Dimebon and Tacrine Inhibit Neurotoxic Action of β-Amyloid in Culture and Block L-type Ca^{2+} Channels

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Dimebon, a Russian-made drug, inhibited toxic effects of β-amyloid on cultured neurons. Excessive accumulation of β-amyloid in the brain is characteristic of Alzheimer dementias. Antialzheimer preparations tacrine and dimebon improve survival of cerebellar granule cells during long-term incubation with $A\beta_{25-35}$, the neurotoxic fragment of β-amyloid. Both preparations can block potential-dependent Ca^{2+} entry into neurons by about 20%, which is explained by their selective action on L-type Ca^{2+} channels. It was assumed that the neuroprotective effect of dimebon and tacrine against $A\beta_{25-35}$ partially depends on inhibition of potential-dependent Ca^{2+} entry.

Key Words: *dimebon; tacrine; $A\beta_{25-35}$, cerebellar granule cells; L-type Ca^{2+} channels*

Impairment of cognitive functions during Alzheimer-type dementias (ATD) is related to degeneration of acetylcholine neurons in the brain. It can be compensated with drugs maintaining the work of postsynaptic receptors. Progression of ATD leads to elimination of nervous cells of other types together with their receptors, so the search for drugs inhibiting neurodegeneration is actual [1]. A Russian-made preparation dimebon (3,6-dimethyl-9-(2-methyl-pyridyl-5)-ethyl-1,2,3,4-tetrahydro-γ-carboline dihydrochloride) previously used as an antihistamine drug, improves cognitive functions in ATD patients [2]. Systemic administration of dimebon improved active avoidance conditioning in rats with partial chronic deprivation of cholinergic functions in the brain; by its effect on learning dimebon is comparable to tacrine used in ATD [3,11]. The major role in the development of pathological process during ATD is played by excessive accumulation of β-amyloid in the brain. β-Amyloid and its synthetic fragments induce degeneration of cultured neurons [9]. It was hypothesized that the neurotoxic effect of β-amyloid is associated with disturbances in intracellular calcium homeostasis [1,9]. Some agents, *e.g.* inhibitors of potential-dependent calcium channels, protect cultured neurons in the presence of β-amyloid [6,15].

Our aim was to study the effect of antialzheimer preparations dimebon and tacrine on neurodegeneration of cultured neurons induced by $A\beta_{25-35}$, a synthetic neurotoxic fragment of β-amyloid, and to examine the potency of these preparations to inhibit potential-dependent Ca^{2+} entry into neurons.

MATERIALS AND METHODS

Cerebellar granule cells (CGC) were isolated from the brain of 7-8-day-old rats by trypsinization and pipetting [14]. The obtained cell suspension ($2.5\text{-}5\times10^6$ cell/ml) was transferred to a 24-well plate (Nunc, 1 ml per well). For electrophysiological studies, polylysine-coated coverslips were placed in some wells. The culture medium contained Eagle's minimum essential medium and Dulbecco's F12 medium (1:1) and was supplemented with 10% fetal bovine serum, glutamine (2 mM), gentamicin (50 μg/ml), glucose (15 mM),

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KCl (up to 25 mM), and pH 7.0-7.2 (all reagents were from Sigma). The cells were cultured in a CO_2-incubator at 37°C and 100% humidity. After 48 h, 80% culture medium was replaced with a fresh portion of the same medium containing cytosine arabinoside (10 μg/ml) and Supplement N1 (1 ml/100 ml), but without serum and Dulbecco's medium. $A\beta_{25-35}$ (Bachem) was dissolved in water (1 mM) and kept 48 h at 37°C for aggregation. The state of cultured cells was daily assessed under an Axiovert-25C inverted microscope equipped with a video monitor. Survival of cultured neurons was assessed spectrophotometrically by the content of lactate dehydrogenase [10]. For electrophysiological study, the glasses with cultures were placed in a medium containing (in mM): 130 TEA, 4 KCl, 10 CsCl, 2 $MgCl_2$, 20 $BaCl_2$, 10 $C_2H_{12}O_6$, 10 HEPES(Cs); pH 7.4. Membrane ionic currents in whole cell were recorded by the patch-clamp method [8]. The micropipettes were filled with a solution containing (in mM): 121 CsCl, 2 $MgCl_2$, 10 HEPES(K), 1 $CaCl_2$, 12 EDTA; pH 7.2. The concentration of free Ca^{2+} was 0.01 μM. To increase the amplitude of ionic currents in calcium channels, $CaCl_2$ in bathing solution was replaced with $BaCl_2$, because calcium channels are more permeable for Ba^{2+} than for Ca^{2+} ions. The currents were activated by short-term stepping of membrane potential (MP) from holding potential -80 mV to maximum 60 mV with 20-mV steps. The amplitude of Ba^{2+} currents in calcium channels was measured as the difference between the baseline and the peak (*i.e.*, minimum) ionic currents. The effect of blockers was assessed from changes in ionic current amplitude in comparison with the control. The amplitude of ionic current under normal conditions was taken as 1, and the effect of test agents was expressed in relative units.

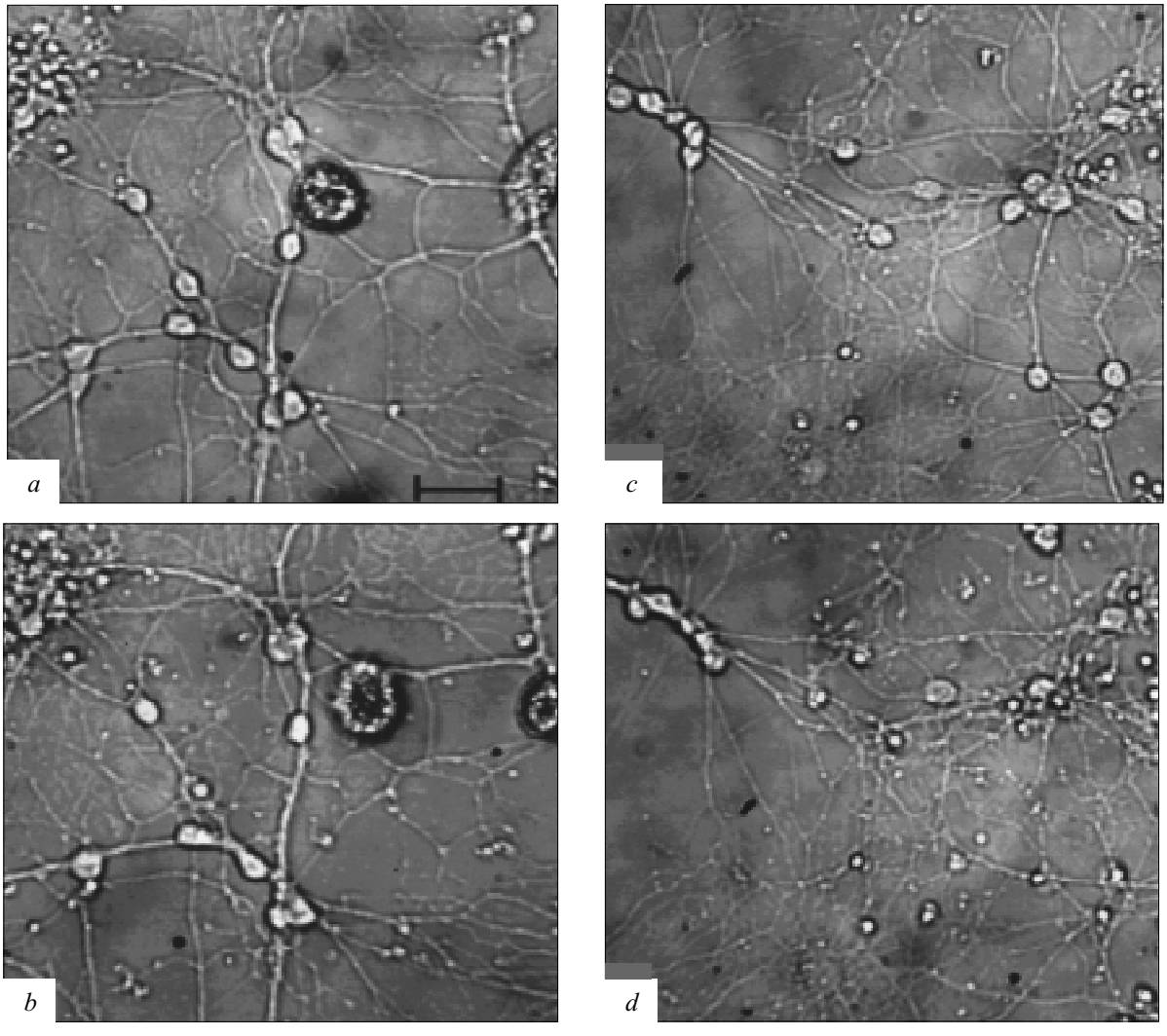

Fig. 1. Neurotoxic effect of $A\beta_{25-35}$ (50 μM) on cultured cerebellar granule cells. Intravital dark field microscopy (scale 25 μ). *a*) 7-day-old culture *in vitro*; *b*) the same field 48 h after addition of $A\beta_{25-35}$; *c*) 8-day-old culture *in vitro* 24 h after addition of $A\beta_{25-35}$; *d*) the same field after 72 h.

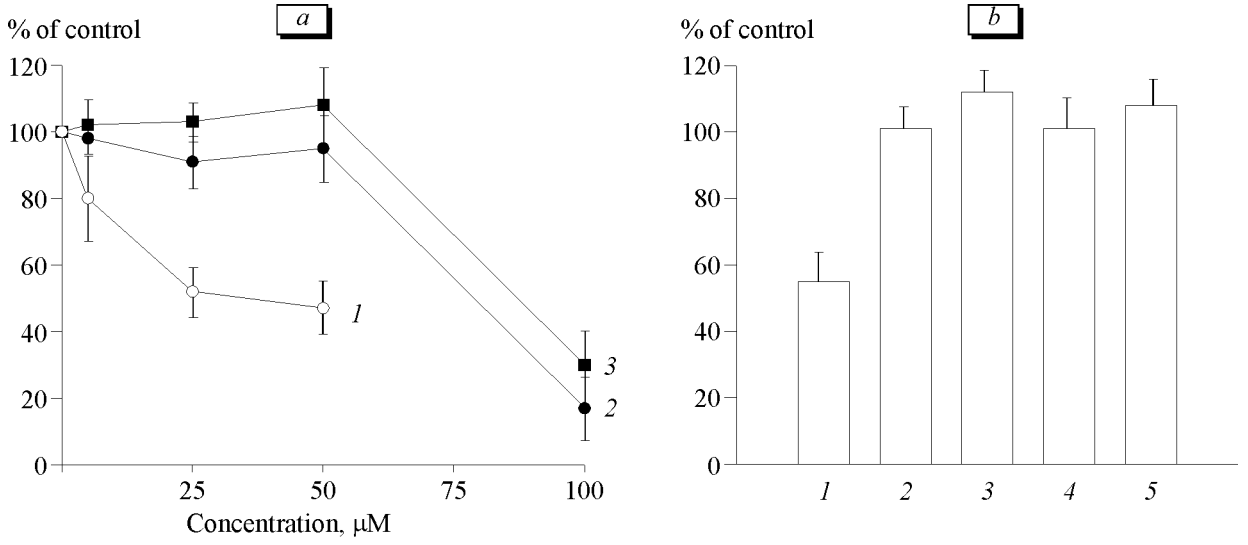

Fig. 2. Effect of dimebon, tacrine, and $A\beta_{25-35}$ on survival of cerebellar granule cells during 3-day incubation. *a*) individual effects of $A\beta_{25-35}$ (*1*), dimebon (*2*), and tacrine (*3*) applied in various concentrations; *b*) effect of dimebon and tacrine on toxicity of $A\beta_{25-35}$ during individual or combined incubation of the following substances in equimolar concentrations (25 μM): *1*) $A\beta_{25-35}$, *2*) $A\beta_{25-35}$+dimebon, *3*) $A\beta_{25-35}$+tacrine, *4*) dimebon, *5*) tacrine. Ordinate: mean survival of neurons evaluated by the content of lactate dehydrogenase. *b*) all differences with $A\beta_{25-35}$ are significant.

RESULTS

On day 7 in culture, the neurons formed long branched processes (Fig. 1, *a*) and in control cultures, the neurons survived to incubation day 21. Tacrine and dimebon were toxic for 2-day-old cultures even in doses below 25 μM, while mature CGC (incubation days 7-8) survived long-term incubation with these agents in concentrations of up to 50 μM (Fig. 2, *a*). It is known that early cultures of CGC are most vulnerable to nonspecific action of physiologically active substances; maturation of protective systems and related receptor structures modifying neuronal responses to chemicals is completed by days 7-8 in culture [5]. In further experiments we used only mature CGC cultures. Degradation of CGC induced by $A\beta_{25-35}$ was accompanied by morphological alterations (fragmentation of processes, uneven soma boundaries, and shrinkage of neurons, Fig. 1, *b-d*). A pronounced decrease in the number of neurons was observed after 3-day incubation in the presence of 50 μM $A\beta_{25-35}$ (Fig. 1, *b*) and virtually all neurons died by days 4-5 (Fig. 1, *d*).

Judging from the content of lactate dehydrogenase, $A\beta_{25-35}$ induced dose-dependent death of neurons after 3-day incubation. The rate of neuronal death only slightly varied in the concentration range of 25-50 μM (Fig. 2, *a*), therefore in further experiments $A\beta_{25-35}$ was used in a concentration of 25 μM. When used with 25 μM $A\beta_{25-35}$, dimebon and tacrine in equimolar concentrations increased survival of neurons by about 45% (Fig. 2, *b*).

Since the main electrophysiological features of CGC developed by incubation days 5-6 [12], we used neurons after 6 days *in vitro*. Step depolarization of CGC membrane activated inward ionic currents. Since bathing solution contained blockers of potassium permeability (TEA and CsCl) and contained no Na^+ ions, the observed potential-activated inward currents were carried out by Ba^{2+} ions through calcium channels. These currents were completely blocked by $CdCl_2$ (1 mM), which confirmed the role of calcium channels in these currents. The currents induced by step depolarization were characterized by low amplitude at MP below -40 mV, but increased at more positive MP (Fig. 3, *a*). This voltage threshold corresponds to the high-threshold calcium channels. The maximum inward currents were recorded at MP=0 mV. The current-voltage relations (CVR) showed that the amplitude of ionic current decreased with increasing positive MP (Fig. 3, *d*, *e*). Dimebon and tacrine (5-200 μM) blocked potential-dependent currents through calcium channels by on average 20% (Fig. 3, *b*, *c*), and the maximum blocking effect was observed 1-3 min after drug application. At positive MP both agents produced a more pronounced inhibition of ionic currents. In the presence of dimebon and tacrine, CVR practically followed the control curve at negative MP, but lay above the control curve at positive MP (Fig. 3, *d*, *e*). The blocking effect of test drugs persisted even after their removal from bathing solution: 5-10-min washout produced only partial restoration of ionic currents in calcium channels. Nimodipine (10 μM), a selective blocker of L-type calcium channels [13], blocked ionic

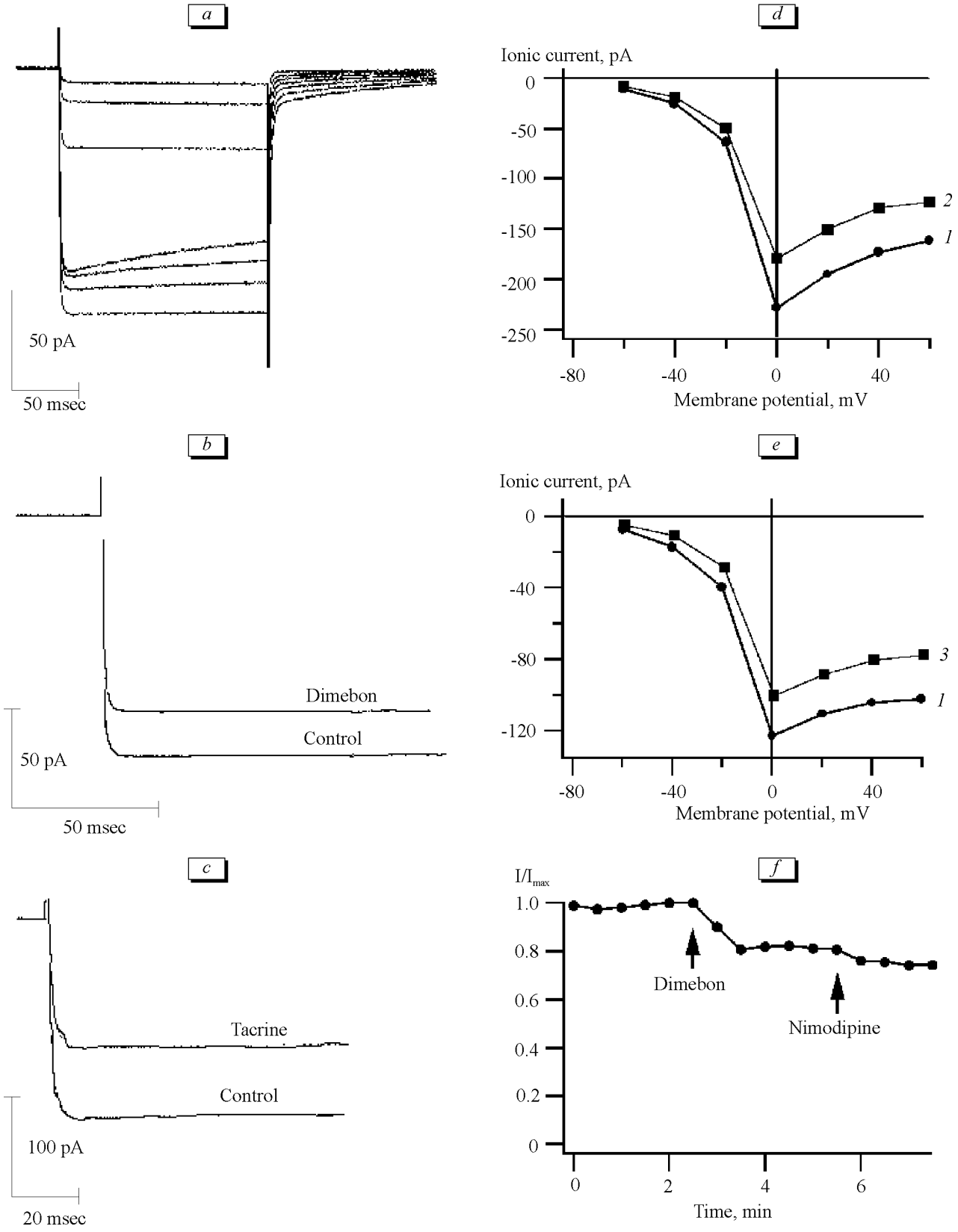

Fig. 3. Effect of dimebon and tacrine on inward currents (*a*-*c*) and current-voltage relations (*d*, *e*) in cerebellar granule cells. Inward currents: *a*) normal, *b*) effect of dimebon, *c*) effect of tacrin; current-voltage-relationships: control (*1*), in the presence of tacrine (*2*) or dimebon (*3*); *f*) changes in calcium permeability of cerebellar granule cells after successive addition of dimebon and nimodipine (time of application are shown by arrows). The currents were activated every 30 sec by stepping membrane potential from -80 to +10 mV.

currents by on average 20%. When added to washing solution after attaining the maximum of dimebon-induced inhibitory effect, nimodipine had practically no effect on residual ionic currents (Fig. 3, *f*).

Cultured neurons are widely used to study the mechanisms of action of β-amyloid and the search for potential inhibitors of its neurotoxic effect [6,9]. The peculiarities of the effect of β-amyloid on cultured neurons (relatively high concentrations and long-term exposure) were reported elsewhere [6,9]. The long-term exposure of neurons to $A\beta_{25-35}$ is necessary to reveal its toxic effect; in particular, it can stimulate generation and accumulation of free radicals, which promote cell death [9,15]. During long-term incubation, the calcium-dependent phase of neurotoxic effect of β-amyloid becomes apparent; this can be prevented by L-type calcium channel blockers, *e.g.* nicardipine [4] or nimodipine [6,15]. It was demonstrated that tacrine inhibits Ca^{2+} entry into nerve cells via L-type calcium channels [7]. This property together with inhibition of acetylcholine esterase can underlie the anti-alzheimer effect of tacrine. Similar inhibitory effects of dimebon and nimodipine (max about 20%) and the absence of additivity of their effects in successive application suggest that these drugs share the common targets on CGC membrane, L-type calcium channels. The maximum blocking effects of dimebon, tacrine, and nimodipine were about 20% total calcium permeability of the membrane. Incomplete blockade of calcium channels was due to the presence of several types of potential-dependent calcium channels in CGC membrane (L-type Ca^{2+} channels constitute 15-25% channels) [12]. It was hypothesized that $A\beta_{25-35}$-induced peroxidation can change the ratio of cations in cells, which induces opening of potential-dependent calcium channels, disturbances in calcium homeostasis, and neuronal death [15]. Dimebon and tacrine inhibit the toxic effect of $A\beta_{25-35}$ by about 45% (Fig. 2, *b*), although it remains unclear, whether their protective effect on cultured neurons is realized exclusively via partial blockade (20%) of calcium currents. Therefore, this problem calls for further studies. Here we showed that dimebon and tacrine exert neuroprotective effect against $A\beta_{25-35}$ and inhibit Ca^{2+} entry into neurons through L-type potential-dependent calcium channels. Probably, these properties underlie the therapeutic effects of the examined drugs in ATD patients with disturbed cognitive functions.

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