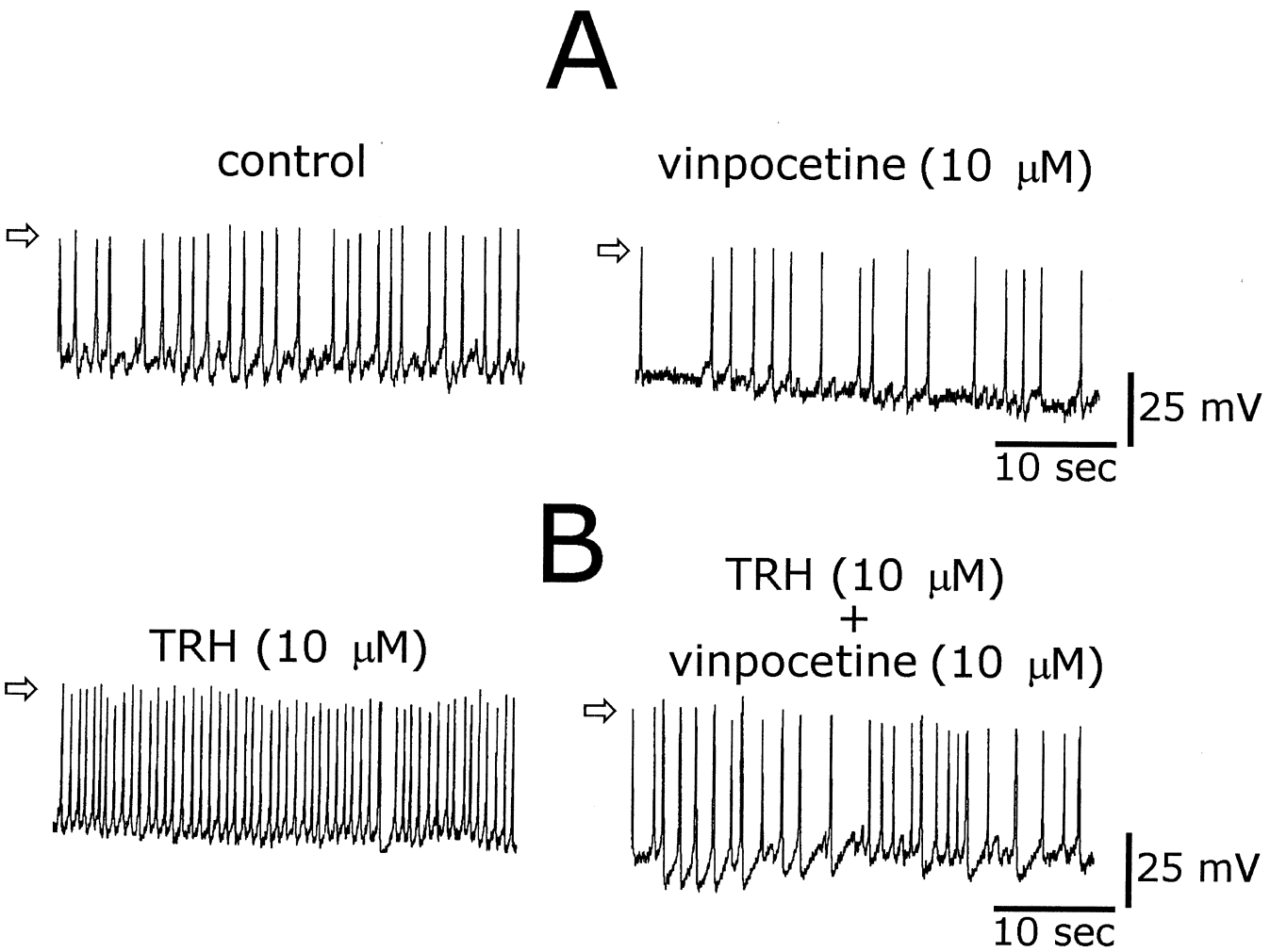


Fig. 9. Effect of vinpocetine on the firing of action potentials in GH_3 cells. Cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$ and 1 μM tetrodotoxin. (A) The original potential trace showing the effect of vinpocetine (10 μM) on spontaneous action potentials of GH_3 cells. The potential trace shown in the left panel is control, white that in the right panel was obtained 2 min after addition of vinpocetine (10 μM). (B) The original potential trace showing the inhibitory effect of vinpocetine on the TRH-induced increase in the firing of action potentials. The potential trace shown in the left panel was recorded in the presence of TRH (10 μM), and that in the right panel was after the addition of vinpocetine (10 μM), but still in the presence of TRH (10 μM). Open arrows indicate 0 mV potential.

intracellular Na^+ [12], but lower than that needed to suppress voltage-dependent Na^+ channels [1,3,11]. However, vinpocetine was found to be potent in inhibiting cell death induced by veratridine with an IC_{50} value of about 0.5 μM. Therefore, it remains to be clarified whether the stimulation of $I_{K(Ca)}$ caused by vinpocetine may contribute to its neuroprotective action.

The present results appear to conflict with a previous report demonstrating the inhibitory effect of vinpocetine on voltage-dependent K^+ current in snail neurons [13]. In our study, we found no significant effect of vinpocetine (10 μM) on voltage-dependent K^+ current in GH_3 cells (data not shown). Furthermore, vinpocetine decreased the firing of spontaneous action potentials and did not prolong the action potential duration (data not shown), but did hyperpolarize the cells. These results suggest that inhibition of the voltage-dependent K^+ current by vinpocetine seems to be minor in GH_3 cells. However, it was found in both GH_3 and PC12 cells that the presence of vinpocetine increased the opening probability of BK_{Ca} channels, but did not affect their single-channel conductance. Vinpocetine-induced channel activity is the consequence of an increase in mean open time and a decrease in mean closed time. Vinpocetine also shifted the activation curve of BK_{Ca} channels to less positive potential by approximately 10 mV in GH_3 cells. Taken together, when membrane potential is not controlled as in *in vitro* patch-clamp studies, this increase in K^+ channel activity caused by vinpocetine may hyperpolarize the membrane and inactivate voltage-gated Ca^{2+} or Na^+ channels, thereby causing a reduction in the firing of action potentials [17,30].

Previous reports have demonstrated that the activity of BK_{Ca} channels could be regulated by intracellular cyclic GMP in pituitary tumor cells [16] and mesangial cells [31].

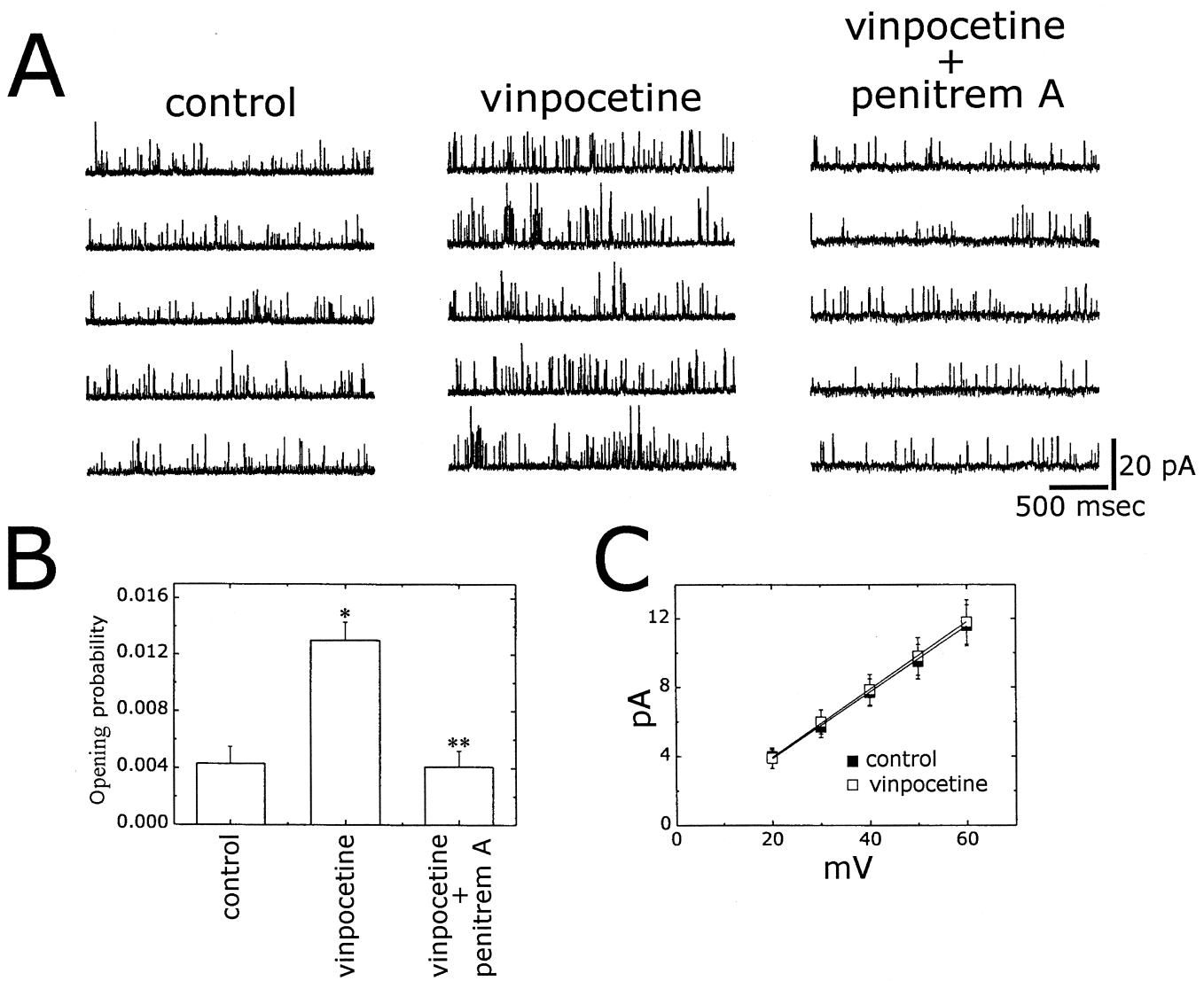


Fig. 10. Stimulatory effect of vinpocetine on the activity of BK_{Ca} channels in pheochromocytoma PC12 cells. Original current traces (A) and the bar graph (B) show the effect of vinpocetine (10 μM) and penitrem A (1 μM) on the activity of BK_{Ca} channels. Upward deflections shown in panel A are due to channel opening. The experiment was conducted in excised membrane patches. Holding potential was +60 mV and bath soliution contained 0.1 μM Ca^{2+}. There was an increase in the probability of the channels being open after the addition of vinpocetine (10 μM), but a decrease with the subsequent addition of penitrem A (1 μM). Each point shown in B represents the mean ± SEM (N = 4–6). *, significant difference from control group ($P < .05$). **, significant difference from vinpocetine-plus-penitrem A group ($P < .05$). (C) Current-voltage relationships of BK_{Ca} channels in the absence (■) and presence of (□) of vinpocetine (10 μM). Each point represents the mean ± SEM (N = 5–7). Note that the single-channel conductances in the absence and presence of vinpocetine are nearly identical.

The present study provides three additional lines of evidence to suggest that the increase in the amplitude of $I_{K(Ca)}$ caused by vinpocetine is in part dependent on the availability of intracellular Ca^{2+} concentrations. First, in the whole-cell configuration, when patch pipettes were filled with a higher concentration of EGTA (10 mM) in which intracellular Ca^{2+} concentrations were strongly buffered, the stimulatory effect of $I_{K(Ca)}$ caused by vinpocetine still existed, but was diminished. Second, further application of 8-bromo cyclic GMP or YC-1 in the continued presence of vinpocetine did not produce additional increases in the amplitude of $I_{K(Ca)}$. Third, in single-channel experiments with the cell-attached mode, the application of vinpocetine into the bath significantly increased the activity of BK_{Ca} channels. These observations led us to speculate that the observed effects of vinpocetine, 8-bromo cyclic GMP, and YC-1 on the stimulation of $I_{K(Ca)}$ in GH_3 cells may involve intracellular signal transduction via a similar mechanism [15,16, 32].

The reason why vinpocetine, 8-bromo cyclic GMP, or YC-1 can activate $I_{K(Ca)}$ in GH_3 cells is currently unclear. However, it might be associated with the elevation in intracellular Ca^{2+} via either the influx of extracellular Ca^{2+} into the cells through voltage- or receptor-gated channels or the release of Ca^{2+} from intracellular stores. Several studies have reported that the nitric oxide–cyclic GMP pathway

might involve the regulation of Ca^{2+} entry [33]. This pathway was thought to facilitate Ca^{2+} influx in various cell types, including cone photoreceptors [34], retinal Müller cells [35], neuroblastoma cells [32], and GH_3 cells [15]. In GH_3 cells, a previous report also showed that an increase in intracellular cyclic GMP might facilitate Ca^{2+} influx into cells that had been induced by depleted Ca^{2+} stores [14].

More interestingly, in the experiments with excised membrane patches, vinpocetine, when applied intracellularly, was found, unlike 8-bromo cyclic GMP or YC-1, to stimulate the activity of BK_{Ca} channels. These findings may be interpreted to mean that vinpocetine-induced channel activity is not entirely dependent on intracellular Ca^{2+} that is induced by an elevation in cyclic GMP and that it may interact with some aspects of BK_{Ca} channels, thus leading to an increase in channel activity. This view is supported by the observation that vinpocetine was more potent than 8-bromo cyclic GMP or YC-1 in reducing the frequency of spontaneous action potentials in GH_3 cells (data not shown). However, it is unlikely that the vinpocetine-stimulated increase in whole-cell currents is dependent on the increased availability of intracellular Ca^{2+} primarily resulting from increased influx through voltage-gated Ca^{2+} channels, because vinpocetine had no effect on L-type Ca^{2+} current in GH_3 cells. Although the mechanism of action of vinpocetine has not yet been determined, the fact that it can stimulate BK_{Ca} channel activity in the inside-out patches suggests that vinpocetine either directly modulates the channel or at least interacts with a closely associated structure. Such interaction may induce a conformational change in channel proteins that affects gating properties, resulting in an increase in channel activity. In fact, based on the gating scheme shown in the present study, vinpocetine was also found to affect both the equilibrium dissociation and gating constants of BK_{Ca} channels. Whatever the mechanisms involved, the ability of vinpocetine to activate BK_{Ca} channels in neurons or neuroendocrine cells should be noted with caution in relation to its use as an inhibitor of type 1 cyclic GMP phosphodiesterase [8,9,15,36].

Vinpocetine can modulate the membrane potential of GH_3 cells by a direct stimulation of BK_{Ca} channels and an indirect increase in intracellular Ca^{2+} via the inhibition of cyclic GMP phosphodiesterase. Despite the fact that vinpocetine can suppress voltage-dependent Na^+ or Ca^{2+} channels [1,11,12], the vinpocetine-mediated regulation of the firing action potentials observed in GH_3 cells does not appear to be a consequence of its inhibition of Na^+ channels. There are three possible explanations for this interpretation. First, the repetitive firing of action potentials present in GH_3 cells is insensitive to tetrodotoxin and inhibited by nifedipine, a blocker of Ca^{2+} channels [18,21]. Second, the firing rate in cells preincubated with tetrodotoxin can be reduced by vinpocetine. Third, vinpocetine (10 μM) had no effect on $I_{Ca,L}$ in GH_3 cells.

A previous report at our laboratory has demonstrated the inhibitory effect of clotrimazole on BK_{Ca} channel activity in GH_3 cells [18]. However, a large number of studies have shown that clotrimazole is an inhibitor of Ca^{2+}-activated K^+ channels of intermediate conductance. More importantly, four types of BK_{Ca} channel β-subunits have recently been cloned, and the differential expression of these subunits may alter the sensitivity of BK_{Ca} channels to inhibitors [37,38]. Thus, the effect of iberiotoxin on vinpocetine-stimulated BK_{Ca} channel activity was also examined. The results showed that both clotrimazole and iberiotoxin suppressed the vinpocetine-stimulated activity of BK_{Ca} channels. Nevertheless, it remains to be clarified whether different accessory β-subunits may affect the stimulatory effect of vinpocetine on BK_{Ca} channel activity.

In summary, the data presented here tend to demonstrate that vinpocetine may exert a dual effect on the activity of BK_{Ca} channels. This effect results from an indirect elevation in intracellular Ca^{2+} concentrations that was induced by an increase in intracellular cyclic GMP and a direct stimulation of BK_{Ca} channels that is independent of intracellular Ca^{2+}. The indirect effect appears to involve the cyclic GMP second messenger system, since it can be mimicked by the presence of either 8-bromo cyclic GMP or YC-1. However, the direct effect shown in both GH_3 and PC12 cells may arise from an interaction between vinpocetine and the channel protein(s).

Acknowledgments

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