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Differential Effects of Lipid-soluble Toxins on Sodium Channels and L-type Calcium Channels in Frog Ventricular Cells

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ABSTRACT

The effect of grayanotoxin I (GTX I), veratridine and aconitine with either an external or internal concentration of 100 μM on L-type calcium (Ca) channels was studied using the whole cell patch clamp and internal dialysis methods. The experimental conditions for the modification of sodium (Na) channels induced by the internal application of these toxins was determined by showing sustained inward currents with depolarizing repetitive pulses. These toxins failed to generate any change in Ca channels under the same experimental protocol as for Na channels. However, external application of these toxins caused a moderate block of the Ca channels without changing the kinetics.

Key words: *Na channel, L-type Ca channel, Lipid-soluble toxins, Myocardium*

Lipid-soluble toxins, such as grayanotoxins, veratridine and aconitine, specifically affect the functioning of fast Na channels in such a way that voltage-dependent activation is shifted in a hyperpolarizing direction and inactivation is slowed down[8,9,16,17]. It has been suggested that the site of action of these toxins is localized in the intracellular phase[14]. Besides these unique actions to Na channel, Romey and Lazdunski (1982)[12] have shown that, on bath application, these toxins block Ca channels in the same range of concentration as they modify Na channels in N1E-115 neuroblastoma cells. A similar finding has been reported by Enyeart et al (1987)[2] who showed that veratridine blocks slowly inactivating Ca channels in pituitary GH_4C_1 cells. Another interesting observation, provided by Romey and Lazdunski (1982)[12], was that the suppressive effects of externally applied veratridine on Ca channel were easily removed by washing. On the other hand, Seyama et al (1988)[14] have made the observation that in Na channels the pharmacological action generated by veratridine was hardly eliminated by perfusing a toxin-free solution for several hours. This striking difference in the mode of action between Ca and Na channels raises the question that the site of action in Ca channels may be on the external side.

This study was designed to show what kind of pharmacological action these toxins exert on L-type Ca channels and on which side the site of action is localized. We carried out experiments on frog ventricular cells, because voltage clamp experiments have revealed the existence of two voltage-dependent transient inward currents: a fast Na current which is tetrodotoxin (TTX) -sensitive at ID_{50} = 0.027 μM[18] and an L-type Ca current showing voltage-dependent and time-dependent inactivation[3,19].

MATERIAL AND METHODS

Cell preparations

Single ventricular cells were obtained from the hearts of bullfrogs (*Rana catesbeiana*) using essentially the same technique as described previously[15]. Briefly, the heart was mounted on a Langendorff apparatus and perfused retrogradely via the aorta with a Ca^{2+}-free solution containing collagenase (0.025 mg/ml, Yakult, Tokyo, Japan and 0.35 mg/ml, Wako Pure Chemical Industries Ltd, Osaka, Japan), trypsin type III (0.06 mg/ml, Sigma Ltd. St. Louis, MO, USA) and crystallized bovine albumine (0.6 mg/ml, Seikagaku Corporation, Tokyo, Japan) for 20 min at 32°C. The dispersed cells were kept in a solution at a low [Ca^{2+}] (200 μM) for 30 min and then centrifuged for 1 min at 65×*g*. After eliminating all the debris, the collected cells were maintained in Leibowitz's L-15 medium (Gibco Laboratories, Gaithersburg, MD, USA) for experimental use.

Solutions

For recording of the activity of Na channels, the composition of the external solution (in mM) was NaCl 45.4, CsCl 5.4, $MgSO_4 \cdot 7H_2O$ 3.0, glucose 5.5, *N*-methyl-*D*-glucamine 68.1 and 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES) 5.0. The pH of the external solution was adjusted

to 7.2 with HCl. The composition of the internal solution (in mM) was CsCl 30, NaCl 10, *l*-aspartic acid 100, adenosine 5'-triphosphate Mg salt (Mg-ATP) 5.0, glucose 10.0, HEPES 10.0 and ethylene bis (oxonitrilo) tetraacetate (EGTA) 5.0. The internal solution was adjusted to pH=7.0 with CsOH.

On measuring the activity of Ca channels, the composition of the external solution (in mM) was NaCl 113.5, CsCl 5.4, $CaCl_2 \cdot 2H_2O$ 2.0, HEPES 5.0, 0.3 μM TTX and 1 μM isoproterenol. The pH of the external solution was adjusted to 7.2 with NaOH. The composition of the internal solution (in mM) was CsCl 100, $CaCl_2$ 0.51, $MgCl_2$ 9.1, Na_2 creatine-phosphate 3.0, tris (hydroxymethyl) aminomethan ATP salt (TRIS-ATP) 3.0, 1, 2-bis (2-aminophenoxy) ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid (BAPTA) 10.0, ethylenediaminetetraacetic acid (EDTA) 5.0 and HEPES 10. The pH of the internal solution was adjusted to 7.0 with CsOH. The pCa and pMg of the internal solution were calculated to be 8 and 3, respectively, according to Schoenmakers' program[13]. The GTX I was kindly provided by Emeritus Professor J. Iwasa of Okayama University. The veratridine and aconitine were purchased from Sigma Ltd.

Whole-cell recording

Whole-cell patch pipettes with a tip resistance less than 2 MΩ were used for obtaining optimum voltage control. Whole-cell currents were filtered at 5 KHz (step pulse) or 1 KHz (ramp pulse) through the low-pass filter (4-pole Bessel filter with a cut-off frequency of 5 or 1 KHz) of an Axopatch 200A amplifier (Axon Instruments, Foster City, CA, USA). The membrane currents were converted into digital signals at a sampling frequency of 4~50 kHz with a 12-bits analog-to-digital converter (TL-1 DMA Interface, Axon Instruments) controlled with pCLAMP software (Axon Instruments), and were stored in disks for later analysis. All the experiments were conducted at room temperature (23–26°C). Data were presented as mean ± SD (n=number of observations), unless otherwise stated.

RESULTS

Effect of lipid-soluble toxins on Na channel

Internal application

To produce GTX I-induced modification of Na channels, 30 repetitive conditioning pulses to 0 mV of 4 msec in duration from the holding potential of –110 mV at the frequency of 4 Hz were employed[17] (see inset figure in Fig. 1). To estimate the extent of modification of Na channels, we used either (1) ordinary rectangular pulses of 12 msec or 160 msec in duration from –130 mV up to 80 mV or (2) an isosceles triangular-shaped pulse (ramp pulse)[4] with a slope of ±1.25 V/sec from –140 mV to 80 mV. No matter which method we used, after repetitive conditioning pulses, the membranes were subjected to hyperpolarization to –140 mV for 10 msec in order to shut the Na channels, which had been open during the repetitive pulsing. In Fig. 1, representative data for sodium current (I_{Na}) in response to rectangular pulses at the very beginning of recording (A-1) and 11 min after establishing the patch clamp mode (A-2) are displayed. In Fig. 1B, the peak values of the family of I_{Na} in A-1 (open squares) taken as control and those in A-2 (filled circles) are plotted against the stimulating membrane potentials. The characteristic properties of sustained I_{Na} in GTX I medium were demonstrated by plotting the steady state value of I_{Na} at the end of a 160 msec stimulating pulse (open circle). The GTX I induced, slow sustained current started to flow at the membrane potential of –90 mV.

The voltage clamp experiment using a ramp pulse yielded characteristic current-voltage relationships qualitatively similar to those in Fig.1B. The record in Fig. 1C was taken 30 sec after establishing the whole-cell configuration. The routine procedure for the modification of Na channels was applied. In the rising phase of the ramp pulse, a sharp downward deflection of the current trace appeared as the Na channels were activated and this was followed by a rapid inactivation. In the falling phase of the ramp pulse, because of the full inactivation of the Na channels, a linear trace would have been observed if GTX I had not reached the site of action. However, we could barely recognize a slight downward bulging pattern, indicating that GTX I had begun to affect the Na channels to a marginal extent. In the upper graph in Fig. 1D, both records were taken 330 sec after a rupture of the patch membrane. The record in solid line (*a*) was taken without repetitive conditioning pulses, on the expectation that no modification would proceed, while the record in dotted line (*b*) was made with repetitive conditioning pulses. A striking difference became obvious: in the rising phase of the ramp pulse, the inward current started to flow around –90 mV and kept flowing up to 40 mV, which is close to E_{Na}, and in the falling phase of the triangular pulse, the inward current appeared at the membrane potential of 40 mV and kept flowing down to –100 mV. The difference in the I–V curve with and without repetitive pulses (*b*–*a*) manifested the characteristic pattern of the sustained current. Both rising and falling ramp pulses gave rise to a qualitatively similar I–V curve for sustained inward currents. Because of mismatching peak I_{Na} in (a) with that in (b) in Fig. 1D, there is a dent in the I–V curve after subtraction for the rising ramp pulse. However, since transient sodium conductance was totally inactivated during the rising ramp pulse, the

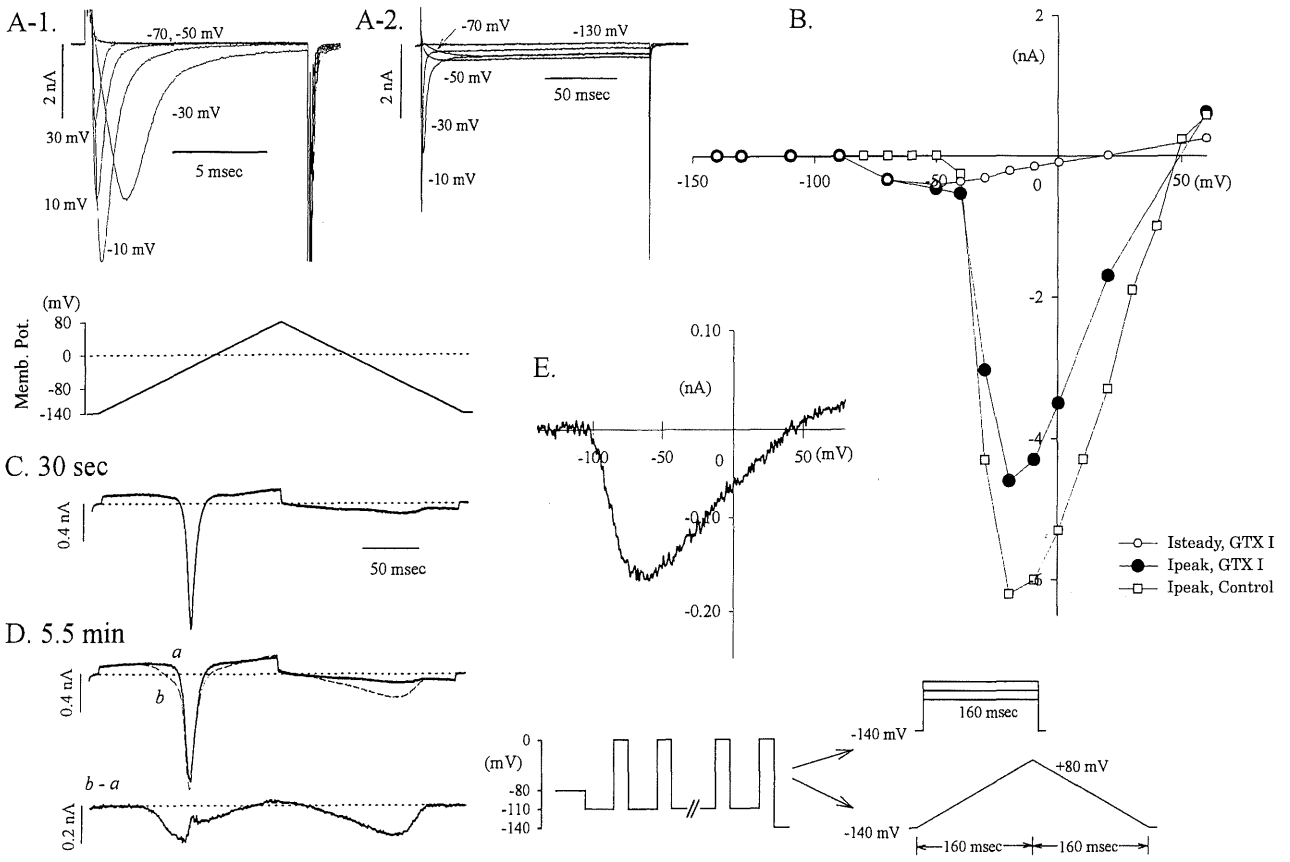

Fig. 1. Slow sustained I_{Na} flowing through Na channels modified by internally applied 100 μM GTX I. Repetitive conditioning pulses were given at 4 Hz. Pulse protocol is given in the inset. In A-1 and A-2, the response of Na channel to a rectangular pulse under a whole cell patch clamp is shown. Numerals in each trace indicate the membrane potential during the test pulse. In B, the I–V curves were constructed by plotting peak and steady state I_{Na} in GTX I against the membrane potentials during the test pulse. In C, the record with ramp pulse following repetitive conditioning pulses was made 30 sec after the establishment of the whole-cell configuration. In D, I_{Na} in response to the ramp pulse under voltage clamp conditions is shown. The trace in solid line (*a*) in the upper graph indicates the record without conditioning pulses and that in dotted line (*b*) the record with conditioning pulses. The difference in the two records (*b–a*) is shown in the lower graph. In E, the current record in the right hand half in the lower trace in D is plotted inversely to adjust the voltage axis to that in B. The reversal potential is well in accord with the value of 40 mV calculated by the Nernst equation.

smooth I–V curve for inward currents on the falling ramp pulse was solely made up of GTX I-induced sustained current. In Fig. 1E, the right-hand half of the I–V curve (*b–a* in Fig. 1D), corresponding to the falling ramp pulse, is plotted inversely to match the voltage axis in Fig. 1E with that in Fig. 1B shown in the open square.

The membrane potential at which the GTX I modified channels shut off in the falling phase of the ramp in Fig. 1E was around –100 mV, while that in the rectangular pulse experiment was around –90 mV in Fig. 1B. This difference can be explained by a slowing down of the activation process through GTX I modification (e.g., current trace for the pulse to –70 mV in A-2). As the membrane potential gradually decreases in the falling ramp pulse, a slow deactivation process may leave the Na channels open down to the membrane potential of around –100 mV. These characteristic patterns indicate that the slow sustained current keeps flowing at a wide membrane potential range. The rest of the lipid-soluble toxins gave rise to a modification of sodium channel similar to that by GTX I (data not shown) . These results made clear that the protocol using the ramp pulse is more suitable for elucidating the properties of Na channels modified by GTX I than that with a conventional rectangular pulse, because (1) the GTX I modification of Na channel is a slow process, (2) the ramp pulse experiment can monitor subtle changes in the behavior of Na channels in response to continuous change in the membrane potential, and (3) we were able to record membrane currents modified by the toxin at a wide range of membrane potentials with one ramp pulse following the conditioning pulses.

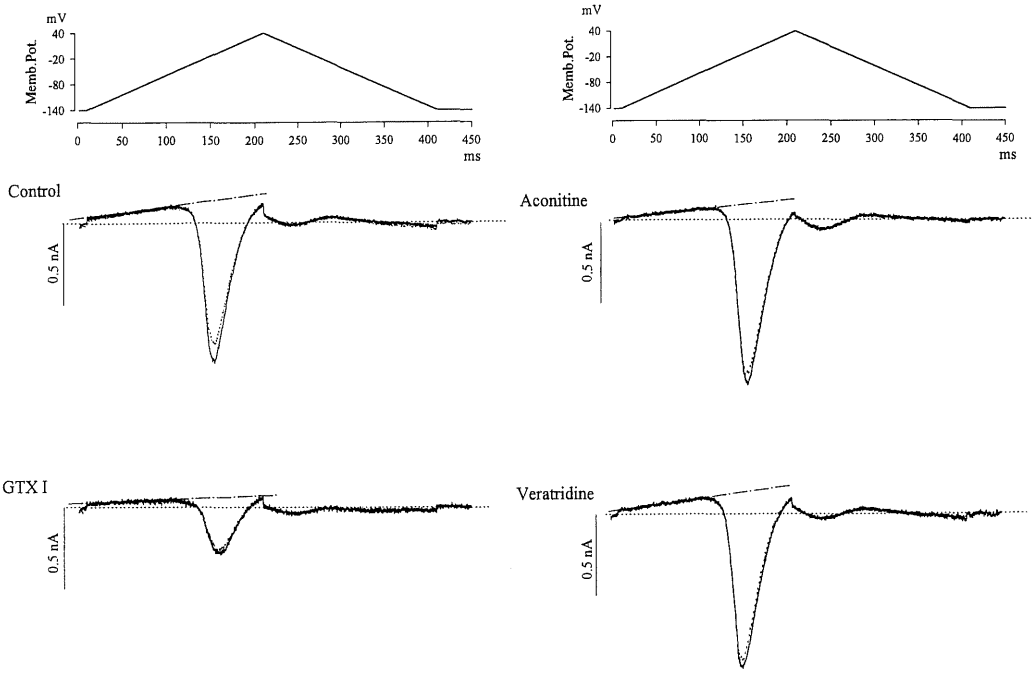

Fig. 2. Effects of GTX I, aconitine and veratridine of 100 μM on I_{Ca}.
They were dialysed through a pipette. The diffusion time to channels was separately estimated to be 2–3 min, using I_{Na} as an index. Records were taken 5 min after rupture of the cell membrane in a patch was established. Thirty repetitive conditioning pulses of 20 msec in duration were given at 2 Hz. The pattern of the I–V curves with (in dotted line) and without (in solid line) a repetitive conditioning pulse for modification is quite alike. The leakage currents (in dot-and-dash line) were obtained by adopting the straight line to the I–V curve at the membrane potentials where the Ca channels are in a closed state. The slight depression of peak I_{Ca} is due to residual inactivation caused by repetitive pulsing. Peak I_{Ca} after the repetitive pulsing was referred to that in control and was estimated separately to be 95.3 ± 4.0% (n=3).

Otherwise we would have needed to apply the conditioning pulses repeatedly before each rectangular test pulse.

External application

The site of action of these toxins has been confirmed by the following experimental findings[14]: 1) external application of these toxins to squid giant axon under nonperfused conditions caused substantial membrane depolarization, but intracellular perfusion of the fibers retarded this depolarization appreciably; 2) tritium-labeled grayanotoxin in the external medium could penetrate through the cell membrane, and 3) the most hydrophilic grayanotoxin analogue was effective only when applied internally. Taking into account this situation, we took GTX I as a representative toxin in this group and examined its effect applied externally on Na channels in frog ventricular cells. When GTX I was applied externally for 10–30 min, we were unable to observe any change in the I–V curve for Na channel under whole cell configuration with the routine procedure for the GTX I-modification. However, when the experiment was performed on cells which had been soaked in external solution containing 100 μM GTX I for 39 min, we were able to observe the characteristic sustained inward currents which commenced at –90 mV. The most likely explanation for the failure to observe GTX I-modification in the cells which had not been soaked in GTX I medium is that (1) because GTX I has difficulty in permeating through the cell membrane, it may need a long time to accumulate in the cells, and (2) GTX I once loaded under whole cell configuration may be diluted by the internal solution in the pipette. The time when GTX I-modification was detected was in the range of several tens of minutes in external application, while in internal application it was in the range of several tens of seconds. This enormous difference in response time is well explained by the notion presented above: the site of action is located in the intracellular phase.

Effect of lipid-soluble toxins on Ca channel

Internal application

The protocol for examining the effect of the three lipid-soluble toxins on Ca channels was essentially the same as that for the Na channels, as in inset in Fig. 1. Suppression of I_{Na} was carried out by applying 0.3 μM TTX in the external medium. The slope of the ramp pulse was adjusted

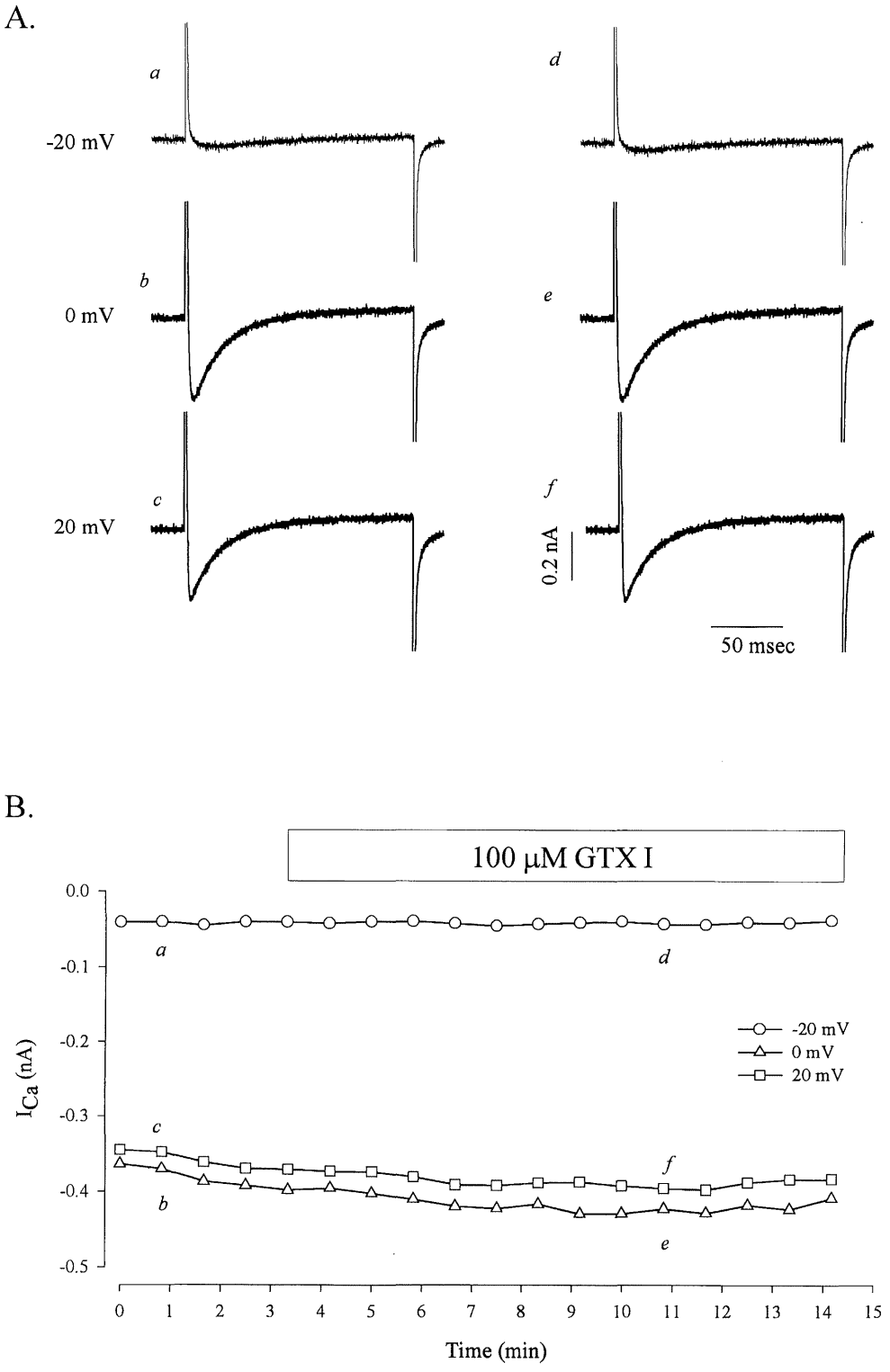

Fig. 3. No effect of internally applied GTX I on I_{Ca}.
Step depolarization to –20, 0 and 20 mV from the holding potential of –80 mV were given every 50 sec. A: original records of I_{Ca} before and after GTX I was given by the internal perfusion method. B: time course of the change in I_{Ca} during perfusion of GTX I. Alphabets from *a* to *f* indicate the times when data were collected and correspond to those in A.

for Ca channel to ±0.9 V/sec. Because in separate experiments we had confirmed that these toxins exert no change in the I–V curve in the control medium, for the sake of clarity the Ca channels were activated by applying 1 μM isoproterenol. The bulging pattern in the I–V curve in the falling phase of the ramp pulse was due to certain Ca channels being still in the open state at the membrane potential of 40 mV, because the I–V curves for leakage current do not cross the

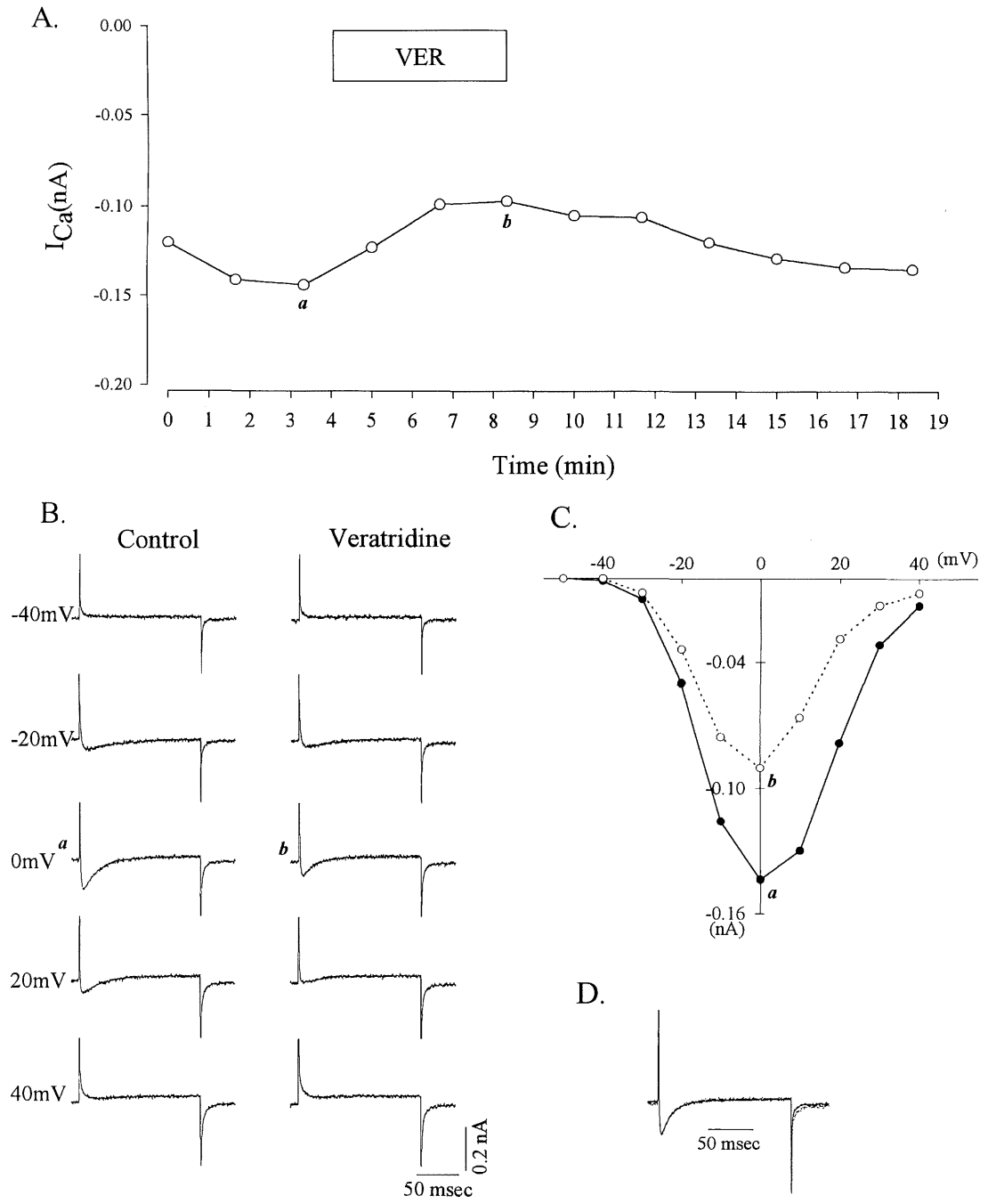

Fig. 4. Suppression of I_{Ca} caused by 100 μM veratridine in the external medium.
A: time course of the change in I_{Ca} during the application of veratridine in the external medium. Ca currents were monitored by giving a test pulse to 0 mV from the holding potential of –80 mV every 100 sec. B: original records of I_{Ca} in the control and veratridine medium. Various amplitudes of test pulse of 150 ms in duration were given. C: effect of external veratridine on the I–V curve. Data for I–V curves were collected before (*a*) and after (*b*) the application of veratridine. D: no effect of veratridine on the time course of I_{Ca}. I_{Ca} in response to a test pulse to 0 mV after veratridine matched with I_{Ca} in control by a scaling factor of 1.6.

current traces for Ca channels (see Fig. 2). This residual component of open Ca channel became inactivated and deactivated as the membranes were gradually hyperpolarized, causing a downwardly concave I–V curve in the falling ramp phase. The I–V curves with and without conditioning pulses were identical, indicating that these toxins do not alter the activity of Ca channels. However, there remained a possibility that the conditioning pulse used might not have been appropriate for the GTX I-modification of Ca channels. We thus employed several different protocols for the conditioning pulse. First, the numbers of conditioning pulses to 0 mV of 20

msec in duration were extended to either 100 or 300 at 2 Hz. In another trial, 30 conditioning pulses of the same configuration were given at either 4 Hz or 10 Hz. Furthermore, 30 conditioning pulses, which elongated from 20 msec to 150 msec, were given at 2 Hz, and the duration of the single conditioning pulse to 0 mV was changed from 20 ms up to 30 s. All these attempts failed to induce a change in I_{Ca} qualitatively similar to that in I_{Na}. Since all these toxins had been injected into a pipette, there was a good chance of missing a tonic action of these toxins to Ca channels. To eliminate this possibility, we continuously monitored the change in amplitude of I_{Ca} during the internal perfusion of 100 μM GTX I. The time course of change in I_{Ca} when the toxins were applied intracellularly by the internal perfusion method is displayed in Fig. 3, which shows that a change in the amplitude of I_{Ca} was not recognized. The other toxins also produced no change in I_{Ca} under the same experimental conditions. Thus, we conclude that none of these lipid-soluble toxins affects the activity of Ca channels from the internal surface.

External application

The whole cell Ca currents were continuously monitored and current amplitude was plotted as a function of time (Fig. 4A). After application of 100 μM veratridine there was a decrease in the I_{Ca} amplitude. When this toxin was withdrawn from the external medium, the Ca currents previously suppressed were reversibly restored to control value. The relative values of I_{Ca} in 100 μM toxin compared to those in control were estimated to be 0.58 ± 0.05 (n=4) in veratridine, 0.66 ± 0.05 (n=4) in aconitine, and 0.89 ± 0.09 (n=4) in GTX I, respectively. In the meantime, we examined the I–V curves before and after the application of veratridine. The calcium currents in the medium containing the toxin were suppressed without shifting along the membrane potential axis (Fig. 4C) and without changing the time course (Fig. 4D). In separate experiments, the I_{Ca} amplitude with and without thirty conditioning pulses to 0 mV of 20 msec in duration was found to remain unchanged.

DISCUSSION

The present study has unveiled the characteristic action of lipid-soluble toxins on Na channels and Ca channels in frog ventricular cells. First, lipid-soluble toxins modify Na channels only when a test depolarizing pulse is preceded by repetitive conditioning pulses and the toxins are applied intracellularly. Second, the Na channels modified by these toxins can generate a slow, sustained current at membrane potentials more negative than the normal threshold potential for Na channels. Third, when these toxins are applied externally, Ca channels are reversibly blocked without any change in kinetic properties. Fourth, when applied internally, ramp pulse experiments yield superimposable I–V curves for the L-type Ca channel with or without repetitive conditioning pulses. We can summarize these findings as follows; the lipid-soluble toxins specifically modify Na channels from the internal side in frog ventricular cells but they moderately block L-type Ca channel only from the external surface. These pharmacological properties are useful for differentiating the molecular moiety responsible for the gating of Na channels from that of Ca channel.

The results on Ca channels in this study are in accord with those by Romey and Lazdunski[12], who claimed that these lipid-soluble toxins block voltage-dependent Ca channel in differentiated N1E-115 neuroblastoma cells in a toxin concentration range comparable for Na channels. However, there are several qualitative as well as quantitative differences. For example, the concentration of veratridine required for half-maximal suppression of I_{Ca} is 26 μM in N1E-115[12], while 100 μM veratridine blocked I_{Ca} only by 42% in frog ventricular cells. In N1E-115 cells, the veratridine-block of the Ca channel was accompanied by a shifting of the I–V curve in the hyperpolarizing direction[12], while in frog ventricular cells the block occurred without a change in kinetics. One hundred μM aconitine was reported to be ineffective[12] in N1E-115 while a 34% block of I_{Ca} occurred in frog ventricular cells. All these differences are most likely due to a difference in the type of Ca channels. Fishman and Spector (1981)[5] have provided evidence that N1E-115 cells contain a type of Ca channel which is not sensitive to organic Ca channel blockers. More detailed analyses conducted by Narahashi, Tsunoo and Yoshii[10] have revealed that N1E-115 contains two types of Ca channels, which were later identified as T and L-type Ca channels[1,11]. The Ca channels which Romey and Lazdunski[12] studied were therefore most likely the T-type, because the channel which commenced activation at –50 mV inactivated rapidly. Kongsamut et al (1985)[7] have demonstrated that in N1E-115 veratridine does not suppress $^{45}Ca^{2+}$ uptake induced by 50 mM K^+, which indicates that there are Ca channels with a slow gating mechanism which is insensitive to veratridine. The Ca channels in frog ventricular cells we have used so far are different, and have been classified as L-type by Yamaoka and Seyama (1996)[19] because an organic Ca antagonist, nifedipine, completely blocks these Ca channels. Enyeart et al (1987)[2] observed a similar block of Ca channels by veratridine in pituitary GH_4C_1 cells.

It is worth noting that the molecule retaining the weakest blocking capability is GTX I which has no charge on it. Although charges on the ter-

tiary amine group are extremely weak, veratridine and aconitine which belong to the alkaloids have faint positive charges. This suggests that one of the factors for blocking Ca channels from the external surface may be the presence of a negative charge around the orifice of L-type Ca channel. This supposition is supported by the finding of Heinemann et al[6] that the substitution of lysine at position 1422 in repeat III and/or alanine at position 1714 in repeat IV of Na channel for glutamic acid converts the ion selectivity of Na channel into that of Ca channel, suggesting that Ca channels hold more negative charges in pores critical to ion selection than Na channels. Given the blocking mechanism of these toxins, it is reasonable to suppose that they may pop in pores close to the ion selective filter in Ca channel from the external surface because of the traction of negative charges at position 1422 and/or position 1714.

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