ACTION OF TRIFLUOPERAZINE ON PHYSICOCHEMICAL PROPERTIES AND THE PROTON PUMP OF RAT BRAIN SYNAPTIC VESICLE MEMBRANES

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Neuroleptics of the phenothiazine series and, in particular, trifluoperazine (TF), are widely used as specific antagonists of calmodulin (CM), for they block its various physiological effects in different cells, including in neurons and synaptic structures. It has been shown [9, 10] that TF, by interacting with CM, inhibits Ca-dependent phosphorylation of membrane proteins of brain synaptic vesicles (SV), transmitter release from isolated brain SV in the presence of Ca^{++}, CM, and Mg-ATP, and interaction of isolated SV with the fraction of synaptic junctions in the presence of the above-mentioned effectors.

Meanwhile TF (like other neuroleptics of the phenothiazine series) may also have a membranotropic action, which is not mediated through CM. Evidence of this is given by the blocking by TF of active uptake of biogenic amines by the corresponding brain nerve endings [4], and direct inhibition of the Ca-ATPase of the sarcoplasmic reticulum, by-passing the CM-dependent stage [2].

In connection with the facts described above, the investigation described below was undertaken to study the various pathways of action of TF on some physicochemical properties and on the functioning of the H^+ pump of membranes of isolated SV of the rat brain.

EXPERIMENTAL METHOD

The SV fraction was isolated from whole brain (without the cerebellum) of rats weighing 150-200 g by the method in [1] with certain modifications. Unpurified synaptosomes (10,000 g, 10 min) were subjected to osmotic shock, by suspending the residue in 10 mM Tris-HCl, pH 7.4 (in the proportion of 4 ml of solution to the residue obtained from 1 g of brain tissue). After freezing to −20°C and thawing the suspension, in which the EDTA concentration was adjusted to 1 mM, was centrifuged for 30 min at 18,000 g, after which the supernatant was centrifuged for 40 min at 55,000 g. The resulting supernatant was centrifuged for 40 min at 120,000 g and the residue constituted the SV fraction. This fraction was suspended in 0.25 M sucrose containing 20 mM Tris-HCl, pH 7.4. The preparation used had been frozen and thawed only once. The protein concentration was determined by Lowry's method.

The intensity of scattering of light (I_{sc}) of the SV suspension in storage medium was measured at an angle of 90°, at a wavelength of 650 nm (37°C), with constant mixing, on an MPF-4 spectrofluorometer (Hitachi, Japan) [1]. Activity of the H^+ pump was judged from Mg-ATP-induced H^+ uptake by isolated SV by means of the fluorescent probe, acridine orange [7]. Mg-ATPase activity was determined by the method described previously [%] in the presence of 0.3 mM N-ethylmaleimide and in the absence of this thiol reagent. The difference between them corresponds to H^+-ATPase activity. The TF used was of Soviet manufacture.

EXPERIMENTAL RESULTS

With an increase in the TF concentration from 10 to 120 μM a steady and considerable increase in I_{sc} of the SV suspension (20 μg protein/ml) was observed compared with the control (Fig. 1a). Within this range of TF concentrations I_{sc} of the SV suspension rose rapidly (in 1-2 min) to its peak value, after which it remained unchanged for 30-40 min of incubation. In

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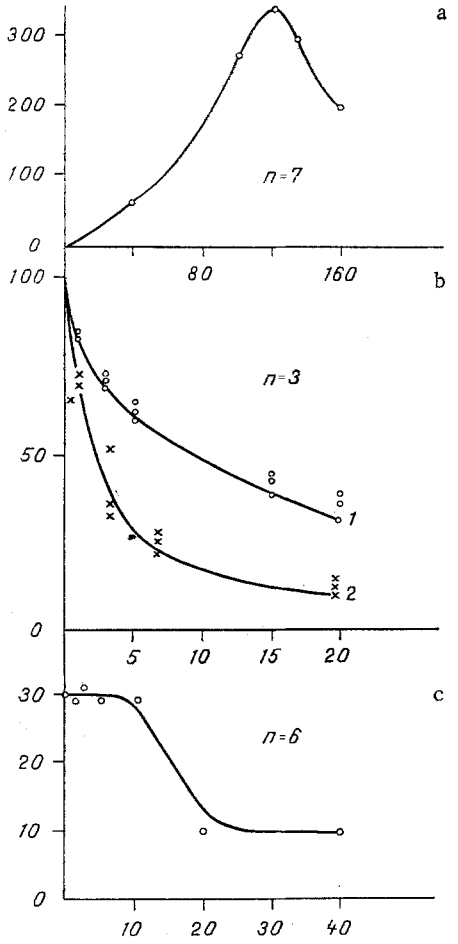

Fig. 1. Changes in I_{sc} (a, c) and sedimentation (b) of brain SV suspension under the influence of TF. Abscissa: a) TF concentration (in μM), b) duration of centrifugation (in min), c) protein concentration of SV suspension (in μg/ml); ordinate: a, c) change in I_{sc} (in % of initial value) after addition of TF, b) I_{sc} of supernatant after centrifugation of SV suspension in storage medium at 55,000 g. a, b) SV protein concentration 20 μg/ml, c) TF concentration 20 μM. During incubation of SV suspensions for 40 min without TF, initial value of I_{sc} was unchanged. 1) Control; 2) TF (60 μM); n) number of experiments.

the presence of higher TF concentrations (120-160 μM) this effect became weaker, so that the maximum of the action of TF appeared at a 120 μM (Fig. 1a). Incidentally TF, in high concentrations, caused I_{sc} of the suspension to fall gradually after its rapid rise, so that a steady-state value was reached 40 min after addition of TF. This final and steady-state value of I_{sc} was used when calculating the results shown in Fig. 1a. If the TF concentration exceeded 200 μM and pH exceeded 7.4, the appearance of a residue was observed.

The considerable increase in I_{sc}, by analogy with the effect of Ca-induced aggregation described by the writers previously [1], suggested that TF also induces aggregation of SV. To test this hypothesis, we studied changes in sedimentation of the SV suspension in a buffered sucrose medium during the action of TF (60 μM). TF considerably accelerated sedimentation of the SV suspension at 55,000 g (**Fig. 1b**). The results thus did not contradict the hypothesis but, at the same time, they could also be explained by a change in the surface properties of the SV membranes.

Dependence of the intensity of the effect of TF (20 μM) on the SV content in the incubation medium was studied. With an increase in the SV content in the samples (1-40 μg protein in 1 μl) a linear increase in I_{sc} was observed, which the writers observed previously [1]. The degree of aggregation (ΔI_{sc}) is a hyperbolic function of the concentration of the object

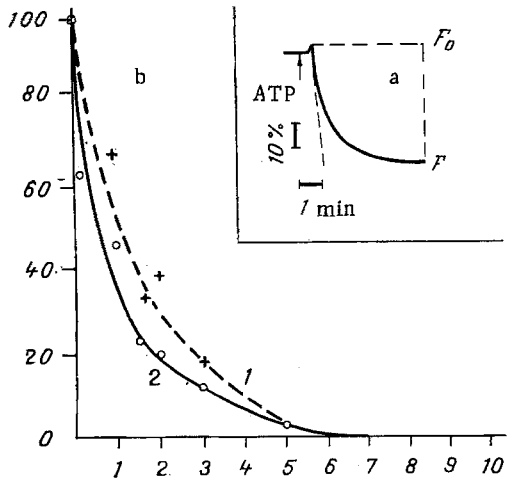

Fig. 2. Action of TF on active H^+ transport across SV membrane. a: abscissa, time (in min); ordinate, intensity of fluorescence (I_{fl}) of 2 μM acridine orange (in per cent of original value [7]). Typical curve of change in I_{fl} shown after addition of 1 mM Mg-ATP to SV suspension (15 μg protein/ml, 150 mM KCl, 20 mM HEPES-Tris, pH 7.4, 25°C). Initial velocity and total accumulation of H^+ calculated from the graph [3]; b: abscissa, TF concentration (in μM); ordinate, initial velocity (1) and total accumulation of H^+ (2), expressed in % of control in absence of TF. Results of 3 experiments shown.

[6], and as a result, the intensity of the effect ought to increase under these circumstances. In this part of the experiments (Fig. 1c), for each point the value of I_{sc} of the initial suspension of SV without TF was taken as 100%. As will be clear from Fig. 1c, within the range of SV protein concentrations from 1 to 10 μg/ml the intensity of the TF effect was constant, but in the presence of higher concentrations of SV protein (up to 40 μg/ml) a decrease of this parameter was observed. This indicated that a definite relationship between concentrations of TF and SV is necessary for maximal manifestation of the effect of TF (at least 2 μmoles of reagent to 1 mg of SV protein). Thus the hypothesis that TF can induce aggregation of a suspension of SV does not explain all of the facts obtained. It may be that TF has a membranotropic action, leading to a change in the refractive index of the membranes and, correspondingly, to a change in I_{sc}. This agrees with the results showing that TF (20-100 μM) considerably reduces Ca-induced SV aggregation, recorded [1] as an increase in I_{sc} of the SV suspension during the action of 1 mM calcium.

An important component of the SV membrane is the proton pump, which maintains an acid medium inside the vesicles [5, 7]. Changes in fluorescence of acridine orange, induced by addition of Mg-ATP to the SV suspension, enables the activity of the H^+ pump to be characterized quantitatively on the basis of the initial rate of quenching of fluorescence, which is proportional to the velocity of H^+ transport, and the total accumulation of protons $F_0 - F/F$, which is proportional to the H^+ concentration gradient achieved across the SV membrane [3], as shown in Fig. 2a. TF in micromolar concentrations inhibited active H^+ transport (Fig. 2b), reducing both the velocity and total accumulation of protons ($K_{0.5}$ = 1 μM).

There are two possible mechanisms of the effects of TF observed: 1) TF directly inhibits activity of the H^+ pump, and this must be manifested as parallel inhibition of corresponding ATPase activity; 2) the effect may be due to the weakly basic properties of TF which, in the undissociated, uncharged form, is able to pass through the membrane, and, becoming protonated inside the vesicles in the weakly acid medium, abolishes the H^+ gradient. Many weak bases, especially ammonium salts, act similarly [7].

Measurements of ATPase activity in the presence of 5 μM TF showed that it increased the ATPase activity of SV membranes, which is sensitive to N-ethylmaleimide, slightly (by 20.3%, n = 3), but did not affect its basal activity. This uncoupling effect of TF on H^+-ATPase rules out the possibility of direct inhibition of the H^+ pump and, consequently, inhibition of H^+ transport takes place through a mechanism of penetrating weak bases. The high efficiency

of TF in this process must be noted: as reflected in their $K_{0.5}$ values they are located among the strongest acidotropic substances [8], and are exceeded only by chloroquine (quingamin).

TP is known to disturb the functions of lysosomes in liver cells, acting through an increase of pH inside the lysosomes [13], without affecting the mg-ATPase activity of the chromatin granules [12]. The neuroleptic chlorpromazine, which is related to TF, lowers the transmembrane potential of the chromaffin granules of the adrenals, formed during operation of the H^+ pump [11] and causes rapid dissociation of the pH gradient on addition to coated vesicles from bovine brain immediately after ATP [14].

Thus TF, in micromolar concentrations, affects various functions of brain SV, evidently bypassing the CM stage. These facts must be taken into account when TF is used as an antagonist of the biological effect of CM in cells, and also during the study of the mechanism of action of neuroleptics.

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