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Comparison of effects of tetrandrine on ionic channels of isolated rat neurohypophysial terminals and Y1 mouse adrenocortical tumor cells

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ABSTRACT The effects of tetrandrine on the voltage-gated Ca^{2+}, K^+, and Na^+ channels of the isolated rat neurohypophysial nerve terminals and Y1 mouse adrenocortical tumor cells were studied using standard and perforated patch-clamp techniques. Among the inward ionic currents, the T-type Ca^{2+} channel current (I_{Ca}) in the Y1 cell line was strongly inhibited (by 52.9%) by a low concentration (13 μmol·L^{-1}) of externally applied tetrandrine. The L-type I_{Ca} in the isolated nerve terminals was also strongly inhibited (by 54.2%) by externally applied tetrandrine 33 μmol·L^{-1}, whereas the N-type I_{Ca} and Na^+ current were not significantly affected by the same dose of the alkaloid. While it had no effect on the fast, transient K^+ channels, external application of tetrandrine 1 μmol·L^{-1} blocked a slowly-gating, maxi Ca^{2+}-activated K^+ ($K^+_{(Ca)}$) channel in the nerve terminals, decreasing its P_o by 84.4%. Conclusions: (1) The order of the sensitivities of ionic channels to tetrandrine is $K^+_{(Ca)}$>T->L->N-type Ca^{2+}>Na^+; (2) tetrandrine serves as a specific blocker of the slowly-gating $K^+_{(Ca)}$ channel.

KEY WORDS tetrandrine; posterior pituitary; cultured tumor cells; calcium channels; potassium channels; sodium channels; patch-clamp technic; nerve endings; electrophysiology

Tetrandrine, a bis-benzylisoquinoline alkaloid (6,6′,7,12-tetramethoxy-2,2′-dimethylberbaman), has been clinically used as an antihypertensive and anti-arrhythmic agent. It exerted a negative inotropic effect on the myocardium and blocked high K^+-evoked contraction of arterial strips, decreased the amplitude of the Ca^{2+}-mediated action potential, and inhibited the inward Ca^{2+} current in cardiac Purkinje fibers[1-2]. Patch-clamp studies have demonstrated that tetrandrine blocked more than one type of voltage-gated Ca^{2+} channel, as well as a Ca^{2+}-activated K^+ channel[3-5].

Taking advantage of the isolated nerve terminals and cultured Y1 cells, we 1) investigated the effects of tetrandrine on the L-, N-, and T-type Ca^{2+} currents, using perforated patch-clamp technique to avoid the 'rundown' of Ca^{2+} currents usually seen with standard whole-cell recordings; and 2) utilized standard whole-cell and outside-out patch-clamp techniques to observe its actions on the transient outward K^+ and Ca^{2+}-activated K^+ channel currents.

MATERIALS AND METHODS

Preparation of nerve terminals and Y1 cells All peptidergic nerve terminals were freshly isolated from ♂ CD rat neurohypophyses. As previously described[6], a CO_2-anesthetized rat was decapitated, and the pituitary gland was excised. The posterior pituitary was homogenized in a solution containing: sucrose 270 mmol·L^{-1}; Tris-HEPES 10 mmol·L^{-1}; EGTA 10 μmol·L^{-1}, pH=6.8, and then transferred to a 35 mm, 0.1% poly-l-lysine coated Petri dish, which was then mounted on the movable stage of an inverted microscope (Nikon, Tokyo, Japan), upon which the isolated neurohypophysial terminals, with the characteristics of golden color, spherical shape, and lack of nuclei, could be indentified. Terminals were then perfused with a physiological solution containing: NaCl 145; KCl 5; $CaCl_2$ 2.2; $MgCl_2$ 1; glucose 15; Na-HEPES 10 mmol·L^{-1}; pH=7. Y1 mouse adrenocortical tumor cells were obtained from Dr Bernard Schimmer, University of Toronto, Canada. They were grown in Ham's F10 medium supplemented with 15% heat-inactivated horse

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serum, 2.5% heat-inactivated fetal calf serum, penicillin 100 U / streptomycin 100 μg per ml and incubated in 5% CO_2 at 37℃. Cells used for electrophysiology were detached with 0.05% trypsin / EGTA 0.53 mol· L^{-1} 1-3 d beforehand and plated sparsely onto a 35 mm Petri dish in supplemented Ham's F10 without antibiotics. The incubation medium was replaced with unsupplemented Ham's F10, 3-4 h before recording, to provide conditions which did not promote flat attachment of cells to the substrate. The cells in the dish were perfused with the physiological solution as mentioned above. The volume of solution in the dish was kept at 2 ml.

Electrophysiological recording After about 1 h of perfusion, terminals of 6-8 μm and Y1 cells of 10-15 μm in diameter were chosen for patch-clamping. A soft-glass pipette (Drummond Scientific Co, Broomall PA, USA), which had been double pulled (David Kopf 700C, Tujunga CA, USA) and fire-polished on a microforge (Narashige, Kyoto, Japan), was pressed onto the surface of a cell or terminal, and suction was applied until a giga-seal was formed. For recording Ca^{2+} current (I_{Ca}) and Na^+ current (I_{Na}), using the perforated whole-cell patch-clamp method, a modified physiological solution in which Ba^{2+} ($BaCl_2$ 5 mmol· L^{-1}) replaced Ca^{2+} as the channel ion carrier was used to perfuse the terminals or cells. The pipette was filled with a solution containing: Cs-glutamate 100; TEA-Cl 20; $CaCl_2$ 2; $MgCl_2$ 1; HEPES 10 mmol· L^{-1}; amphotericin B 200-240 μg· L^{-1}; pH = 7.4. Because amphotericin B permeabilizes the membrane patch drawn into the pipette, membrane resistance can be reduced that we can record whole-cell I_{Ca} and I_{Na} currents through the patch without 'rundown' caused by dialysis of the cytoplasm[7]. Monovalent cations such as Cs^+ in the pipette solution flow into cells or terminals through the amphotericin B-permeablized membrane to replace K^+ and prevent outward currents through K^+ channels. For recording Ca^{2+}-activated K^+ channel currents ($I_{K(Ca)}$) or fast, transient K^+ current (I_A) using standard outside-out patch or whole-cell configurations, the bath solution was similar to the physiological solution but the concentration of $CaCl_2$ was increased to 10 mmol· L^{-1}. The pipette was filled with a solution contaning: KCl 130; $CaCl_2$ 1.997 and EGTA 2 (giving 10 μmol· L^{-1} free Ca^{2+}); K-HEPES 10; *N*-methyl-*D*-glucamine 20 mmol· L^{-1}; pH = 7.2. Withdrawal of the pipette from the terminal, after achieving the whole-cell configuration by rupturing the membrane, led to formation of an outside-out patch[8]. All the currents were recorded using a List EPC-7 amplifier (List Electronic, Darmstadt, Germany) and filtered at 2 kHz by an 8-pole Bessel filter (Frequency Devices Inc, Haverhill MA, USA). The single channel currents were sampled at 10 kHz and stored on hard disks, and then analysed using pClamp software (Axon Instrument inc, Burlingame CA, USA).

Data analysis Data were expressed as $\bar{x} \pm s$. The *t* test was used to analyze statistical significance of paired or unpaired data. The determination of the channel open probabillity (P_o) was calculated from all-points amplitude histograms, based on the area under each peak fit by the Gaussion distributions.

Drugs Tetrandrine, a product of Jinhua Pharmaceuticals Inc (Jinhua, Zhejiang Province, China), was dissolved in distilled water after acidification with HCl (pH = 3), and then neutralized with NaOH to pH = 6 to obtain a 10 mmol· L^{-1} stock solution. All the other chemicals used were obtained from Sigma Chemical Co (St Louis MO, USA).

RESULTS

Effects of tetrandrine on inward ionic currents Low-voltage-threshold, T-type I_{Ca}[9] was maximally elicited from the Y1 cells by a depolarization from a holding potential (HP) = -90 mV to a step potential (SP) = -20 mV (Fig 1A). External application of tetrandrine 13 μmol· L^{-1} (Fig 1A) led to a significant decrease in the amplitude of T-type I_{Ca} by 52.9% (from 154± 23.5 pA of the control to 72.5± 14.3 pA of the tetrandrine-treated, $n = 3$, $P < 0.05$).

N-type and L-type high-voltage-threshold I_{Ca}[9] have been observed in the neurohypophysial

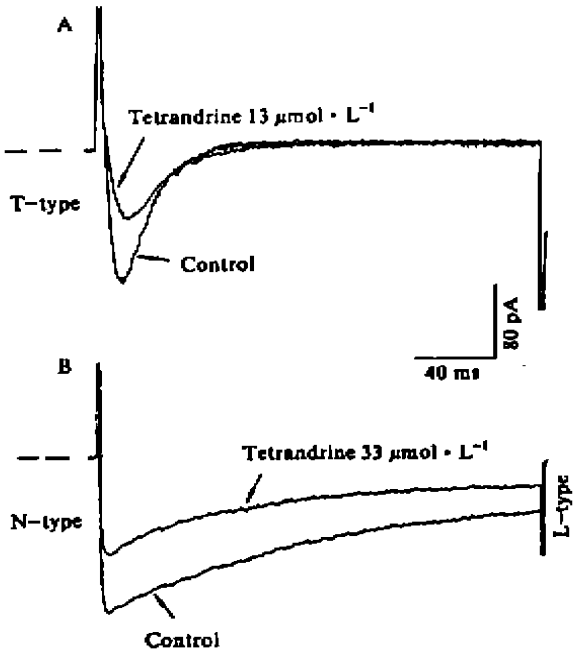

Fig 1. Inhibition of T-type and L-type I_{Ca} by tetrandrine. A. T-type I_{Ca} was recorded from Y1 adrenocortical tumor cell by a depolarization from −90 mV to −20 mV. Tetrandrine 13 μmol · L^{-1} inhibited the current (from experiments M2824c21, M2824c25). B. Both N-type and L-type I_{Ca} were maximally elicited from a neurohypophysial nerve terminal by depolarizing from −90 mV to +10 mV. The amplitude of I_{Ca} at the end of the depolarizing pulse is considered to be that of L-type I_{Ca}, while the difference in amplitude between the L-type I_{Ca} and the I_{Ca} peak is the inactivating N-type I_{Ca}. Tetrandrine 33 μmol · L^{-1} caused about 54% inhibition of the L-type I_{Ca} (from experiments G2721c01, G2727c05). The dashed lines on the left stand for baselines of currents.

nerve terminals[5,10]. Our study, using the perforated whole-cell patch-clamp technique on the terminals, demonstrated that the current-voltage relationship and average amplitudes of both types of I_{Ca} were very similar to those recorded with the standard whole-cell patch-clamp method[10]. The advantage of this method is that the I_{Ca} from these small sized terminals remained sustained without 'rundown' for 1–2 h, enabling us to conduct pharmacological studies on the Ca^{2+} channels.

As shown in Fig 1B, both N- and L-type I_{Ca} were maximally elicited by depolarizations from −90 mV to +10 mV[10]. External administration of tetrandrine 33 μmol · L^{-1} (Fig 1B) led to inhibition of the L-type I_{Ca} by 54.2% (from 48.5± 17.1 pA of the control to 22.2± 10.6 pA of the tetrandrine-treated, $n=4$, $P<0.01$). The N-type I_{Ca} in the terminal was not significantly inhibited by the same concentration of tetrandrine (by only 16.7%, from 110.5± 24.1 pA of control to 92± 23.5 pA, $n=4$, $P>0.05$). In the absence and presence of tetrandrine 33 μmol · L^{-1}, no significant difference in the amplitude of I_{Na} from different nerve terminals was seen (differed by 8.7%, from 624± 186 pA of 15 controls to 678± 281 pA of 11 tetrandrine-treated, $P>0.05$) (Fig 3).

Effects of tetrandrine on outward K^+ channel

Using the standard whole-cell patch-clamp technique, two kinds of outward K^+ channel currents, I_A and $I_{K(Ca)}$, have been previously reported[11,12] from the neurohypophysial nerve terminals. The former could be blocked by 4-aminopyridine, whereas the latter was sensitive to Ba^{2+}. We examined tetrandrine's effects on these two currents, finding that the whole-cell I_A was insensitive to tetrandrine 1 μmol · L^{-1}, but, in contrast, the Ca^{2+}-activated K^+ ($K^+_{(Ca)}$) channel was extraordinarily sensitive to the alkaloid. Compared to the amplitude of I_A in the control (452± 87 pA, $n=5$), the amplitude of I_A in the presence of tetrandrine 1 μmol · L^{-1} (Fig 3) was just slightly different (491± 154, an 8.6% increase, $n=4$, $P>0.05$). The control single $K^+_{(Ca)}$ channel currents, which were elicited by a depolarization from HP=−50 mV to SP=+40 mV using an outside-out patch from a terminal, are slowly-gating, ie, with few transitions between the open and closed states (Fig 2). In sharp contrast to its effect on I_A, tetrandrine 1 μmol · L^{-1} strongly blocked the $K^+_{(Ca)}$ channel (lower panel in Fig 2). The P_o within bursts of $K^+_{(Ca)}$ channel decreased by 84.4% in the presence of tetrandrine 1 μmol · L^{-1} from 0.88± 0.03 to 0.14± 0.01, $n=3$, $P<0.01$). Tetrandrine blocked the channel by producing many more transitions between the open and closed states, ie, a flickery block (Fig 2).

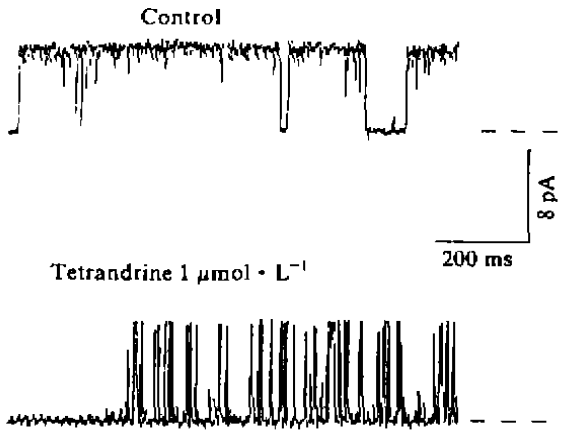

Fig 2. Blocking effect of tetrandrine on $K^+_{(Ca)}$ channel. The upper panel is the control, single $K^+_{(Ca)}$ channel current trace recorded from an outside-out patch of a terminal, which was elicited by a depolarization from −50 mV to +40 mV. Free Ca^{2+} was 10 μmol · L^{-1} in the pipette solution. The lower panel is a trace from the same $K^+_{(Ca)}$ channel in the presence of tetrandrine 1 μmol · L^{-1}. Many strong flickery blocks occurred without any influence on the amplitude of the currents. The dashed lines on the right were the baselines of the currents.

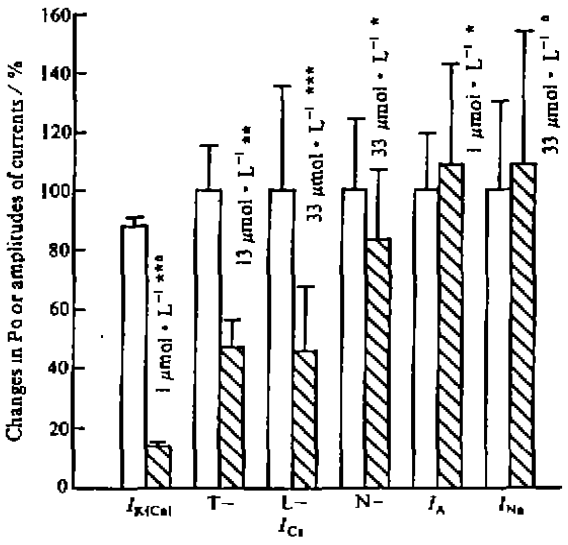

Fig 3. Blocking effects on K^+, Ca^{2+}, and Na^+ channels. Control (blank) and tetrandrine (hatched) were plotted against % changes in P_o of single $K^+_{(Ca)}$ or amplitude of whole-cell currents. $\bar{x} \pm s$. $^*P > 0.05$, $^{**}P < 0.05$, $^{***}P < 0.01$.

Fig 3 summarizes the different blocking effects by tetrandrine on K^+, Ca^{2+}, and Na^+ channels. It is evident that among these channels the $K^+_{(Ca)}$ channel is the most sensitive to tetrandrine; another type of outward K^+ channel, the A-channel, in contrast, is not affected by the same concentration of tetrandrine. Among the inward ionic currents, the T- and L-type Ca^{2+} channel are, respectively, the first and second most sensitive to tetrandrine. The N-type Ca^{2+} currents and Na^+ currents were basically unaffected by a high concentration of tetrandrine (33 μmol · L^{-1}).

DISCUSSION

The present results indicate that tetrandrine blocks $K^+_{(Ca)}$, T-type, and L-type Ca^{2+} channels but that the blocking potencies vary. The order of sensitivities of the ionic channels to tetrandrine are: $K^+_{(Ca)} > T- > L- > N$-type $Ca^{2+} > Na^+$ channels. Since we tested tetrandrine effects on I_A only at 1 μmol · L^{-1}, the question of whether higher concentrations could produce any inhibition on the I_A is still open.

Ca^{2+} channel block The blocking effects on T-type and L-type Ca^{2+} channels by tetrandrine have been previously determined by standard patch-clamp experiments[3-5]. Tetrandrine inhibited T-type I_{Ca} in neuroblastoma cells with an $IC_{50} = 2.5$ μmol · L^{-1} [3]. Tetrandrine 4 μmol · L^{-1} inhibited L-type I_{Ca} in GH_3 anterior pituitary cells by more than 50% without affecting T-type I_{Ca}[4]. Our previous results with the standard patch-clamp technique[5] indicated that the IC_{50} for L-type I_{Ca} by tetrandrine in the neurohypophysial nerve terminals was approximately 10 μmol · L^{-1}. The present data, with the perforated patch-clamp method, agree that tetrandrine blocks both T-type and L-type Ca^{2+} channels. These data, however, also show that the IC_{50} for the T- and L-type I_{Ca} by the alkaloid are approximately 13 and 33 μmol · L^{-1},

respectively, much higher than those reported with typical whole-cell patch-clamp approaches. One possible reason to explain the difference is that the dialysis of the cell or terminal contents using the standard whole-cell patch-clamp method leads to elimination of enzyme machinery and materials with subsequent chemical and physical changes in the channels, making them more sensitive to tetrandrine.

$K^+_{(Ca)}$ channel block Tetrandrine, a promising specific type II $K^+_{(Ca)}$ channel blocker, produced flickery blockade of the terminal $K^+_{(Ca)}$ channel, which is similar to that by tetraethylammonium (TEA). TEA, however, resulted in more frequent flickery blocks of the channel than tetrandrine[12-14], suggesting that residence time for TEA inside the channel is shorter than that for tetrandrine. Other $K^+_{(Ca)}$ channel blockers such as Ba^{2+} and charybdotoxin, block the channel by terminating the burst for seconds[12,14,15], indicating that they reside inside the channel longer than tetrandrine. The fact that tetrandrine had little effects on the $K^+_{(Ca)}$ channel from the cytoplasmic side of the terminal suggests that its binding site in the channel is located around the outer mouth of the channel[5]. Although the relevance of the $K^+_{(Ca)}$ channel block by tetrandrine to its antihypertensive action is not yet clear, this finding will certainly shed light[5] on the mechanisms underlying clinical effects of tetrandrine.

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粉防己碱对分离大鼠神经垂体末梢及 Y1 小鼠肾上腺皮质瘤细胞离子通道作用之比较

R971.9

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摘要 本文采用标准及穿孔膜片钳技术研究了粉防己碱对分离大鼠神经垂体末梢及 Y1 小鼠肾上腺皮质瘤细胞的电压控门的钙道、钾道及钠道的作用. 细胞外的低浓度粉防己碱(13 μmol·L^{-1})可强抑制 Y1 细胞株上的 T 型钙道电流 52.9%. 粉防己碱 33 μmol·L^{-1} 细胞外使用时亦可强抑制分离的神经垂体末梢 L 型钙道电流 54.2%，但对其 N-型钙道电流无明显影响. 1 μmol·L^{-1} 粉防己碱在细胞外可阻断神经垂体末梢的慢控门、大电导、钙激活的钾道，使其开放概率降低 84.4%. 同一剂量的粉防己碱不影响快瞬时的钾道. 本文结论为(1) 离子通道对粉防己碱敏感性的顺序是：钙激活钾道 > T 型钙道 > L 型钙道 > N 型钙道 > 钠道；(2) 粉防己碱可为一慢控门的钙激活钾道的特异性阻断剂.

关键词 粉防己碱；后叶垂体；培养的肿瘤细胞；钙通道；钾通道；钠通道；膜片钳技术；神经末梢；电生理学

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Relation of age to effects of phentolamine and phenylephrine on heart

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ABSTRACT In the present study, the responses of neonatal and adult dog hearts to phenylephrine (PE) and phentolaminie (PA) were investigated in order to determine the influence of age factors. PA resulted in the slowing of the heart rate, the prolongation of corrected sinus node recovery time, atrial refractory period (ARP), ventricular effective refractory period (VERP), and ventricular functional refractory period (VFRP), and a significant inhibition of conducting tissues in neonatal dogs. In contrast, no such effects were seen in adult dogs. PE had a positive chronotropic effect in neonatal dogs but no such effect in adult dogs. In conclusion, α-adrenoceptors played an important excitatory role in the neonatal dog heart.

KEY WORDS phentolamine; phenylephrine; propranolol; heart rate; blood pressure; electrocardiography

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There has been an increasing interest in the understanding of the functional significance of neonatal myocardial α-adrenoceptors. Most investigators focused their attention to chronotropic effects of α-adrenocepors on *in vivo* myocardium. They suggested that α-adrenergic stimulation in immature cardiomyocytes results in a positive chronotropic effect. In contrast, α-adrenergic stimulation may result in a decrease of spontaneous HR in adult myocardium[1–3]. The conversion of a positive chronotropic effect is related to the postnatal increase in autonomic innervation[4] specifically, the adrenergic system.