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Heart rate changes in partially restrained rats during behaviorally and pharmacologically evoked emotional states

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Abstract. The effect of fear and relief from fear on heart rate (HR) was studied in partially restrained adult male rats. The emotional state was influenced behaviorally with the use of Pavlovian aversive conditioning procedure, and pharmacologically by injections of the selected anxiolytics and anxiogenics. A signal of danger (DS) - light or tone, preceded tail-shock (excitatory trials), while a signal of safety (SS), respectively tone or light, overlapped last 3s of 5s DS and predicted an omission of this expected aversive event (inhibitory trials). To assess the stability of HR changes to DS and SS we analyzed whether and how the experimental conditions (modality and position of signals, the ratio of numbers of excitatory to inhibitory trials in the session) influenced HR. HR changes to DS were different in pattern, stability and direction when compared to HR changes in response to SS. Reactions to DS, although accompanied mainly by conditioned bradycardia, were not consistent and depended on experimental conditions. However, the SS always evoked conditioned tachycardia. Anxiolitycs - benzodiazepines (diazepam and midazolam) and buspirone - influenced HR in nonconsistent manner. Anxiogenics - PTZ and FG7142 were without significant effects on HR. HR could not be trusted therefore as direct index of pharmacologically induced emotional states. The results are discussed in the context of possible biobehavioral meaning of HR changes in response to danger and safety and their reliability as fear/relief correlates.

Key words: heart rate, fear, relief, anxiolytics, anxiogenics

INTRODUCTION

Fear is component of natural reactions to threat. It can be evoked by innate species-specific stimuli signaling danger, by novelty or uncertainty, and by stimuli associated through learning with pain or other aversive event (Bolles and Fanselow 1980, Graeff 1990, Fanselow 1994, LeDoux 1995,1997). Incorrect triggering, control or expression of fear reduces the chance of survival of an organism. In humans fear/anxiety disorders are the symptoms of serious psychiatric diseases (generalized anxiety, neurosis, phobia, panic attacks, obsessive-compulsive disorders, post-traumatic stress disorder or depression). The terms fear and anxiety are often used interchangeably, however, historically they can be differentiated on the basis of the presence or absence of cues.

One of the standard procedures for studying mechanisms of fear and anxiety is Pavlovian aversive conditioning (see LeDoux 1997). In a typical fear conditioning experiment the animal is exposed to an innocuous stimulus e.g. tone or light (conditioned stimulus CS) followed by aversive stimulation e.g. electric shock (unconditioned stimulus US). Conditioning occurs usually after few pairings and CS acquires the capacity to activate the state of fear.

From our point of view, very important advantage of this procedure is the possibility of eliciting not only the state of conditioned fear but also its inhibition. In this case a neutral stimulus, paired with the omission of shock, can play a role of a predictor of safety. Presentation of fear conditioned stimuli (danger signals DS) and conditioned inhibitors (safety signals SS) in the same experimental session allows to observe, record and compare under strictly controlled conditions, various physiological and behavioral changes concomitant to two opposite emotional states - fear, and its inhibition - the state of relief (e.g. Dess and Soltysik 1989). Since there is no direct method to determine the fear state either its onset or its inhibition, many different experimental paradigms, based on the measurable changes in the physiological state or behavior of the subject exposed to the threatening stimuli (conditioned or unconditioned) are used. For example the elicitation or modulation of fear/anxiety state can be assessed by behavioral defense responses (e.g., freezing) (e.g. Blanchard and Blanchard 1969, Bouton and Bolles 1980), changes in endocrine and visceral organs or systems (e.g., van de Kar et al. 1991), changes in the reactivity to pain stimuli (e.g. Fanselow and Helmstetter, 1988), potentiation of some reflexes (startle reflex, eyeblink reflex) (e.g. Davis 1986), suppression of appetitive, innate or operant behaviors (e.g. Estes and Skinner 1941), as well as changes in respiration (Soltysik et al. 1988) or vocalization (e.g. Cuomo et al. 1992, Borszcz 1995). More recently (due to fNMR and PET techniques) the pictures of changes in the activity of particular brain structures, are also used in neurobiological research (e.g. LaBar et al. 1998). In spite of such a multitude of experimental fear measures there is no agreement as to their reliability, and sensitivity to different classes of anxiolytic and anxiogenic drugs.

One of the most often used and, at the same time, most controversial correlates of fear is heart rate (HR). In the fear conditioning procedure HR changes to the DS are used as an index of emotional learning, although their bio-behavioral meaning is still not clear. Heart rate is discussed in the literature in the context of emotions and/or motivation (Gantt 1960, Overmier 1966, Malmo and Belanger 1967, Young and Leaton 1996), attention or receptivity to environmental events (Lacey and Lacey 1974,1978), or as a byproduct of a behavior (somatic activity) (Obrist et al. 1974, Obrist 1976).

The present experiment was undertaken in order to describe precisely the pattern of heart rate reactions to the danger signal evoking the state of fear and to the safety signal eliciting, as we assume, the state of relief. It is known that heart rate responses do not differentiate positive and negative emotions. The same direction of conditioned HR changes might be observed in case of aversive as well as appetitive reinforcement (Powell et al. 1993). We were interested however, whether the induction of conditioned fear and its active inhibition will affect HR changes in different manner.

It has been found that HR changes in response to danger signal depend on experimental arrangement (restrained vs. freely moving animals). Conditioned bradycardia in reaction to DS has been recorded in restrained adult rats when either the tail or foot-shock was used as the unconditioned stimulus (Fitzgerald and Hoffman 1976, Martin and Fitzgerald 1980, Hunt et al. 1997). The opposite effect i.e. conditioned tachycardia has been described in freely moving animals when foot shock served as the reinforcement (Martin and Fitzgerald 1980, Iwata et al. 1986, Iwata and LeDoux 1988). Little is known about the influence of experimental conditions on HR changes in rats evoked by safety stimulus. Therefore we wanted to assess the stability of HR reactions to both stimuli - DS and SS by analyzing the effect

of their modality and placement and the ratio of inhibitory to excitatory trials in the session. Effect of conditioned inhibition on HR has been usually observed in various species in the differential conditioning procedure when one stimulus (CS+) was paired with electric shock while the second (CS-) was not (Powell et al. 1971, Martin and Fitzgerald 1980, Teich et al. 1988, Buchanan 1991, Chachich and Powell 1998). In this procedure, however, the excitatory context for the learning of inhibition may be provided only by the contextual cues of experimental situation. In our experiment SS overlapped the last 3 s of 5 s DS. By using explicit conditioned inhibitor procedure the HR correlates of active inhibition of conditioned fear evoked by DS, and not only by contextual cues could be observed. To our knowledge there is no data concerning the HR changes in rats in response to SS in an experimental design such as ours.

Recording of locomotion on the treadmill was performed to find out whether the observed HR changes correlate with the overt somatic behavior. Simultaneously, in the same experiment, other then heart rate correlates of conditioned fear (respiration and ultrasonic vocalization) were measured and the pattern of their changes in response to danger and safety signals and the comparison of their stability will be a subject of another paper.

The pharmacological part of the present experiment was aimed to test the influence of selected anxiolytic and anxiogenic agents on HR reactions in response to DS and SS (or their influence on baseline i.e. pre-DS HR). We were interested whether the direction of these effects was corresponding to pharmacological profiles of used drugs (anxiolytic or anxiogenic) in comparison with the effects of DS and SS. It might be expected that anxiolytics inhibit the induction of fear by DS and/or enhance active inhibition of this emotional state by SS. On the contrary, anxiogenic drugs are expected to enhance the effect of DS and/or block the inhibition of fear by SS. It was interesting to see if the engagement of specific types of receptors play any role in these mechanisms, therefore drugs of the same pharmacological profile but from different pharmacological groups were used.

METHODS

Animals

The experiment was performed on 21 male Wistar rats weighing between 350 and 450 g. The animals were housed individually in standard laboratory conditions with food and water *ad libitum.*

Surgery

The implantation of chronic heart rate electrodes was performed under chloral hydrate (360 mg/kg body weight) anesthesia. The electrodes were inserted beneath the skin on the left forelimb and right haunch. The wires, connecting electrodes to the socket placed on the head of an animal, run under the skin. During the same surgical procedure the electrodes for chronic bipolar recordings of diaphragm EMG activity (not analyzed here) were also implanted. The implantation of the electrodes did not restrict the freedom of animals' movements.

Training

For 3 days (30 min per day) each rat was familiarized with the special apparatus (Soltysik et al. 1996). This apparatus enabled the partially restrained animals to run on the treadmill, and allowed the experimenter to detect the "locomotion" of an animal. The adaptation procedure served to attenuate tonic fear linked with the new situation and restriction of movements. On the first day of experiments only the responses to new stimuli (5 s tone, 5 s light) were assessed. These stimuli were used later as the signals of danger (DS) or safety (SS). The next day rats were divided to three groups. In the first group tone served as DS and light as SS. In the second and third groups light signaled the danger and tone safety. The only difference between two last groups was the placement of the loudspeaker, either in front or at the back of the animals. Pavlovian aversive conditioning was performed in the following 2 weeks. Daily training consisted of 10 trials separated by the intervals varied randomly between 90 s-150 s. Each trial lasted 15 s: 5 s prior to the onset of the DS, 5s DS period and the final 5s after DS offset. The DS was followed by the tailshock (3 mA, 100 ms) which was used as unconditioned stimulus (US). In inhibitory trials the last 3 s of the DS were overlapped by safety signal (SS). The SS was a predictor of an omission of the, otherwise expected, aversive US. This procedure enables the experimenter to elicit the rapid changes of animals' emotional state (from fear to relief). While the first session consisted only of 10 DS-US trials, in the sessions 2-14 five DS-US and five DS-SS trials were randomly scheduled (phase 1 of the experiment). In order to asses the effect of anxiety level

on observed reactions, after 2 weeks the proportion of excitatory to inhibitory trials in the session was changed from 1:1 to 1:2, by adding 5 additional inhibitory trials in each session (phase 2 of the experiment). After a week of the training, with this 1:2 ratio of excitatory to inhibitory trials testing the influence of anxiolytic and anxiogenic drugs on HR started. The sessions with each drug were separated by sessions without any injections.

Drugs

The following drugs were used: diazepam (Sigma, 1 and 5 mg/kg), midazolam (LaRoche, 2 mg/kg), buspirone (Sigma, 1 and 5 mg/kg), pentylenetetrazole (Sigma, 5 and 10 mg/kg) and FG7142 (RBI, 5mg/kg). Diazepam was suspended in 0.9% saline and 1% of Tween 80 and dispersed by ultrasounds. Midazolam, buspirone and pentylenetetrazole were dissolved in 0.9% saline. FG7142 was administrated in complex with 2-hydroxypropyl-β-cyclodextrin in the form of 17.5-mM water suspension. As a control - injection of 0.9% saline was used. Drugs (or saline) were injected intraperitoneally (i.p.), once a week, 30 minutes before testing, in a volume of 2 ml/kg.

Data acquisition and analysis

The animals restrained in the special apparatus were placed in sound-attenuating chamber. The camcorder mounted in the chamber enabled the experimenter to observe the behavior of animals on the monitor. During each trial the EKG signal from implanted electrodes was amplified, filtered, rectified, integrated, digitized and recorded on the computer disk. Analysis of the data was performed off line. The inter-beat intervals were calculated from raw data and converted to beats-per-minute measure of HR. The mean 15 s HR patterns for all groups of rats in excitatory and inhibitory trials were calculated. For further quantitative analysis we defined reaction to DS as the mean change in HR during 1s interval between 3.5-4.5 s from the onset of DS related to pre-DS HR. The reaction to SS was defined as the mean difference in HR in inhibitory and excitatory trials in the same period of time (3.5-4.5 s from DS onset). The effects of the following factors were tested: the presence and modality of DS and SS, the administration of anxiolytic and anxiogenic drugs. In the first phase of the experiment HR was successfully recorded from all 21 animals while in the second phase from 20 rats. "Locomotion" of the animals was also recorded. The apparatus enabled the detection of the belt movement by a photocell activated through the holes in the circular sidewall of the horizontal cylinder - the part of the tread-belt mechanism. The signal from the photocell was transformed, digitized and recorded on the computer disk simultaneously with the HR signal. To test the effect of modality and position of used signals and the effect of the phase of the experiment on analyzed reactions, we used ANOVA and post-hoc Duncan tests. ANOVA and Duncan tests were also used to compare the effects of used drugs to effects of saline on pre-DS HR, HR reaction to DS and HR reaction to SS. Additionally two-tailed t-test was used to find out if the particular mean HR reaction to DS or SS in each experimental group and phase was statistically significant.

RESULTS

The mean patterns of HR changes in response to signals of danger (DS) and safety (SS) in fear conditioning procedure for 3 groups of rats and 2 phases of the experiment are presented on Fig. 1. To focus the attention on the analyzed reactions, the figure shows only 6 s interval of 15 s trial: 1 s immediately preceding the DS and 5 consecutive seconds when DS or DS/SS compound was presented. Generally, although the reactions to the onset of DS were different and depended of DS modality, during the last 3 s of DS decrease in HR was observed. This decrease lead to obvious bradycardia in relation to pre-DS period or only to return to pre-DS HR (when the onset of DS evoked increase in HR).

In the group 1, (where tone served as DS and light as SS) the onset of the DS induced increase in HR in both phases of the experiment (i.e. irrespective of the proportion of numbers of excitatory to inhibitory trials in the session). During the last 3 s of DS, clear progressive decrease in HR resulting in return to the baseline value is seen but only in the second phase. In the groups 2 and 3 where the opposite modality of signals was applied (light-DS and tone-SS), generally the conditioned bradycardia was observed in response to DS. Only in the group 3 in the first phase of the experiment this effect is not seen, because the baseline (pre-DS) HR was there very low (compare HR patterns in phase 1 and phase 2 for group 3). The analysis of pre-DS HR by two-way mixed design ANOVA (3 groups x 2 phases) showed significant group and phase interaction ($F_{2,17} = 5.83$, $P<0.02$). The separate ANOVAs performed for both phases of the experiment demonstrated significant group

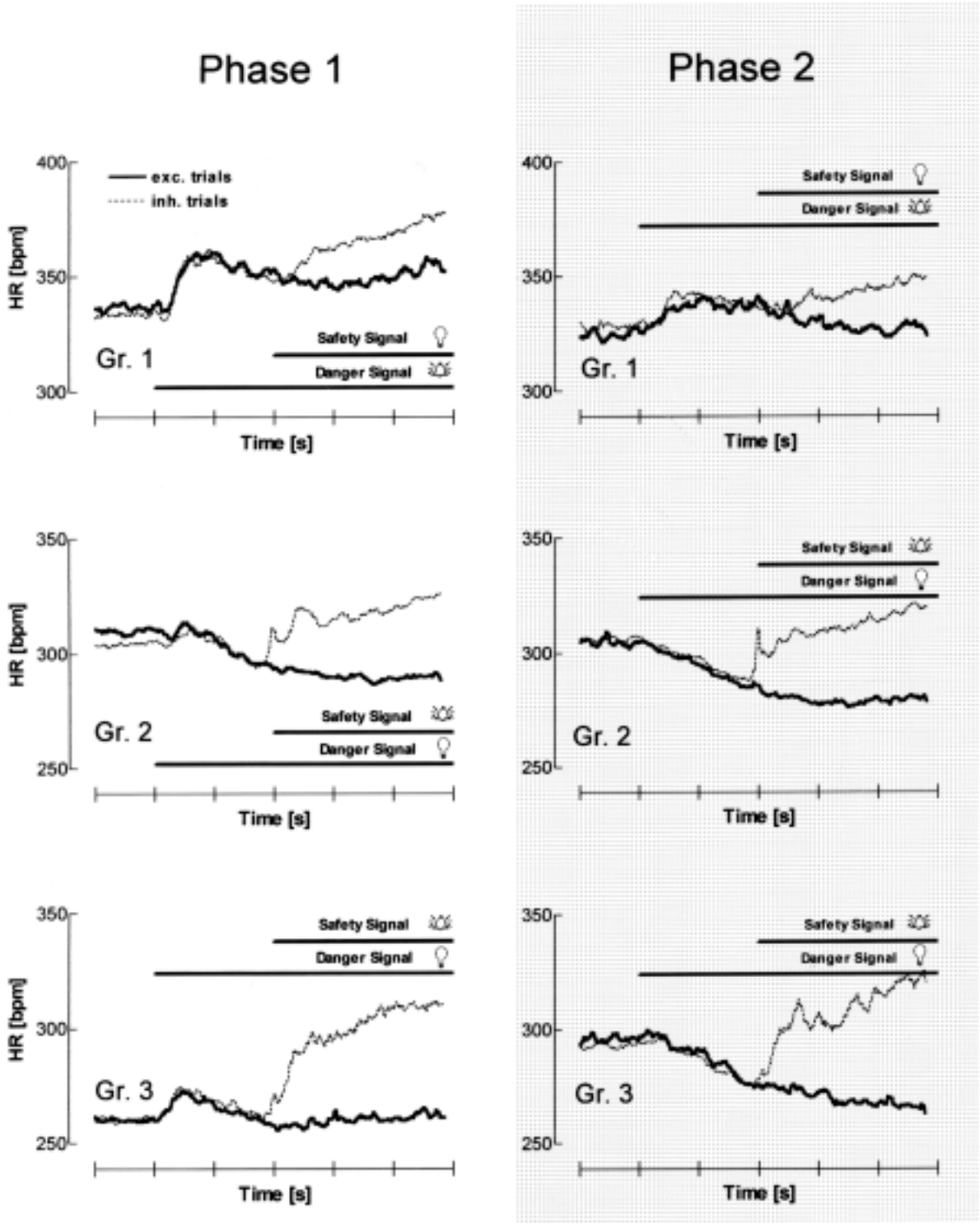

Fig. 1. Patterns of HR reactions to signals of danger (DS) and safety (SS). Phase 1, the first part of an experiment when the proportion of numbers of excitatory trials to inhibitory trials was 1:1 (the mean pattern of HR reactions was calculated for sessions 7-14); Phase 2, the second part of an experiment when proportion of the numbers of excitatory to inhibitory trials was equal to 1:2 (the mean pattern of HR reactions was calculated for 6 sessions immediately preceeding drug sessions). Gr. 1, group 1 (DS=TONE, SS=LIGHT); Gr. 2, group 2 (DS=LIGHT, SS=TONE in front of the rat); Gr. 3, group 3 (DS=LIGHT, SS=TONE from the back).

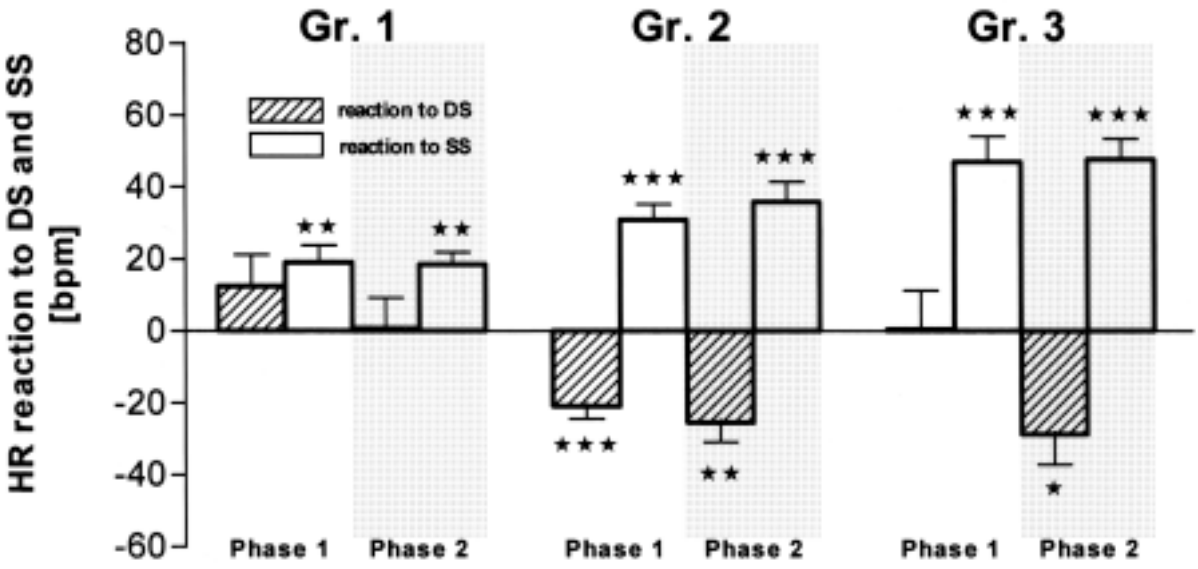

Fig. 2. Mean HR reactions (± SEM) to signals of danger (DS) and signals of safety (SS); *P<0.05, **P<0.01, ***P<0.001 (t-test).

effect but only in the phase 1 ($F_{2,17}$ = 24.7, P<0.00001). This was mainly due to low pre-DS HR in the group 3.

Reaction to signal of safety was qualitatively identical in all 3 groups of animals and both phases of experiment. It consisted in progressive increase in HR during SS presentation. The strength of observed effect was greatest in tone-SS group when the signal of safety was presented from the back of an animal. The use of light as DS and tone as SS allowed for better dissociation of HR changes in response to fear and relief (danger/safety). To assess quantitatively these qualitative observations the reactions to DS and SS were further analyzed according to definitions presented in the section "Methods". These definitions were based on the assumption that conditioned fear should progressively increase during DS presentation and the increase should be expressed best at the end of DS duration just before aversive stimulation. Mean HR reactions to DS and SS are presented on Fig. 2. They were analyzed separately by two-way mixed design ANOVA (3 groups x 2 phases). The analysis of the reactions to DS showed statistical significance of group x phases interactions ($F_{2,17}$ = 7.28, P<0.005), and both factors alone: groups ($F_{2,17}$ = 4.08, P<0.04), and phases ($F_{1,17}$ = 33.67, P<0.0002). Because of significant interactions the group differences were additionally analyzed separately for both phases of the experiment with the use of one-way ANOVA. Again for both phases the group differences were statistically significant (phase 1: $F_{2,18}$ = 4.15, P<0.04; phase 2: $F_{2,17}$ = 4.44, P<0.03). It can be said that the best conditions to observe conditioned bradycardia in our experiment was light-DS in the second phase, when the ratio of the excitatory to inhibitory trials in the session was equal to 1:2. Signal of Safety (SS) induced increase in HR. The strength of reaction was dependent on experimental group (modality of stimuli and position of SS) ($F_{2,17}$ = 8.96, P<0.003) but not on the phase of experiment.

In order to check the possible influence of the new stimuli (light or tone presented separately) on the heart rate, the results of the experimental session preceding the proper conditioning (see Methods section) were analyzed. The two-way mixed design ANOVA (3 groups x 2 modalities) performed for all 21 rats showed significant interaction of group and the signal modality ($F_{2,18}$ = 4.65, P<0.03). The separate analysis performed for light and tone-stimuli showed significant group differences only for tone-stimuli ($F_{1,12}$ = 7.78, P<0.02). Performed post hoc Duncan tests demonstrated that HR changes induced by tone presented from the back (bradycardia in group 3) are different from HR changes induced by tone presented in front of the rat (tachycardia in group 1 and group 2) (Fig. 3).

The effects of drugs on baseline HR were analyzed in the groups 1 and 2, where the same agents were used, by two-way ANOVA (2 groups x 6 saline and drugs) and separately in the group 3 by one-way ANOVA (6 saline and drugs). The results are presented on Fig. 4. Neither group effect nor interaction was shown by the first

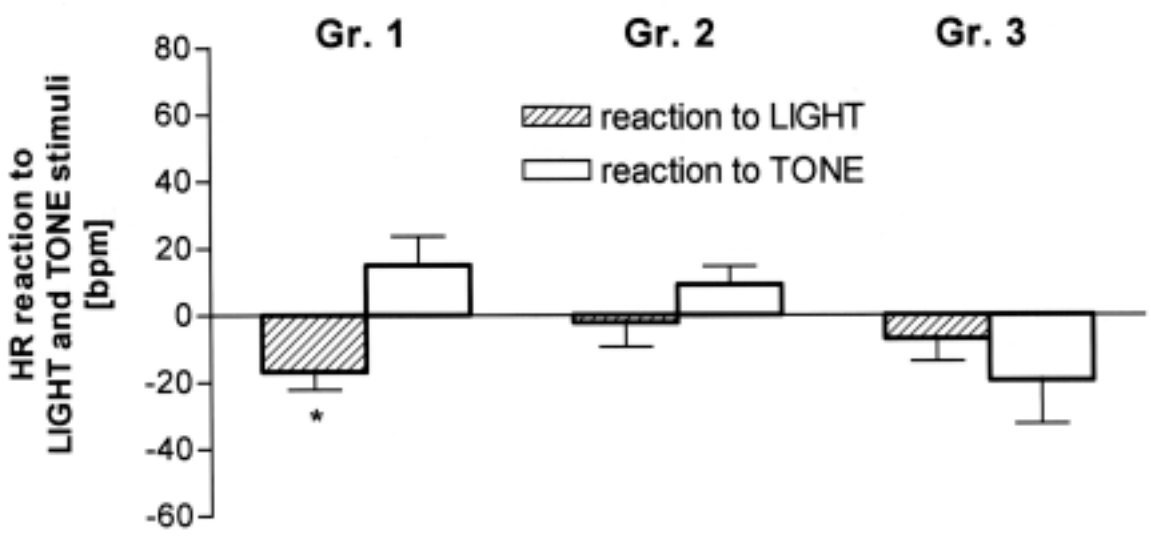

Fig. 3. Mean HR reactions (±SEM) to LIGHT and TONE stimuli before conditioning; **P*<0.05 (t-test).

ANOVA. Only the drug effect was statistically significant ($F_{5,55}$ = 4.77, *P*<0.001). Performed post-hoc Duncan tests showed that DZP (5 mg/kg) significantly (*P*<0.003) increased HR comparing to saline, and that the other drugs were without effect. In the group 3 also DZP injection (5 mg/kg) increased baseline HR (*P*<0.001), but the other anxiolytic agent - buspirone (5 mg/kg) had opposite effect (*P*<0.001). Anxiogenic drugs (PTZ and FG7142) did not exert any significant effect on pre-DS HR independently of modality and position of applied conditioned stimuli. The reactions to DS in all analyzed groups of rats were insensitive to drugs action (Fig. 5) except midazolam (2 mg/kg) which decreased conditioned bradycardia in the group 2 (*P*<0.05). As it is shown on the Fig. 6 the reactions to SS were more sensitive to drugs. Again, midazolam

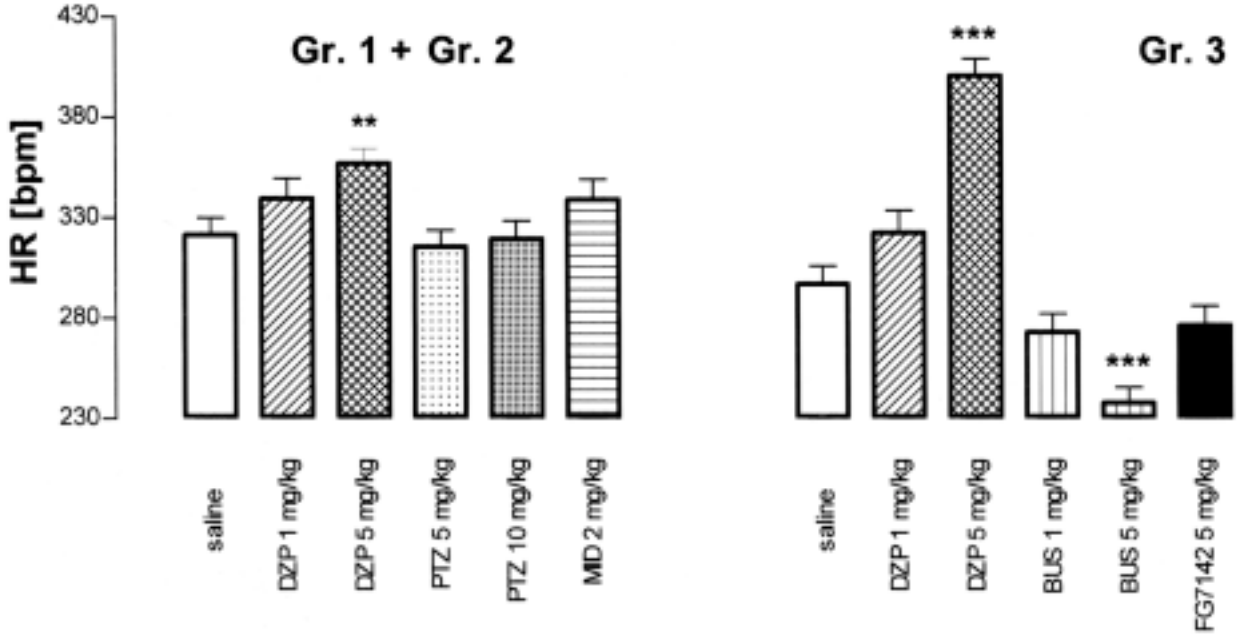

Fig. 4. The effect of selected anxiolytic and anxiogenic drugs on pre-DS HR; ***P*<0.01, ****P*<0.001 - drug effect in comparison to saline (ANOVA and post-hoc Duncan tests).

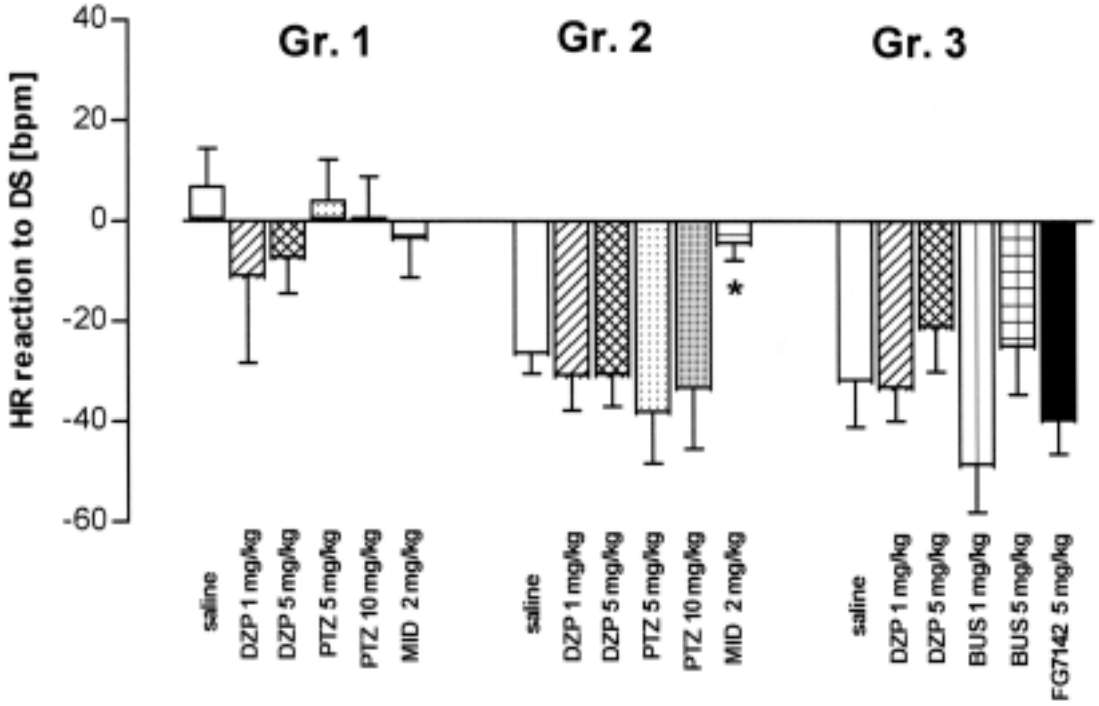

Fig. 5. The effect of selected anxiolytic and anxiogenic drugs on HR reaction to DS; *P<0.05 - drug effect in comparison to saline (ANOVA and post-hoct Duncan tests).

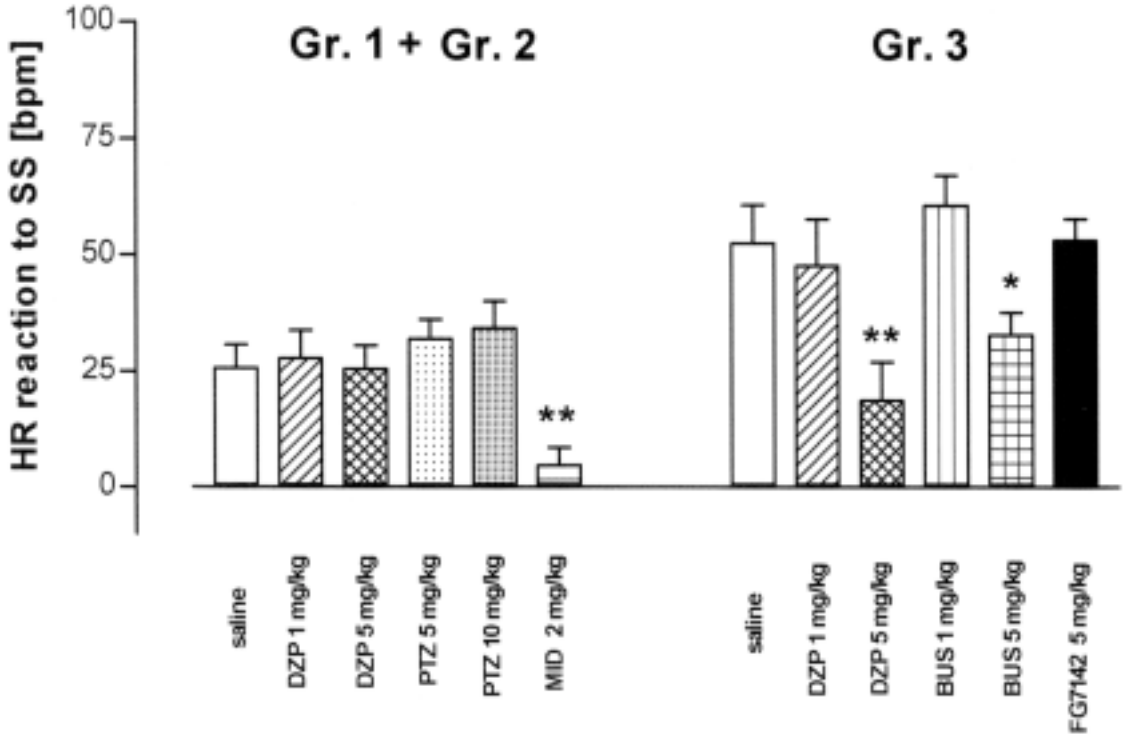

Fig. 6. The effect of selected anxiolytic and anxiogenic drugs on HR reactions to SS; *P<0.05, **P<0.01- drug effect in comparison to saline (ANOVA and post-hoc Duncan tests).

(2 mg/kg) suppressed the increase in HR in combined groups 1 and 2 ($P<0.01$). In the group 3 diazepam (5 mg/kg) ($P<0.01$) and buspirone (5 mg/kg) ($P<0.05$) suppressed the increase in HR in response to SS. PTZ and FG7142 did not influenced significantly the increase of HR induced by SS.

DISCUSSION

The results of our study indicate that HR changes in response to danger signal are different in the pattern, stability and direction when compared to HR changes in the response to safety signal. The difference in direction is especially pronounced in the phase 2 when the level of general anxiety accompanying the experimental situation is presumably decreased. During last 3 s of DS (in excitatory trials) progressing bradycardia was observed, while DS/SS compound (in inhibitory trials) evoked always tachycardia (Fig. 1). As it has been found also by other authors (Powell et al. 1971, Teyler 1971, Martin and Fitzgerald 1980, Sigmundi and Bolles 1983), HR response to conditioned stimuli signaling danger strongly depends on the experimental conditions. As our analysis showed this is reflected in the pattern of heart rate changes. In our experiment light-DS induced decrease in HR (bradycardia) while the response to tone-DS started with rapid HR acceleration (tachycardia), followed by progressive deceleration resulting merely in the return to the baseline HR (see Fig. 1 and Fig. 2). The conditioned bradycardia was better expressed in phase 2, when the anxiety level was diminished by decrease of the proportion of excitatory to inhibitory trials in the session. As it is seen on Fig. 1 and Fig. 2 the modality of conditioned stimuli clearly affects heart rate reactions to DS. This is in agreement with the results of other authors indicating that the modality of conditioned stimuli influence the conditioned reactions in both: classical (Powell et al. 1971, Holland 1977, Jacobs and LoLordo 1980, Sigmundi and Bolles 1983) and instrumental conditioning (Werka and Zieliński 1998). It is known that the nodal brain structure related to fear reactions (conditioned as well as unconditioned) is amygdala (Davis 1992,1994, LeDoux 1997). The importance of amygdala in HR conditioning is widely accepted (Gentile et al. 1986, McCabe et al. 1992, Chachich and Powell 1998, Antoniadis and McDonald 2000). Both anatomical and electrophysiological data indicate different distribution of sensory input of visual and auditory stimuli from cortical and subcortical systems to the particular nuclei of amygdala in rats (see Ono et al. 1995). This can, at least partly, explain different processing of visual and auditory stimuli, particularly the time needed to form an association with aversive events in amygdala or its strength. The difference in direction of HR changes to stimuli of different modalities is sometimes explained by difference in behavior (level of somatic activity) in the association forming time (see Powell et al. 1971). Another, non-mutually exclusive interpretation of the observed, in our experiment, influence of DS modality is the difference in "unconditioned" effects of the used stimuli. The temporary increase in HR immediately after the onset of DS does not have to reflect the fact that DS predicted an aversive stimulation, but it can be related to some unspecific arousal (not linked with the meaning of the signal). We showed in our experiment that the differences in HR in response to the stimulus even before conditioning might depend on its modality or position in relation to the rat. It is well known that neutral stimulus of medium strength might evoke HR reaction in stressed animals. In this case, however, it would be rather temporary, whereas the effect of conditioned fear should progressively rise during DS presentation and reach its full expression at the end of the signal - just before aversive stimulation. Partial restraint and presence of aversive stimulation during each session undoubtedly stressed our rats. The decrease of anxiety level in phase 2 of the experiment resulted in suppression or total disappearance of the mentioned above temporary increase in HR and, in consequence, conditioned bradycardia was expressed more clearly. Thus, it is possible that temporary increase in HR in response to onset of auditory was unconditioned alpha reaction that masked conditioned effect of DS (bradycardia).

According to literature the direction of HR reaction to DS is different in freely moving and restrained rats (respectively tachycardia and bradycardia). However, in restrained rats usually the tail-shock is used as unconditioned stimulus, (Fitzgerald and Hoffman 1976, Martin and Fitzgerald 1980), while in freely moving animals the foot-shock through the grid-floor is applied (Martin and Fitzgerald 1980, Iwata et al. 1986, Iwata and LeDoux, 1988). Using the specially designed apparatus (Soltysik et al. 1996) we were able to investigate conditioned HR reaction in the new experimental situation i.e in the partially restrained rats with the possibility to "run" on the treadmill with the tail-shock used as unconditioned stimulus. This apparatus assured the relative freedom of movements and fixed position of the body securing the

stable relationships of the receptive organs to the sources of stimuli. Our results showed that tail-shock is effective in inducing conditioned bradycardia in such a situation.

The effect of restraint on the direction of HR changes in response to DS is sometimes explained by its influence on somatic reactions. According to one of the basic conceptions linking somatic activity and HR, the same nervous process can commonly direct both of them (Obrist et al. 1974).

As it has been noticed by Powell et al. (1971) the freely moving rats use to jump during foot-shock through the grid-floor in an open field. This reaction is effective in diminishing an aversive strength of unconditioned stimulation. Therefore, the conditioned stimulus signaling danger may lead to increased somatic activity resulting in an increased HR. The behavior of restrained animals is different because they can neither avoid the stimulation nor escape or diminish its strength. In such situation an arrest of ongoing behavior and immobility is usually observed. Our results suggest that inevitability of stimulation rather than the lack of movements is the crucial factor for direction of HR changes in response to DS in fear conditioning procedure.

As it has been mentioned in the Introduction, the bio-behavioral significance of heart rate response to danger stimuli is a matter of discussion. Besides changes in somatic activity i.e. activity of striate muscles (Obrist et al. 1974, Obrist 1976) the possible interpretations involve attention (Lacey and Lacey 1974,1978), interaction of attention and somatic activity (Powell et al. 1990, 1993), motivational-emotional processes (Gantt 1960, Overmier 1966, Malmo and Belamger 1967, Young and Leaton 1996) or defense reaction (Martin and Fitzgerald 1980). All these concepts have different level of generality.

The conditioned bradycardia we observed in response to DS can not be easily explained by decrease in overt somatic activity i.e. locomotion on the treadmill because in trained rats vigorous locomotion followed always and exclusively the aversive stimulation and was present otherwise very rarely. As it seems, locomotor activity was not the crucial factor influencing the heart rate in our experiment. For example the highest pre-DS HR was observed in the group 1, while the pre-DS locomotion level in this group was relatively lower than in the group 2. We did not measure the covert somatic activity but as Martin and Fitzgerald (1980) have demonstrated, the conditioned bradycardia may be concomitant also to an increase in EMG activity.

Bradycardia as an autonomic correlate of passive defense reaction seems to explain better the heart rate changes in response to danger stimuli observed in our rats. The defense reaction, i.e. species-specific integrated cardiovascular, visceromotor, somatomotor and antinociceptive response, is activated by fear and serves to avoid confrontation with the noxious stimulus or provide a proper adjustment to its onset. The form of defense depends on the situation of the animal (e.g. in nature on the distance from the predator, in experimental conditions on the degree of restraint and possibility to avoid, escape or to diminish the aversive stimulation). Two forms of defense reactions have been described: active and passive. Passive form of defense is often observed in small rodents. In nature, for example, when the predator approaches too close, rat in response to direct danger will change the form of reaction from passive i.e. freezing, which serves to hide, to active one i.e. escape if possible, or defensive attack. The change of the form of reaction in the proper moment requires good assessment of the situation, therefore an increased attention. It has been found that active defense is accompanied by increase in HR (see Hilton 1982, Yardley and Hilton 1986). Although the cardiovascular concomitants of passive defense are not precisely described, there are data demonstrating simultaneous appearance of natural freezing behavior and bradycardia in birds (Steen et al. 1988), cats (Adams et al. 1971), kittens (Soltysik et al. 1982), wild rodents (Hofer 1970). The coexistence of freezing and conditioned bradycardia in restrained rats in response to a discrete stimulus was shown by Hunt et al. 1997. There are also data indicating that in freely moving rats, when the freezing is induced by contextual conditioned fear (environment paired with an aversive stimulation), this behavior is coupled with HR deceleration (Carrive 2000).

In our rats during DS presentation, in addition to bradycardia, freezing was observed. The ongoing behavior (USV 22-kHZ vocalization) was totally suppressed. SS presentation, on the contrary, was accompanied by head movements, sniffing and return to pre-DS vocalization. Some locomotion was also observed in the tone-SS group when the safety signal was presented from the back of the animal. The response to DS in our rats could be characterized therefore as passive defense reaction with the conditioned bradycardia as an autonomic correlate what is in agreement with Martin and Fitzgerald (1980). The concept of bradycardia as autonomic correlate typical for passive form of defense re-

action allows also to explain the differentiated effects on HR such factors like species, restraint, and, to some degree, the influence of other circumstances accompanying the experimental situations.

As it is shown on the Fig. 1 the signal of safety caused in all groups and in both phases of the experiment increase in the heart rate. HR changes were not only opposite in direction to the reaction observed in response to danger stimulus, but also they formed stable and homogenous pattern. During DS/SS presentation HR progressively rose until the end of the stimulus. They were also qualitatively independent on the properties of conditioned stimuli. The diversity of reactions to DS as compared to the stability of reaction to SS may suggest competition of some neurobehavioral processes in the first case and their cooperation in the second. As it is well known the activation of the parasympathetic division of the autonomic nervous system (ANS) is associated with bradycardia, whereas increase of sympathetic activity with tachycardia. According to literature co-activation of sympathetic and parasymphatetic divisions with the domination of the first one accompany conditioned reaction to DS in freely moving rats (Iwata and LeDoux 1988). As a result of this co-activation conditioned tachycardia occurs. In restrained animals, on the contrary, parasymphatetic activity dominates resulting in conditioned bradycardia, although some decrease in sympathetic activity is also observed (Fitzgerald et al. 1973). It should be noticed however that regardless of the experimental conditions some increase of parasymphatetic activity in response to danger stimulus is always present. It might be suggested therefore that synergistic or competitive changes in the activity of the parasympathetic and sympathetic divisions of ANS depend on the specific experimental situation (possibility of free movements). In the present experiment the rats, although partially restrained, were able to move its head, and limbs (ambulate or even "run" on the treadmill). This experimental design allows to expect rather enhanced then decreased sympathetic activity during DS presentation. It might be supposed that the diversity of the temporal pattern of heart rate changes in response to DS, especially pronounced immediately after the onset of the stimulus, results from competition of both parts of ANS with the parasympathetic dominance. Stable, progressing increase of HR in response to safety signal might be caused by the cooperation of ANS divisions i.e. increase of sympathetic and inhibition of parasympathetic activity. Verification of this hypothesis requires the pharmacological blockade successively for each of the divisions followed by testing its influence on HR reactions.

In the second part of the experiment the effects of anxiolytic and anxiogenic drugs on heart rate in response to danger and safety stimuli and their influence on the baseline (pre-DS) HR were analyzed. The benzodiazepines diazepam and midazolam increased baseline HR. Midazolam in the dose 2 mg/kg i.p. totally inhibited the reaction to conditioned stimuli. Diazepam in the higher dose (5 mg/kg) suppressed both reactions, but was statistically significant only in reducing HR increase in response to SS.

Benzodiazepines might influence the cardiovascular system in two ways - by acting mainly through the nervous system (smaller doses) and directly on the heart muscle (higher doses) (Strubelt 1980). It was demonstrated *in vitro* on ventricular heart myocytes of newborn rats, that diazepam and midazolam produces dose-dependent decrease in frequency and amplitude of their contractions (Nakae et al. 1997). The effect of diazepam on HR and strength of contraction in rats' isolated heart or heart-lung preparation is equivocal (Edoute et al. 1993, Nonaka et al. 1994). The effects of benzodiazepines on cardiovascular system of conscious rats are also not clear. Carpenter et al. (1977) demonstrated that diazepam in dose 1.8 mg/kg was without effect on HR and blood pressure. However, administered when HR and blood pressure were decreased by imipramine, diazepam increased the HR but enhanced the drop of blood pressure. Yang et al. (1987) showed dose-dependent decrease in arterial blood pressure and HR after intravenous injections of diazepam (1-30 mg/kg). Conahan and Vogel (1986) proved that the effects of benzodiazepines on cardiovascular system of conscious rats depend on the emotional state of animals. It was shown that diazepam in the dose over 1 mg/kg increases HR (although it did not influence the blood pressure) probably through the inhibition of parasympathetic influence on heart, while in stressed animals the injection decreases blood pressure and it is without effect on heart rate elevated by the stress. In our experiment the increase in pre-DS heart rate after the injection of diazepam was demonstrated. This result suggests that the low level of pre-DS heart changes is more crucial for the effect of diazepam than the stress, which undoubtedly accompanies the experimental situation. Its presence is confirmed by 22-kHz ultrasonic vocalization (not analyzed in this paper) as well as by the urination and defecation during the sessions. We demonstrated in this paper only small, not statistically significant, sup-

pressing effect of DZP (5 mg/kg) on conditioned bradycardia induced by light-DS in group 3. The suppressing effect of diazepam in conditioning procedure is consistent with the results of Nashimoto et al. (1991). The authors found that after the training of electric foot-shock avoidance, the placement of rats into the shuttle-box induced increase of their HR and this effect was totally inhibited by β-adrenergic blockers. Tone (conditioned stimulus) induced opposite effect (decrease of HR), that was blocked by injections of DZP. The differences in experimental model may explain the differences in strength of DZP effects.

Injections of buspirone ($5HT_{1A}$ agonist), the non-benzodiazepine anxiolytic agent induced, in contrast to DZP, the decrease in pre-DS HR. The observed decrease of HR in rats after injections of buspirone is consistent with the literature (Van de Kar et al. 1985). In the dose of 1 mg/kg it enhanced non-significantly the conditioned bradycardia in response to danger. In the higher dose (5 mg/kg) buspirone strongly diminished pre-DS HR and, supposedly for this reason, the bradycardia in response to DS could not be observed. In dose 5 mg/kg buspirone as well as diazepam, suppressed the increase in HR in response to SS.

The effects of buspirone and diazepam, despite that both drugs share the same pharmacological profile, differ not only in respect to heart rate but also in respect to hormonal changes. The decrease in HR is accompanied by simultaneous increase of catecholamines level (adrenaline, noradrenaline) and the other "stress hormone" - corticosterone (Matheson et al. 1988, Taylor et al. 1989). The effects of benzodiazepines and buspirone on activity of the brain neurotransmitters systems are also different. Benzodiazepines enhance the GABA-ergic activity and suppress the serotoninergic, dopaminergic and adrenergic activity. Buspirone does not influence the GABA-ergic activity, while it inhibits serotoninergic system and enhances dopaminergic and adrenergic activity (Eison and Temple 1986, Goa and Ward 1986). Serotoninergic system is crucial for the proper functioning of central autonomic network and, particularly, for the cardiovascular system. Activation of the $5HT_{1A}$ produces bradycardia and hypotension (Ramage and Fozard 1987, McCall and Clement 1994). There are data, however, indicating that microinjection of buspirone into the forebrain (preoptical area) induce the opposite reaction in conscious rats i.e. hypertension and tachycardia (Szabo et al. 1998). These data together with our data suggest that baseline heart rate cannot be trusted as a reliable index of the emotional state, since two drugs of clinically proved anxiolytic profile act on it in different manner. Only in response to safety signal the effect of both anxiolytics was the same, but they suppressed tachycardia rather then enhanced it - thus the increase of HR in response to safety signal cannot be regarded as direct index of fear reduction by drugs.

In order to enhance the level of emotions that accompany the experimental situation two anxiogenic drugs were used, FG7142 and pentylenetetrazole (PTZ). Injections of FG7142 were performed only in group 3. FG7142 influenced neither the HR reactions to DS or SS nor pre-DS HR. This drug is the inverse agonist of benzodiazepine receptors. The agonists and inverse agonists of benzodiazepine receptors exert generally opposite effect on GABA-ergic transmission. Functionally, their activity is often bi-directional. BDZ agonists exert anxiolytic, while inverse agonists, anxiogenic effect in various animal models. Quigley et al. (1994) testing cardiovascular effects of FG7142 injections on freely moving rats, found that in dose 8 mg/kg the drug causes the decrease in pre-DS HR, but do not influence the blood pressure. It has been also demonstrated that the systemic injection of FG7142 enhances the increase of HR in response to unexpected acoustic stimulus. This enhancement is supposedly related to increased activity of the central cholinergic system. According to Berntson et al. (1996) the effect of FG7142 is mediated by the central muscarinic mechanisms. The cortical cholinergic system of the basal forebrain is involved in the regulation of emotions and agonists and inverse agonists of benzodiazepine receptors modulate its activity (Berntson et al. 1997,1998, Hart et al. 1998). In the model used by Berntson et al. (1996) tachycardic component of defense reaction was further augmented by systemic administration of FG4271. Authors suggest that the drug selectively enhance aspects of anxiety dependent on cortico-cognitive processes. In our experimental model based on passive form of defense reaction bradycardia was observed and this might be the reason for the lack of FG4271 effects on HR in response to danger signal. The conditioned bradycardia is mainly the result of increased parasympathetic activity. Thus, it can be suggested that FG7142 does not enhance the parasympathetic activity in response to stimulus signaling electric shock. The observed small (nonsignificant) decrease in the baseline (pre-DS) HR is consistent with literature (Brentson et al. 1996).

The other anxiogenic drug we applied - pentylenetetrazole (PTZ), (nonspecific stimulant affecting benzodiazepine site in $GABA_A$ complex) even in dose 10 mg/kg influenced neither HR reactions to DS and SS nor basal HR. The lack of effects on HR is interesting, because these injections enhanced 22-kHz USV vocalization. This might indicate the increased level of anxiety and will be described in another paper.

The pharmacological part of our experiment showed therefore that both, anxiolytic and anxiogenic drugs exert diverse influence on the basal heart rate as well as on the heart rate changes in response to danger and safety signals. This suggests that the effects of used drugs depend rather on the receptors involved then on the general pharmacological profile of the drug (anxiolytic/anxiogenic).

In conclusion, the described pattern of heart rate changes in response to signal of danger in partially restrained rats suggest that the HR can not be trusted as a reliable measure of fear because it strongly depends on experimental conditions, while changes in vocalization (not analyzed here) do not. However, our study showed that active inhibition of the fear is accompanied by the consistent increase in HR. The reaction to the signal of safety characterized by stable pattern and resistance to the changes in experimental conditions could be used therefore in research aimed in identifying the neural mechanism of learned alleviation of fear in rats.

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