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Dual Action of d-Tubocurarine on Large-Conductance Ca^{2+}-activated K^+ Channels from Rat Brain Reconstituted into Planar Lipid Bilayer

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Using the planar lipid bilayer method, we investigated the effect of d-tubocurarine (dTC) on the extracellular side of large-conductance Ca^{2+}-activated K^+ channel from rat brain. When the initial open probability (Po) of the channel was relatively high, dTC decreased channel activity in a concentration dependent manner. In contrast, when the initial Po was lower, sub-micro molar dTC increased channel activity by destabilizing the closed states of the channel. Further addition of dTC up to micro molar range decreased channel activity. This dual effect of dTC implicates that there exist at least two different binding sites for dTC.

Key Words: Ca^{2+}-activated K^+ Channel, d-Tubocurarine, Planar lipid bilayer

INTRODUCTION

d-Tubocurarine (dTC) is well known as an open channel blocker of acethylcholine receptor channel. However, it has also been used to block Ca^{2+}-activated K^+ conductance in sympathetic ganglia (Nohmi & Kuba, 1984), chick ciliary-ganglion (Dryer et al, 1991), and hepatocytes (Cook & Haylett, 1985). The apamin sensitive Ca^{2+}-activated K^+ conductance, I_{AHP}, in guinea-pig inferior mesenteric ganglion cells is also blocked by dTC (Dun et al, 1986). Using outside-out patches from rat sympathetic neurons, dTC is shown to block the large-conductance Ca^{2+}-activated K^+ (maxi-K) channels (Smart, 1987). Recently, in an experiment using cloned small-conductance Ca^{2+}-activated K^+ channel, two amino acid residues on either side of deep pore are identified as the determinants of sensitivity to dTC (Ishii et al, 1997). In this paper, we employed planar lipid bilayer method to investigate the effect of dTC on the activity of single maxi-K channels of rat brain. We observed that dTC not only blocks the maxi-K channel from rat brain at micro molar concentration but also activates the channel at sub-micro molar concentration when the initial open probability(Po) is low. This result suggests that there exist at least two different binding sites for dTC: a high affinity site which activates the channel activity upon dTC binding and a low affinity site which depresses the channel activity.

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METHODS

Materials

Phosphatidylethanolamine (PE) and phosphatidylserine (PS) were purchased from Avanti Polar Lipid (Alabaster, USA). dTC and all other chemicals were purchased from Sigma (St. Louis, USA).

Preparation of rat brain plasma membrane vesicles

Rat plasma membrane vesicles were prepared as described (Chung et al, 1991). Briefly, rat brain cortex was excised and immediately homogenized in an ice-cold isotonic sucrose buffer (10 ml/gram of tissue) containing 0.25 M sucrose, 2.5 mM KCl, 0.1 mM

EGTA, 0.1 mM dithiothreitol (DTT), and 20 mM HEPES (pH 7.2). The homogenate was centrifuged at 1,000xg for 2 min, and the supernatant was centrifuged at 13,000xg for 10 min. The pellet was resuspended in a hypotonic lysis buffer containing 0.1 mM DTT, 2.5 mM KCl, 5 mM tris-HCl, 0.1 mM EGTA, and 0.1 mM EDTA (pH 8.2), and allowed to stand on ice for 20 min. The membrane was then rehomogenized and centrifuged at 100,000xg. The pellet was layered under a discontinuous step gradient of Percoll (25, 18, 10, and 0%; v/v). The gradient was centrifuged at 40,000xg for 2 min, and the membrane fraction at the 0 to 10% interface was collected. Percoll was removed by centrifugation at 100,000xg for 50 min. The membrane was stored at -70°C until use.

Single channel recording using planar lipid bilayer reconstitution

We obtained single-channel recordings by incorporating these plasma membrane vesicles into planar lipid bilayers (Shin et al, 1997). Two compartments of the recording chamber were filled with 150 mM KCl, 1 mM EGTA, 1.05 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.2) for the cytosolic (or cis) solution, and 5 mM KCl, 0.1 mM EGTA, 10 mM HEPES (pH 7.2) for the extracellular (or trans) solution, respectively. The bilayer with a capacitance of >200 pF was made around a 250 μm diameter hole by painting with phosphatidylethanolamine and phosphatidylserine mixture (3:1) dissolved in n-decane (20 mg/ml). After the incorporation of a channel, further fusion was suppressed by adding 50 mM KCl to the extracellular side, thereby reducing transmembrane osmotic gradient. Proper amount of EGTA or $CaCl_2$ was added to the cis solution to adjust Po. Voltage was represented as the voltage of the cytosolic side with respect to the extracellular side. Solution changes were made by perfusing extracellular chamber. The current was amplified by an Axopatch 200A amplifier (Axon Instruments, Inc., Foster City, California, USA), and filtered at 1 kHz. Both the current and the voltage were stored using a digital tape recorder DTR 1204 (Biologic, France). Later, recorded data were played back, and digitized using Digidata 1200 (Axon Instruments, Inc.) and a software, Axotape (Axon Instruments, Inc.) at 5 kHz sampling rate. The channel activities were analyzed using a pClamp 6 software (Axon Instruments, Inc.).

RESULTS

Maxi-K channels were identified by two criteria. First, when reconstituted into lipid bilayer, a Maxi-K channel has a single channel conductance of around 220 pS using K^+ as a charge carrier (Reinhart et al, 1989). Second, its activity is dependent upon the calcium concentration in the cis (Magleby & Pallota, 1983; Reinhart et al, 1989).

Fig. 1(A) shows that the addition of 0.35 μM dTC to the extracellular side of the channel decreases maxi-K channel Po from 0.926 to 0.771 (16.7% decrease). In contrast, when the initial Po was set at 0.370, the addition of similar concentration of dTC (0.28 μM) increased channel activity to 0.463 (Fig. 1(B); 25.1% increase). Further application of dTC to this channel up to 14 μM decreased Po of the channel to 0.096 as shown in the figure. Both the activation and inhibition effects of dTC were reversible.

The change of the Maxi-K channel activity was monitored while dTC concentration was increased progressively (Fig. 2). Po calculated for every 1 min was plotted as a function of time. Some fluctuations in Po at a given dTC concentration could be observed, but the increase and the subsequent decrease of channel activity was evident as dTC concentration was increased. The initial Po was about 0.32 before dTC addition, increased to reach a peak after addition of 0.5 μM of dTC, and decreased by further addition of dTC.

Fig. 3 is Po plots as a function of dTC concentration from six different single channel experiments. In this figure, Po profiles look categorically different between two groups. In one group with the initial Po (before dTC addition) set relatively high (circle, triangle, and square), Po either remained unchanged or decreased little from the initial Po at around 0.5 μM dTC. In the other group with the initial Po set relatively low (diamond, inverse triangle, and hexagon), the Po increased to reach the maximum at around 0.5 μM dTC as described in Fig. 2. As dTC concentration increased further, Po decreased in both groups. Similar activation by sub-micromolar dTC was observed in 9 other channels (41.08 ± 38.48% increase, mean s.d.).

Kinetic analysis were performed in four single channel data (Table 1). Channel I corresponds to the group with relatively high initial Po, and channel II, III, and IV to the other group with relatively low initial Po as categorized in Fig. 3. In all of these

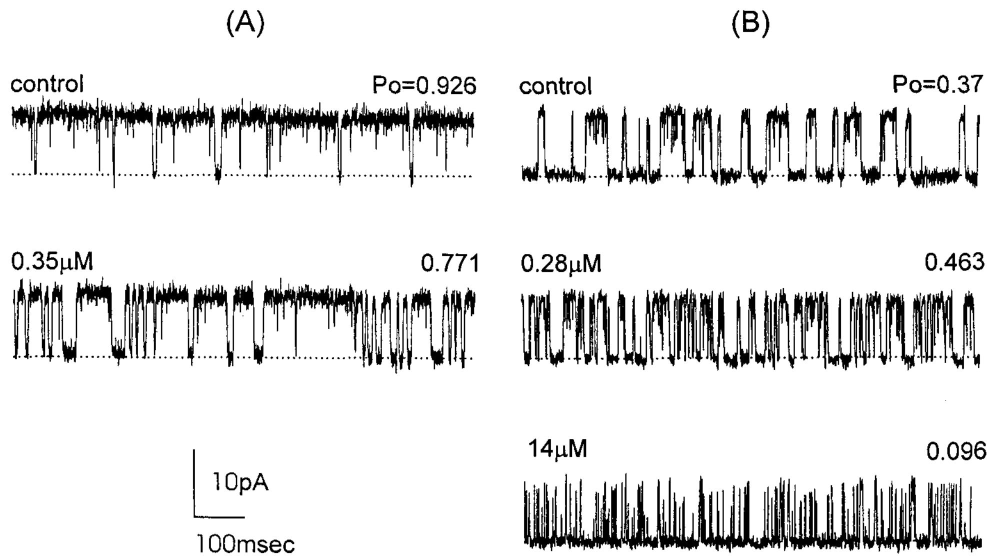

Fig. 1. dTC increases and decreases maxi-K channel activity. Representative traces of the maxi-K channel activities from two different experiments are shown. Channel openings are upward deflections, and the dotted lines represent the closed levels. The initial Po were set to (A) 0.926 and (B) 0.37, respectively. dTC was applied to the extracellular side of the channels. dTC concentration and Po is indicated on each traces. The calculated free Ca^{2+} concentration was 3.66 μM for (A) and 1.47 μM for (B). The membranes were held at depolarizing voltage of +15 mV.

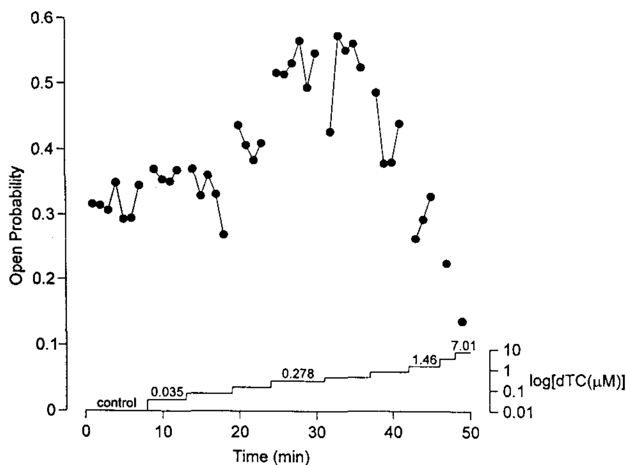

Fig. 2. Po of the maxi-K channel progressively increases and decreases by increasing concentration of dTC. Po of maxi-K channel was plotted every one minute. In the lower part of figure some of dTC concentration are shown. The free Ca^{2+} concentration was 1.47 μM.

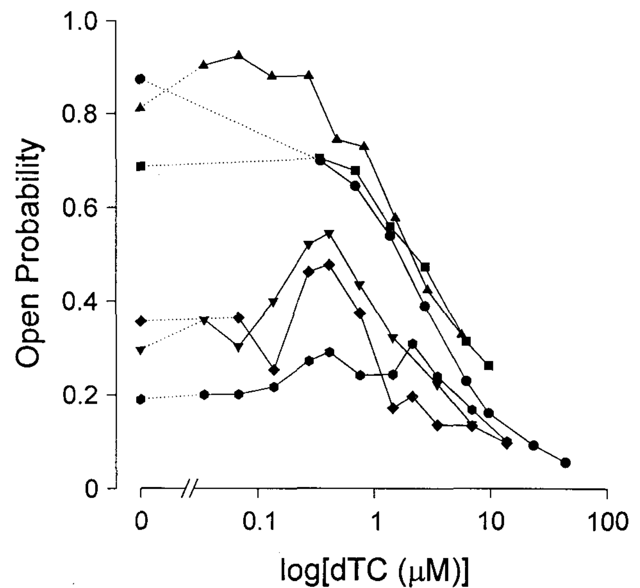

Fig. 3. The dTC effects on the channel activity depend on the initial Po. Po was plotted as a function of dTC concentration from six different single channels. Each symbol represents different channels. Free Ca^{2+} concentration was in micromolar range in each channels, and the membrane was held at +15mV.

channels, the open times were well fit with two exponentials and the closed times with two or three exponentials as reported previously (Reinhart et al, 1989). In case of channel I, after addition of 0.42 μM dTC the open times decreased, but the closed times

Table 1. Kinetic analysis of four single channels

Channel #	(Po)	Open time (ms)	Closed time (ms)
channel I			
control	(0.864)	7.7 (6%), 24.5 (94%)	0.37 (43%), 5.5 (57%)
dTC, 0.42 μM	(0.702)	5.1 (15%), 9.7 (85%)	0.37 (15%), 4.0 (81%), 12 (4%)
dTC, 7.0 μM	(0.232)	1.1 (100%)	0.37 (2%), 3.9 (93%), 8.6 (5%)
channel II			
control	(0.296)	9.1 (78%), 14.3 (22%)	0.44 (66%), 51.8 (26%), 110 (8%)
dTC, 0.42 μM	(0.545)	7.3 (95%), 15.6 (95%)	0.44 (54%), 7.7 (33%), 25 (14%)
dTC, 7.0 μM	(0.136)	1.25 (100%)	0.44 (2%), 8.4 (95%), 45 (3%)
channel III			
control	(0.357)	3.4 (7%), 11.8 (94%)	0.66 (68%), 48.7 (25%), 90 (7%)
dTC, 0.42 μM	(0.478)	7.3 (54%), 9.1 (46%)	0.55 (69%), 9.1 (22%), 45 (9%)
dTC, 7.0 μM	(0.133)	1.55 (100%)	0.59 (8%), 8.3 (81%), 37 (11%)
channel IV			
control	(0.190)	3.3 (20%), 9.7 (80%)	0.55 (45%), 13.3 (5%), 68.3 (50%)
dTC, 0.42 μM	(0.291)	6.96 (100%)	0.59 (32%), 9.3 (38%), 45 (30%)
dTC, 7.0 μM	(0.168)	1.65 (100%)	0.73 (5%), 7.6 (85%), 30 (11%)

Channel I represents the experiment where the initial Po was adjusted relatively higher, and channels II, III, and IV represent the experiments with the relatively lower initial Po's. The mean open times and mean closed times were fit with two and three exponentials as described elsewhere (Reinhart et al, 1989), and presented with the weights in parenthesis.

remained unchanged. However, in case of other three channels, the intermediate and long closed times became significantly shorter at the same concentration of dTC, while the open times remained unchanged. When dTC concentration was increased to 7.0 μM, the open time became even shorter in all of four channels, but the closed times did not change any further.

DISCUSSION

In this report, we demonstrated that dTC has a dual effect on the maxi-K channel activity. dTC at micromolar concentration decreased the channel activity as described elsewhere (Smart, 1987). However, when the initial Po was set low, dTC applied at submicromolar concentration increased the channel activity, and subsequent application of dTC up to micromolar concentration inhibited the channel. The activation by sub-micromolar dTC was not due to the fluctuation in the channel activity as evidenced in Fig. 2.

Since the Maxi-K channel activity is dependent upon both the cytosolic calcium concentration and the transmembrane voltage, the low initial Po can be produced by either low calcium, low voltage, or both. Even though we did not systematically sort out the experimental conditions in terms of calcium concentration and voltage, it is observable from kinetic analysis that the two groups (low initial Po and high-initial Po) are distinct in terms of the mean closed times as shown in Table 1. The mean closed times of Channel I were significantly shorter than other three cases. When 0.42 μM of dTC were added, the intermediate and long closed times of the channels with low initial Po's (Channel II, III, and IV) became shorter whereas the mean closed times of the channel with high initial Po (Channel I) remained relatively unchanged. This might be the reason why dTC did not show any activation effect when the initial Po was relatively high. When Po of channel was high, the closed times were already too short to be affected by dTC. Therefore, there would be much less chance for dTC to activate the channel. This result suggests

that binding of dTC to the channel or to regulatory components closely associated to the channel destabilize the closed states of channel without affecting the open states, thereby decreasing the closed times. On the other hand, the inhibition of the channel at micromolar dTC was due to shortening of the open times as described elsewhere (Smart, 1987).

The dual effect of dTC on the channels with low initial Po suggests that there exist at least two different dTC binding sites on the channel. The binding of dTC to the high affinity site activates the channel, and the binding of dTC to the low affinity site induces blocking events. It was not possible to accurately obtain the affinities of dTC either for activation nor for blocking, because even at sub-micromolar concentration the channel was activated as well as blocked by dTC. From Fig. 3, we estimated that the apparent half concentrations for activation (EC_{50}) and inhibition (IC_{50}) were about 0.2 μM, and 3 μM, respectively. The latter was in a similar range of the reported K_i value of 2.4 μM on one of cloned small-conductance Ca^{2+}-activated K^+ channel (Kohler et al, 1996), suggesting that blocking by dTC may be mediated by similar mechanism between two channels.

dTC is a well known anatagonist of nicotinic acetylcholine receptor/channels and also acts as a blocker of Ca^{2+}-activated K^+ channels at micromolar concentration. However, the results from this paper indicate that the drug can activate the channel at sub-micromolar concentration. Therefore, careful consideration should be taken in interpreting data from experiments using dTC.

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