The effect of some peptides from the hibernating brain on Ca^{2+} current in cardiac cells and on the activity of septal neurons

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Abstract The effects of the peptides TSKYR and DY isolated from the brain of hibernating ground squirrels on Ca^{2+} current were studied. TSKYR activated Ca^{2+} current in frog auricle fibers and in single cells from frog ventricle whereas DY blocked Ca^{2+} current in both preparations. In isolated rat and ground squirrel cardiocytes, TSKYR had no effect on Ca^{2+} current, and DY increased it. In brain slices of rat, DY blocked the activity of medial septal neurons. TSKYR increased activity of septal neurons at the initial phase, which was followed by decrease of neuronal activity.

Key words: Hibernation; Peptide; Ca^{2+} current; Cardiocyte; Patch clamp; Septal neuron

1. Introduction

Studies of different aspects of the hibernation phenomenon suggest that specific features of the hibernator cells, organs and the whole organism are controlled by endogenous factors [1,2]. In an attempt to isolate such factors from the brain of hibernators (*Citellus undulatus*) [3,4], we proceeded from the hypothesis that endogenous peptides that affect the amplitude and kinetic characteristics of Ca^{2+} current in cardiocytes may be involved in regulation of the biochemical reactions that determine the entry into, and exit from, hibernation. This hypothesis is based on dramatic changes in cardiac rhythm [5] and voltage-dependent Ca^{2+} currents in cardiocytes of hibernators during the hibernating–awakening cycle [6,7] and the effect of Ca^{2+} channel agonists and antagonists on regulation of metabolism in the cells as well as in the whole organism [8,9]. We identified a series of peptides from the brain of hibernating animals capable of regulating Ca^{2+} current in cardiac cells [4,10]. It is likely that at least two of these peptides, TSKYR (isolated originally [11] from the bovine brain as a weak analgesic) and DY, play some special role at different stages of the hibernating–awakening cycle in hibernators [12]. The peptides TSKYR and DY were identified by their effect on Ca^{2+} current in frog auricle. Therefore it is important to know how these peptides influence Ca^{2+} current in cardiocytes of hibernators and homoiothermal animals. In addition, it is well known that the activity of some brain structures of hibernators undergoes dramatic changes during the hibernating–awakening cycle [13]. Particular attention in hibernation has been paid to neurons of the medial septum regarded as a critical link in control of the forebrain level of activity in hibernating–awakening cycle [14,15]. From this viewpoint it was important to assess the action of TSKYR and DY on the activity of neurons from this area.

The present study is concerned with the effect of TSKYR and DY on voltage-dependent Ca^{2+} currents in cardiac cells of hibernators, rats, amphibians and on the activity of the medial septum–diagonal band complex in slices taken from the rat brain.

2. Materials and methods

2.1. Peptide identification

Sequential separation of brain extract of hibernating ground squirrels was carried out as described in preliminary reports [3,4]. At the initial stages of separation all fractions were tested for hypothermic and antimetabolic activity (changes of body temperature and oxygen consumption in white mice after intraperitoneal injection). By this means the activity fraction containing substances with a molecular mass of 0.5–2 kDa was found. The activity of the initial extract and analogous fractions of non-hibernating ground squirrels in summer was more than 10-fold less. However, the tests on hypothermic and antimetabolic activity are unsuitable for further HPLS separated fractions since, at a certain step, the amounts obtained proved insufficient for sequence determination. We found that the fractions active in hypothermic and antimetabolic tests were also able to alter voltage-dependent Ca^{2+} current in frog auricle trabeculae. It was a good test for the further separation of active 0.5–2 kDa fraction. Fig. 1 shows a reverse-phase HPLC profile of 0.5 mg fraction on Nucleosil $7C_{18}$ column equilibrated with 0.1% TFA in the concentration gradient of acetonitrile (0–60% for 90 min). Elution rate was 0.5 ml/min. Peptide DY was isolated from the zone of chromatogram which contained the substances decreasing the Ca^{2-} current and TSKYR was isolated from the zone containing the substances that activate Ca^{2+} current. Primary structures of peptides were determined by means of gas phase sequenator (Applied Biosystem, model 477). Peptides were synthesized conventionally and all further experiments were performed with synthetic analogs.

2.2. Isolation of heart cells

Ca^{2+}-tolerant cardiocytes were isolated from the hearts of frog (*Rana ridibunda*), rat (Wistar strain) and Yakutsk ground squirrel (*Citellus undulatus*) using the method described previously [7]. Ground squirrels caught in Yakutia in late summer of 1992–1995 were kept under conditions approximating the natural environment. The experiments were performed from December to March on cardiocytes from animals being in two different physiological states: hibernation and active during spontaneous arousal. The body temperature of sleeping ground squirrels was 6–7.5°C and that of awake animals was 37°C.

2.3. Current recordings in isolated single cardiocytes

Currents were recorded using the perforated patch-clamp technique [16]. The bathing solution contained 20 mM glucose, 2 mM $CaCl_2$, 10 mM HEPES, 80 mM NaCl, 20 mM TEA-HCl, 1.2 mM KH_2PO_4, 10 mM CsCl (pH ≈7.3). The pipette solution consisted of 130 mM CsCl, 5 mM $MgCl_2$, 10 mM HEPES (pH ≈7.3). The solutions were filtered through a 0.22 μm filter (Millipore, USA). The pipettes were fabricated from soft molybdenum glass; their resistance was

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1∼5 MΩ. Amphotericin B (0.5 mg), which served as a perforating compound, was dissolved in 9 μl of DMSO. Then 3 ml of the pipette solution were added to the antibiotic solution. The fresh antibiotic-containing pipette solution was used within 2–2.5 h after its preparation. The final concentration of antibiotic in the pipette was close to saturating (150∼170 μg/ml). Vortex mixing or sonication was not used. As perforation occurred, the capacitive current increased, and the access resistance (≈5∼10 MΩ), as well as the time constant of the current resolution, were reduced. Membrane permeabilization was completed within 10∼15 min. After this, the current remained unchanged for about 1.5 h and, in some experiments, for 2.5 h. All experiments were carried out at room temperature (20∼22°C). While measuring Ca^{2+} currents in cardiac cells of rat, rapid Na^{+} current was blocked by tetrodotoxin (10^{-5} M). The measurements of Ca^{2+} current in cardiocytes of ground squirrel (both in hibernation and active states) were performed usually without the blocker of Na^{+} channels according to previous findings [17]. The holding potential was −50 mV in all experiments. In the present study the outward membrane currents was blocked by Cs and TEA and we call the total inward membrane current (evoked by shifting the membrane potential from −50 mV towards depolarization) Ca^{2+} current because the other inward currents in our conditions are held to a minimum.

2.4. Ca^{2+} currents in isolated auricle trabeculae of frog (Rana ridibunda)

Ca^{2+} currents in isolated auricle trabeculae of frog (*Rana ridibunda*) were recorded by the voltage clamp mode and double sucrose gap technique [18]. The modifications of this method used by us were described earlier [19].

The ionic currents were recorded using a PCL-718 data acquisition card (Advantech Co., Ltd.) and an IBM/AT 486 computer. Most experimental data were analyzed using BioQuest software.

2.5. Preparation of slices and recording of septal neuron activity

Adult rats (180–200 g) were used for the experiments. After decapitation, the brain was removed and placed upon an agar–agar block on the vibroslicer table. Slices 400 μm thick were placed into the experimental chamber with circulating standard Ringer-Krebs solution at 30.5±0.5°C oxygenated by carbogen (95% O_2+5% CO_2). Complete change of the medium in the chamber took 1 min. The activity was recorded extracellularly by a tungsten microelectrode (tip diameter 3–5 μm) which under visual control was placed in the medial septal area of the slice. The resistance of the electrodes was 0.8–1.5 MΩ. The amplitude of spikes was conventionally amplified and recorded by a tape recorder. The peptides were added to the incubation medium at the final concentration of 10^{-6} M.

3. Results

3.1. Effect of the peptides on Ca^{2+} current

In mammalian cardiac cells dipeptide DY increased Ca^{2+} currents in 12 of 14 cardiocytes of both ground squirrel (irre-

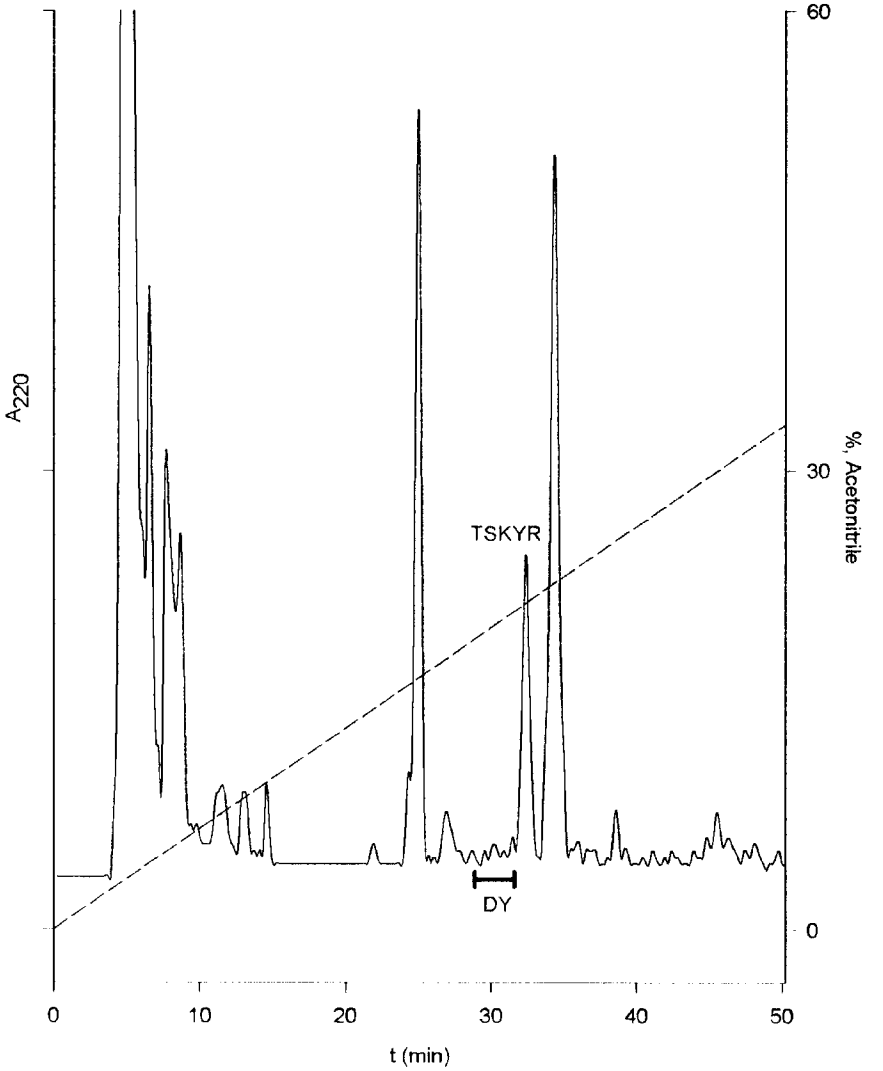

Fig. 1. HPLC profile of the 0.5 mg fraction of the brain from hibernation animals containing substances with a molecular mass of 0.5–2 kDa. The zone of chromatogram containing DY as well the peak corresponding to the TSKYR are shown.

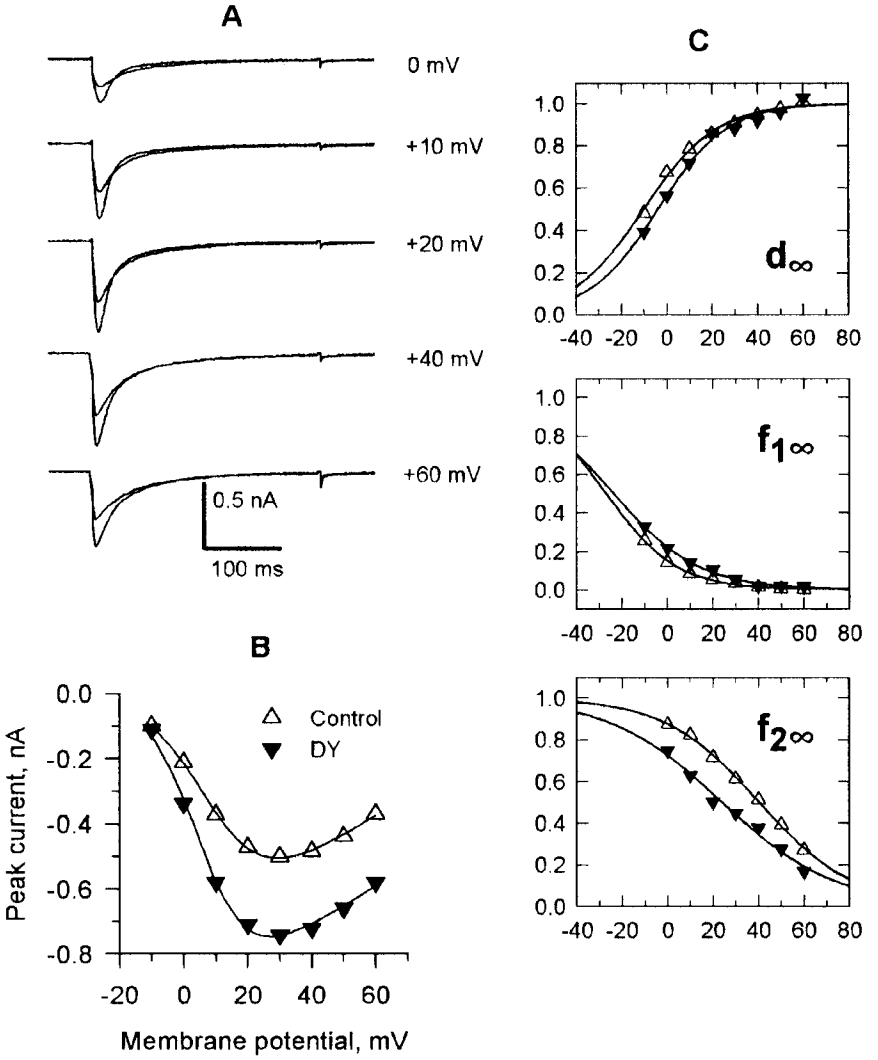

Fig. 2. DY-induced stimulation Ca^{2+} currents. A: Records of the currents on a cell of an active ground squirrel evoked by the shift of the membrane potential from −50 mV up to different levels (indicated at the right) in control (the upper trace) and 50 μM DY treated (the lower trace). B: Voltage-dependence of the peak current values. C: Calculated by Eq. 1 normalized conductances versus membrane potential: (top) activation (d_∞), (middle) slow inactivation ($f_{1\infty}$), (bottom) fast inactivation ($f_{2\infty}$). Similar data were obtained for the cardiac cells of hibernating ground squirrel.

spective of the state) and rat. Fig. 2A,B shows that DY affects both the amplitude and kinetics of Ca^{2+} currents in isolated cardiac cell. We attempted to determine which of the kinetic components of the Ca^{2+} conductance in cardiocytes undergoes the greatest changes under the influence of DY. For this, Ca^{2+} current was represented as

$$I = g_{Ca}\cdot(E-E_{Ca})\cdot\{d_\infty + (d_0-d_\infty)\cdot e^{(-t/\tau_d)}\}^m\cdot \prod_i\{f_{i\infty} + (f_{i0}-f_{i\infty})\cdot e^{(-t/\tau_{fi})}\}^{ni},\quad i=1,2 \tag{1}$$

where E is the membrane potential, g_{Ca} is the conductance, E_{Ca} is the reversal potential (in our calculations, 150 mV), d_∞, $f_{1\infty}$, $f_{2\infty}$ are the normalized steady-state conductances of activation and of slow and fast inactivations, respectively; d_0, f_{10}, f_{20} are the normalized conductances at a holding potential E_0 (in our calculations 1, 0, and 0, respectively); τ_d, τ_{f1}, τ_{f2} are the characteristic times of the variables; $m=2$, $n_1=2$ and $n_2=1$. The choice of this function for the description of calcium current was substantiated in [17]. The variables d_∞, $f_{1\infty}$, $f_{2\infty}$ were determined by minimizing the function $x^2=[I_{exp}-I]^2$ in the time interval between 0 and 300 ms by the method of Nelder-Meed [20]. Here, I_{exp} is the experimental current and I is the goal function. The calculations showed (Fig. 2C) that DY has the greatest effect on the normalized conductance of fast inactivation $f_{2\infty}(E)$; this curve shifts by approximately 15 mV towards hyperpolarization at the peptide concentration of 50 μM; the parameters of the curves $d_\infty(E)$ and $f_{1\infty}(E)$ remain virtually unchanged. It should be noted that the effect of the peptide was reversible almost in all experiments; 10–15 min after washing out the currents were restored (Fig. 3A).

Fig. 3B presents the dose–response curve of DY action on Ca^{2+} current in cardiocytes of active ground squirrel. Approximating the dose dependence of Ca^{2+} current by the Hill function

$$\frac{k_m\cdot(x/k_d)^\eta}{1+(x/k_d)^\eta} \tag{2}$$

(where k_m is the maximum effect, k_d is the dissociation con-

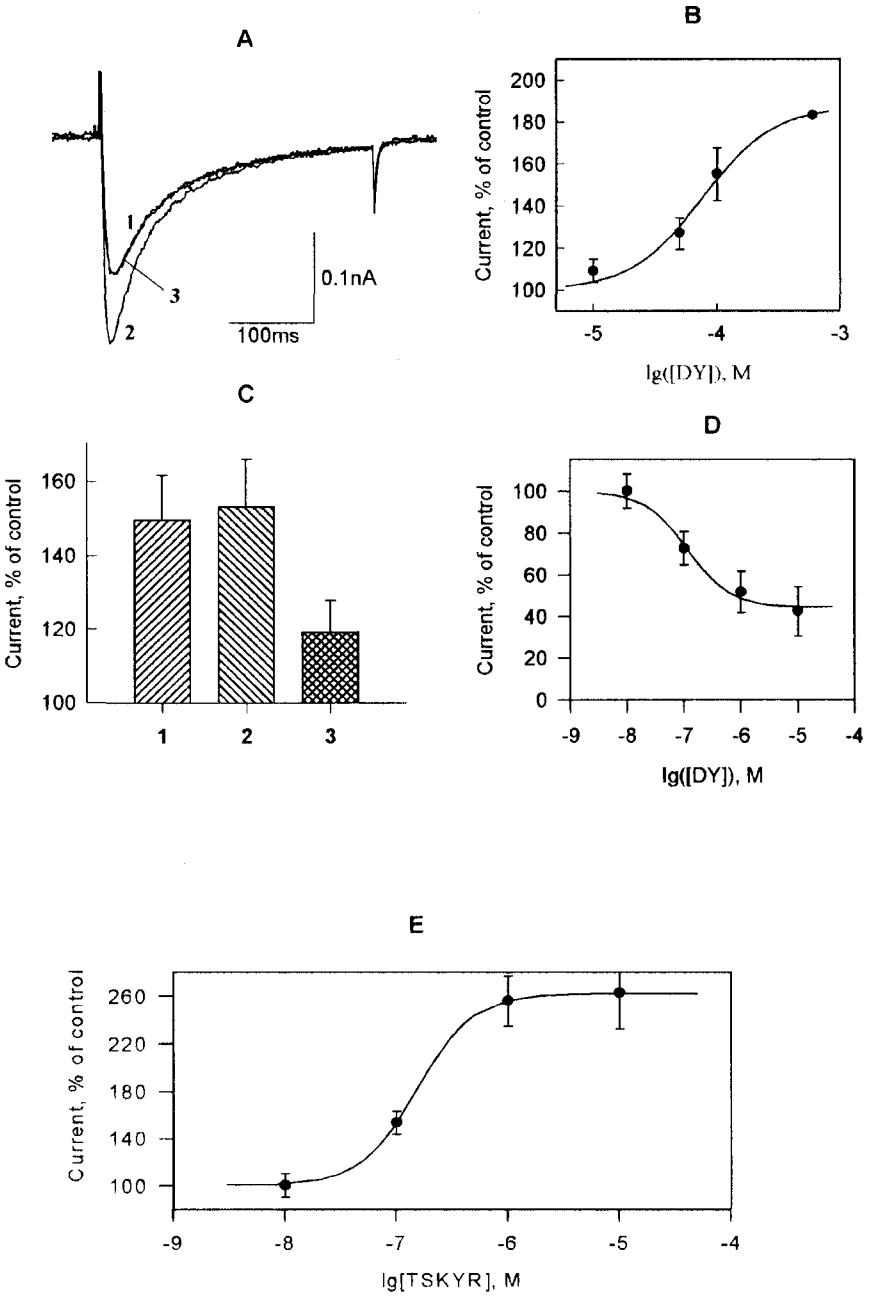

Fig. 3. The species-specific effect DY and TSKYR on Ca^{2+} currents of cardiac cells. A: Peak Ca^{2+} currents on a cell of an active ground squirrel: 1, control; 2, 5 min after administration of 10 μM DY; 3, after washing. The currents were evoked by shifting the membrane potential from −50 mV to 0 mV. B: Dose–response relation for the DY effect on Ca^{2+} currents in cardiocytes of active ground squirrels. C: Effects of 10 μM DY on Ca^{2+}-currents in cardiocytes isolated from different animals (1, hibernating squirrels; 2, active squirrels; 3, rats). D: Dose–response relation for DY effect on Ca^{2+} currents in frog auricle trabecula. E: Dose–response relation of TSKYR action on Ca^{2+} currents in frog auricle trabecula. (TSKYR had no clear effect on Ca^{2+} currents in cardiac cells of hibernating and active ground squirrels and rat.) The continuous lines on the dose–response relations for DY and TSKYR effects were obtained by fitting the experimental points using the Hill Eq. 2.

stant, η is the Hill coefficient characterizing the cooperativity of interaction, x is the concentration of the peptide) yields $k_d = 0.80$ μM and $\eta = 1.5$, $k_m = 87\%$. The Hill coefficient for the cells of rat and hibernating squirrel was the same. Fig. 3C presents the histograms of the Ca^{2+} current changes produced by DY for the active (1, $n = 5$) and hibernating (2, $n = 5$) ground squirrels and for rats (3, $n = 4$). The histograms were constructed using the peaks of Ca^{2+} currents. The peptide concentration in all experiments was 10 μM. The peptide in cardiocytes of hibernators is more than twice as effective as in rat cardiocytes. The efficiency of the peptide in the heart cells of active and hibernating animals is nearly the same.

In isolated auricle trabeculae of frog (*Rana ridibunda*) and amphibian isolated ventricle cells DY decrease (as opposed to mammalian cells) the Ca^{2+} current (Fig. 3D). Approximation of the dose–effect experimental points by the Hill function gives for Ca^{2+} currents in auricle trabeculae $k_d = 0.3$ μM, $\eta = 1.5$, $k_m = 52\%$ ($n = 5$) and indicates that the cooperativity of DY interaction with Ca^{2+} channels of amphibian and

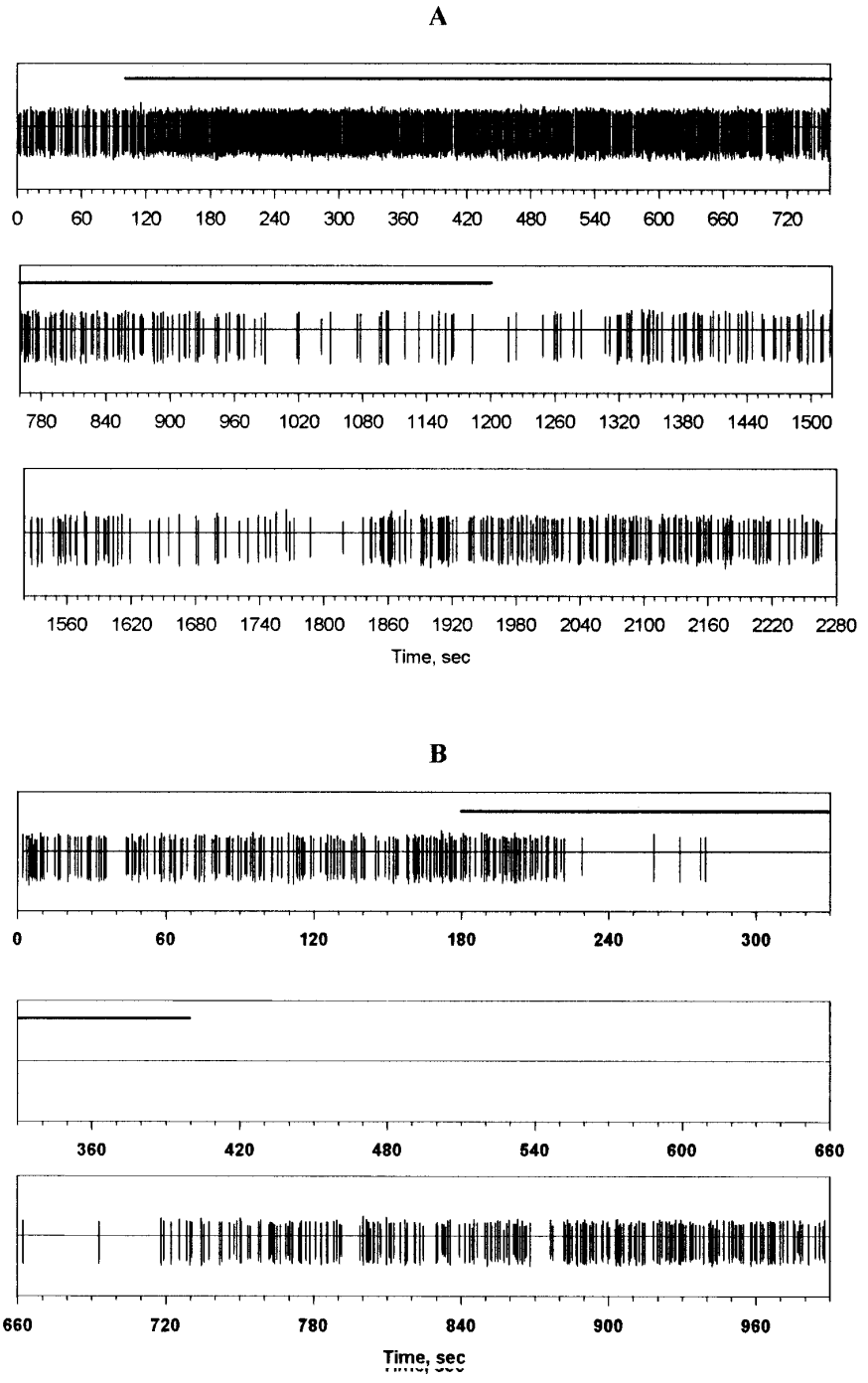

Fig. 4. The action of 1 μM TSKYR (A) and 1 μM DY (B) on neuronal activity of the medial septal brain slices of rat. Peptide application is indicated above the record of spikes by solid lines.

mammalian cardiocytes is the same though the effect on Ca^{2+} current is opposite.

Fig. 3E shows a TSKYR dose–response curve for peak Ca^{2+} currents in frog auricle fibers. The mean values of k_d, η and k_m for frog auricle fibers were 0.45 μM, 1.5, and 58%, respectively ($n=5$). TSKYR had no clear effect on Ca^{2+} currents in the cells of rat ($n=5$) and ground squirrels, either active ($n=6$) or hibernating ($n=5$).

3.2. Action of the peptides on the activity of septal neurons

The typical effects of peptides on spontaneous activity of the rat medial septum–diagonal band complex are shown in Fig. 4. Of the 10 neurons tested eight responded to application of TSKYR (1 μM). The change of spontaneous activity developed 20–30 s after peptide application and was biphasic; after the initial period (5–7 min) of increased discharge frequency, the rate of spikes gradually slowed and (in 5 of 8 neurons) decayed to zero. After washing out TSKYR the frequency of spontaneous activity slowly ($\approx$15 min) returned to initial level (Fig. 4A). DY (1 μM) was effective in 13 of the 20 neurons tested. In this case monophasic decrease of discharge frequency to very low level (5 neurons) or its complete

suppression (8 neurons) was observed (Fig. 4B). This effect was also reversible, 7–9 min after washout activity returned to the control level. The inhibitory action was retained in condition of synaptic blockade by the medium with low Ca^{2+} and high Mg^{2+} concentration.

4. Discussion

The results of this study do not fit into the usual concept of the effect of Ca^{2+} channel agonists and antagonists in cardiac cells. It is known that both in frog auricle fibers and in cardiocytes of rat and ground squirrel at the membrane potential up to −40 mV the main component of Ca^{2+} current flows through the L-type channels [7,21]. However, the pentapeptide TSKYR increases this current in auricle cells of amphibians and virtually does not change it in mammalian cells; the dipeptide DY suppresses the Ca^{2+} current in frog fibers and cells but activates it in rat and ground squirrel cardiocytes. These results show the species-specific action of both peptides on ionic transport in cardiac cells.

Our data show that the peptides isolated from the brain of hibernating animals effectively modulate the level of neuronal spontaneous activity in a non-hibernator (rat). Differential effects of TSKYR and DY on the activity of septal neurons suggest their universal participation in regulation of the central mechanisms determining the level of forebrain arousal, particularly in the control of medial septal complex, which were regarded as 'sentry post' for interbout arousal in hibernators [13–15,22]. The question to be answered is whether the neuroregulatory properties of the peptides are associated with Ca^{2+} current modification in neurons or there is another target for their action.

A certain specificity of the action of peptides on hibernator cells, as compared to non-hibernators, and their neuroregulatory function described above, may be useful in analysis of transition stages in hibernation.

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References

[1] Margules, D. (1979) Neur. Biobehav. Rev. 3, 155–162.
[2] Kondo, N. and Kondo, J. (1992) J. Biol. Chem. 267, 473–478.
[3] Vaskovsky, B.I., Ivanov, V.T., Mikhaleva, I.I., Kolaeva, S.G., Kokoz, Y.M., Svieryev, V.I., Ziganshin, R.H., Ignatiev, D.A., Sukhova, G.S. (1990) Peptides, pp. 302–304, ESCOM, Leiden.
[4] Ziganshin, R., Svieryaev, V., Vaskovsky, B., Mikhaleva, I., Ivanov, V., Kokoz, Y., Alekseev, A., Korystova, A., Sukhova, G., Emelyanova, T. and Usenko, A. (1994) Bioorg. Khim. (Russian) 20, 899–918.
[5] Lyman, C. (1984) Farmacol. Ther. 25, 371–393.
[6] Kondo, N. (1986) Circ. Res. 59, 221–228.
[7] Alekseev, A., Korystova, A., Malyutova, D. and Kokoz, Y. (1994) Biochem. Mol. Biol. Int. 33, 365–376.
[8] Berridge, M. (1993) Nature 361, 315–325.
[9] Driedzic, W. and Gesser, H. (1994) Physiol. Rev. 74, 221–257.
[10] Ziganshin, R., Mikhaleva, I., Ivanov, V., Kokoz, Y., Alekseev, A., Korystova, A., Emelyanova, T. (1996) Adaptations to the Cold (Geiser, F., Hulbent, A., Nicol, S., Eds.) Tenth International Hibernation Symposium, pp. 373–378, University of New England Press, Armidale.
[11] Takagi, H., Shiomi, H., Fukui, K., Hayashi, K., Kiso, Y. and Kitagawa, K. (1982) Life Sci. 31, 16–17.
[12] Ignatiev, D., Zagnoiko, V., Souchova, G., Bokaneva, V. and Souchov, V. (1995) Zhurnal Obshchei Biologii (Russian) 56, 450–467.
[13] Kilduff, T.S., Radeke, C.D., Heller, H.C. (1986) Living in the Cold (H.C. Heller et al., Eds.), pp. 215-224, Elsevier, Amsterdam.
[14] Heller, H. (1979) Annu. Rev. Physiol. 41, 305–321.
[15] Belousov, A., Vinogradova, O. and Pakhotin, P. (1990) Neuroscience 38, 599–608.
[16] Horn, R. and Marty, A. (1988) J. Gen. Physiol. 92, 145–159.
[17] Alekseev, A., Markevich, N., Korystova, A. and Kokoz, Yu. (1995) Biophys. J. 70, 786–797.
[18] Rougier, O., Vassort, G. and Stampfli, R. (1968) Pflügers Arch. 301, 91–108.
[19] Kokoz, Y.M., Alekseev, A.E., Povzun, A.A., Korystova, A.F. and Peres-Saad, H. (1994) FEBS Lett. 337, 277–280.
[20] Himmelblau, D. (1972) Applied Nonlinear Programming, p. 438, McGraw-Hill, Austin, TX.
[21] Campbell, D., Giles, W. and Shibata, E. (1988) J. Physiol. 403, 239–266.
[22] Pakhotin, P., Pakhotina, I. and Belousov, A. (1993) Prog. Neurobiol. 40, 123–161.