Classic conditioning of the ventilatory responses in rats

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Nsegbe, Elise, Guy Vardon, Pierre Perruchet, and Jorge Gallego. Classic conditioning of the ventilatory responses in rats. *J. Appl. Physiol.* 83(4): 1174–1183, 1997.—Recent authors have stressed the role of conditioning in the control of breathing, but experimental evidence of this role is still sparse and contradictory. To establish that classic conditioning of the ventilatory responses can occur in rats, we performed a controlled experiment in which a 1-min tone [conditioned stimulus (CS)] was paired with a hypercapnic stimulus [8.5% CO_2, unconditioned stimulus (US)]. The experimental group (n = 9) received five paired CS-US presentations, followed by one CS alone to test conditioning. This sequence was repeated six times. The control group (n = 7) received the same number of CS and US, but each US was delivered 3 min after the CS. We observed that after the CS alone, breath duration was significantly longer in the experimental than in the control group and mean ventilation was significantly lower, thus showing inhibitory conditioning. This conditioning may have resulted from the association between the CS and the inhibitory and aversive effects of CO_2. The present results confirmed the high sensitivity of the respiratory controller to conditioning processes.

control of breathing; carbon dioxide

RECENT STUDIES provide new experimental evidence that ventilatory activity can be adapted to physiological requirements by learning processes (29, 30). Among these processes, a particular importance is attached to classic conditioning, i.e., the process by which a conditioned stimulus (CS) that has been paired with an unconditioned stimulus of breathing (US) is able to elicit a conditioned ventilatory response (CR) (13, 21, 24, 34). In particular, it has been reported that early conditioning during the postnatal period may have lasting influences on the breathing pattern (31, 32). In general terms, classic conditioning is interpreted as the acquired ability to anticipate forthcoming metabolic needs, which means that a CS signaling a respiratory US such as hypercapnia should normally elicit a conditioned increase in ventilation (33). This prediction was supported by early investigations of respiratory conditioning (16, 26). For example, Pogrebkova (26) reported that in dogs a sound previously paired with a hypercapnic stimulus of 9–10% triggered a conditioned increase in breathing frequency and amplitude.

However, the results of subsequent studies failed to confirm these early findings consistently. Weinstein and Fowle (37) observed no conditioning in pigeons, even after 400 paired presentations of a light or a sound with a 7.4% CO_2 stimulus. A still more contrasted outcome was reported by Biryukov et al. (5). These authors performed a series of experiments in monkeys, dogs, cats, rabbits, guinea pigs, pigeons, turtles, frogs, and rats, in which a sound or a light was paired with a 50% CO_2 stimulus. CRs were observed in most species (although not in cats) after different numbers of paired conditioned and unconditioned stimuli: 3–5 in dogs and monkeys, 6–10 in pigeons, and 20–25 in rabbits. The CR consisted of a decrease in breathing amplitude and frequency, sometimes reaching apnea. By itself, the fact that the CR and the unconditioned ventilatory response (UR) act in opposite directions is not an exception in classic conditioning, because such action has been reported in conditioning of body temperature, blood glucose levels, heart rate, etc.[1] However, in the specific framework of respiratory conditioning, this result was in total conflict, with both with previous findings and the current notion that conditioned ventilatory responses anticipate forthcoming metabolic requirements.

Three factors may account for these controversial data: the absence of appropriate control procedures, the detection of CO_2, and the inhibitory effects of CO_2. First, most early investigators used very few subjects, generally one or two, and evidence for conditioning was based on selected tracings of the ventilatory signal after the CS alone (2, 6, 11, 16, 26, 28, 36). Since these pioneering studies, new methodological concepts have led to substantial changes in conditioning designs (20, 27). According to present criteria, conditioning is not established unless it is shown that the response elicited by the CS is a specific consequence of the pairing of the CS with the US. This is particularly important in respiratory conditioning experiments because repeated exposure to hypercapnia or hypoxia may induce long-term physiological and behavioral changes, which may affect the response to any stimulus, including the CS, independently of any associative process. Without appropriate control procedures, it is impossible to decide whether ventilatory changes result from learning the association between the CS and the US (i.e., conditioning) or from nonassociative processes.

Second, the contradictory results of conditioning studies may be due to the ability to detect the US, especially CO_2, because of its sensory properties rather than its respiratory effects. This may strongly affect conditioning. Previous literature in fact showed that a conditioned activation of breathing occurred when CO_2

[1]The contention that the CR and UR act in opposite directions may in fact result from erroneous identification of the US and UR (10).

was administered intratracheally, i.e., when the probability of detecting CO_2 was low (16, 26), whereas conditioned inhibition of ventilation (5) or no conditioning at all (37) was reported when CO_2 was delivered through the upper airways. In fact, stimulation of the nasal mucosa by CO_2 may act as a CS, in addition to the auditory or visual stimuli experimentally designed to do so. What generally occurs when two CS are presented together with the US is that the "stronger" one, in terms of intensity, salience, and predictive value in relation to the US, may "overshadow" the weaker one (20). The US is preferentially associated with the stronger stimulus, and no CR occurs in response to the weaker stimulus. This may explain the negative outcome of some previous experiments (37).

Third, the contradictory results of conditioning experiments may result from the fact that the CO_2 stimulus, in addition to its stimulatory effects through chemosensitivity, also has inhibitory effects on breathing mediated by an upper airway sensory reflex (1, 4). Conditioning studies have shown that when a US has several different effects, they may be conditioned at different rates (2, 10). This raises the question of whether the inhibitory or stimulatory effect of CO_2 is predominantly conditioned. When CO_2 is delivered through upper airways instead of intratracheally, its inhibitory effects may be at first associated with a CS. This may account for the conditioned inhibition of breathing reported by some previous authors (5).

In view of the above considerations, we carried out a controlled experiment in which rats were submitted to paired presentations of CO_2 stimuli and tones. The control procedure consisted of submitting a control group to the same number of unpaired CS and US. Conditioning was examined by comparing the ventilatory responses to test trials with CS alone at identical times in the two groups. Accordingly, any difference in the response to test trials would unambiguously reveal conditioning. Second, we attempted to avoid the overshadowing of the CS by the CO_2 stimulus by the use of a continuous masking somatosensory stimulus. We postulated that such a stimulus would prevent the early detection of CO_2 and reduce the predictive value of its sensory effect, thus facilitating the CS-US association. This would ensure conditioning, even though CO_2 was delivered through the upper airways. However, no prediction was made about the direction of the CR. We postulated that conditioning would be either inhibitory or stimulatory, depending on which of the effects of CO_2 would be predominantly associated with the CS.

METHODS

Subjects. Sixteen adult male Wistar rats were randomly assigned to the experimental group ($n = 9$, mean wt 208 ± 32 g) or the control group ($n = 7$; 206 ± 37 g). Rats were fed ad libitum and tested at least 5 days after their arrival in the laboratory.

Apparatus. A built-in whole body plethysmograph based on Drorbaugh and Fenn's principle (9) was used for ventilatory measurements. This device consisted of three superimposed communicating cylindrical chambers of 0.27, 0.7, and 4.5 liters, respectively. The upper chamber was used for gas admission and mixing, the second chamber served as reference for pressure measurement, and the third contained the animal. A constant airflow of 2 l/min was delivered through each chamber. This relatively high airflow avoided CO_2 and water accumulation in the animal chamber and maintained a constant temperature. The difference between the pressure in the reference and animal chambers (measured by using a Celesco VR pressure transducer, sensitivity ±2 cmH_2O) was proportional to tidal volume (V_T). The differential pressure signal was filtered (bandwidth 1.15–15 Hz), converted into a digital signal (MacAdios A/D 12-bits converter, GW-Instruments, Somerville, MA) at a sample rate of 100 Hz, and processed to calculate the total duration (T_T) and V_T of each breath (Software Superscope II, GW-Instruments, Somerville, MA). Animal temperature was not recorded, to avoid any invasive measurement, and only the uncalibrated volume signal was used. Two receptacles containing 20 ml of a 50% dilution of acetic acid were placed in the gas admission chamber throughout the experiment, for the purpose of masking the onset of the CO_2 stimulus. On the basis of previous studies (15, 18, 19), we assumed that, at least for small CO_2 concentrations, the masking of CO_2 was effective for the onset of the CO_2 delivery, thus preventing CO_2 onset from signaling the forthcoming higher CO_2 concentrations and their physiological effects.

CS and US. The CS was a 1-min tone (4,000 Hz, 70 dB at 10 cm) delivered by a buzzer placed inside the animal chamber. The US was a hypercapnic mixture created by delivering a constant flow of CO_2 to the plethysmograph. The fraction of CO_2 (F_{CO_2}) in the animal chamber was estimated from the outflow value. It rose linearly up to 8.5% (Fig. 1) and decreased after the closure of the electrovalve, to reach its baseline level in 3–4 min. The residual F_{CO_2} inside the chamber was <0.3%.

Procedure and design. Each animal underwent three sessions (1 session per day), on 3 consecutive days, at the same time of day. The experimental design is summarized in Table 1.

The first session (i.e., *day 1*) served to familiarize each animal with the plethysmograph and to compare the initial values for breathing variables in the experimental and control groups. The procedure was identical for the two groups and consisted of a familiarization period of 140 min, followed by 70 min of baseline measurements. The same sequence was repeated after the two recipients of acetic acid were placed in the plethysmograph.

The second session (i.e., *day 2*) aimed at establishing and testing the conditioning. This session was composed of six phases of 70 min each. Each phase was divided into seven trials of 10 min. No stimulus was delivered during the first trial. The CO_2 stimulus and the tone were delivered once each during the five following trials. In the experimental group,

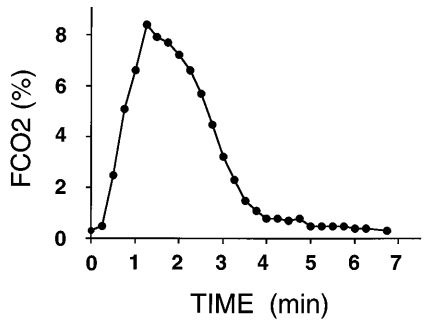

Fig. 1. CO_2 stimulus. F_{CO_2}, fraction of CO_2. Electrovalve controlling CO_2 inflow into animal chamber was opened at time = 0 min and closed at time = 1 min. Residual F_{CO_2} was <0.3%.

Table 1. *Summary of the experimental design*

	Experimental Group	Control Group
Day 1	Familiarization + 70 min recording	
	Familiarization with AA + 70 min recording	Same as experimental group
Day 2	6 Phases comprising 7 trials each (*trials 1–7*)	Same as experimental group
	Trial 1: baseline	Same as experimental group
	Trials 2–6: paired CO_2/tone (acquisition trials)	*Trials 2–6*, unpaired CO_2/tone
	Trial 7: tone alone (test trial)	Same as experimental group
Day 3	6 Phases comprising 7 trials each (*trials 1–7*):	
	Trial 1: baseline	Same as experimental group
	Trials 2–7: tone alone	

Familiarization lasted 140 min. Sessions in *day 2* and *day 3* comprised 6 phases each. Each phase lasted 70 min and comprised 7 trials. Each trial lasted 10 min. AA, acetic acid.

the CO_2 stimulus and the tone were delivered simultaneously. In the control group, each CO_2 stimulus was delivered 3 min after the onset of the tone (i.e., 2 min after the tone was switched off). The last trial of each phase was identical in each group; it consisted of delivery of the tone alone (without CO_2). The between-group comparison of the ventilatory response to the tone alone served to test for conditioning.

The third session (i.e., *day 3*) aimed at assessing the retention of conditioning and testing its extinction. It was identical in the two groups. *Day 3* was the same as *day 2*, except that no CO_2 was delivered to either group.

Data analysis. Dependent variables were the breath-by-breath values of T_T (ms), V_T (arbitrary units), and ventilation ($\dot{V}_I$) calculated as the V_T/T_T ratio (arbitrary units). These variables were averaged over successive 1-min periods. We used repeated-measures analyses of variance (Superanova software, Abacus Concepts, Berkeley, CA) with the group (experimental vs. control) as a between-subject factor. *Phases 1–6* and *trials 2–7* were used as within-subject factors. In some analyses, the 10-min duration of the trials was split over three successive time blocks, thus introducing a new within-subject factor. *Trial 1* (no stimulation) served as baseline period. To take into account the heterogeneous correlations among the repeated measurements with more than two degrees of freedom, we adjusted the degrees of freedom by using the Huynh-Feldt ϵ factor. The within-subject main effects and interactions are reported along with *P* values based on these adjusted degrees of freedom (8).

RESULTS

Spontaneous breathing variables (day 1). Baseline values for the two groups on *day 1* (Table 2) show that the differences between the two groups were not significant, with or without acetic acid. A significant difference between the two latter conditions was observed for $\dot{V}_I$ [$F(1, 897) = 9.41$, $P < 0.009$], but it is unclear whether this difference was specifically caused by acid or by behavioral changes during *day 1*. Group-by-acid interaction was not significant.

Baseline and ventilatory response to CO_2 (day 2). Baseline levels were calculated from *trial 1*, during which neither the tone nor the hypercapnic stimulus was delivered to either group. Differences between groups were not significant. An upward drift in T_T and a downward drift in $\dot{V}_I$ were observed in both groups, as confirmed by a significant main effect of phase for T_T [$F(5, 70) = 8.02$, $P < 0.0001$] and $\dot{V}_I$ [$F(5, 70) = 5.32$, $P < 0.0003$]. The corresponding changes in V_T were not significant.

Maximal responses to CO_2 (*day 2*) were reached within 3 min of the onset of the US (Fig. 2). The increase in $\dot{V}_I$ elicited by hypercapnia was ~100%, with similar contributions by breathing frequency and V_T. The ventilatory responses to CO_2 were almost identical in the two groups, whether or not CO_2 stimuli were paired with tone (between-group comparison yielded nonsignificant differences).

We attempted to establish whether the repetition of the hypercapnic tests changed the pattern of the hypercapnic response. We focused on *trial 6*, which was the last trial with CO_2 before the test trial (*trial 7*) with tone only. Figure 2 shows that the repetition of hypercapnic tests yielded between-group differences in $\dot{V}_I$ at the end of *trial 6*. These differences were analyzed by analysis of variance (ANOVA), for the 10th min of *trial 6*, which was the minute immediately preceding the test trial of each phase (*trial 7*): the $\dot{V}_I$ value for the 10th min decreased significantly as a function of phase in the experimental group [$F(5,40) = 4.59$, $P < 0.006$] but not in the controls [$F < 1$, not significant (NS)]. This was confirmed by marginally significant group-by-phase interaction [$F(5,70) = 2.18$, $P < 0.066$]. Analysis of T_T yielded similar results, although differences in T_T values for *minute 10* were already present in *phases 1–3* (Fig. 2). T_T rose significantly in the experimental group [$F(5,40) = 5.48$, $P < 0.0006$], but not in the controls ($F < 1$, NS). For group-by-phase interaction, it almost reached significance [$F(5,70) = 2.36$, $P < 0.059$]. Contrast analyses between a given phase and all the previous ones showed that $\dot{V}_I$ and T_T did not change significantly in *phases 1–3* but displayed a significant increase in *phase 4* [$F(1,40) = 8.84$, $P < 0.009$ and $F(1,40) = 11.12$, $P < 0.0002$, respectively]. V_T for *minute 10* did not change significantly throughout *phases 1–6*.

CR to the CS. First, the ventilatory response to the CS was analyzed by averaging breathing variables over the 3 min after the onset of the CS (this corresponded to the ascending limb of the hypercapnic response in Fig. 2). Conditioning was assessed by the difference between the responses to the CS alone (*trial 7*) in the experimental and control groups. Figure 3 shows a marked difference between the T_T and $\dot{V}_I$ values for the two groups in *phases 4–6*. For T_T, this difference

Table 2. *Baseline ventilatory data*

		Without Acetic Acid			With Acetic Acid		
Group	*n*	T_T, ms	V_T, AU	$\dot{V}_I$, AU	T_T, ms	V_T, AU	$\dot{V}_I$, AU
Experiment	9	764 ± 276	25 ± 3	40 ± 13	781 ± 241	24 ± 28	36 ± 10
Control	7	697 ± 253	23 ± 4	41 ± 14	766 ± 397	23 ± 4	37 ± 11

Values are group means ± SD; *n*, no. of rats. T_T, total breath duration; V_T, tidal volume; $\dot{V}_I$, mean ventilation; AU, arbitrary units. Between-group differences were not significant. $\dot{V}_I$ was significantly higher with acetic acid than without (see text).

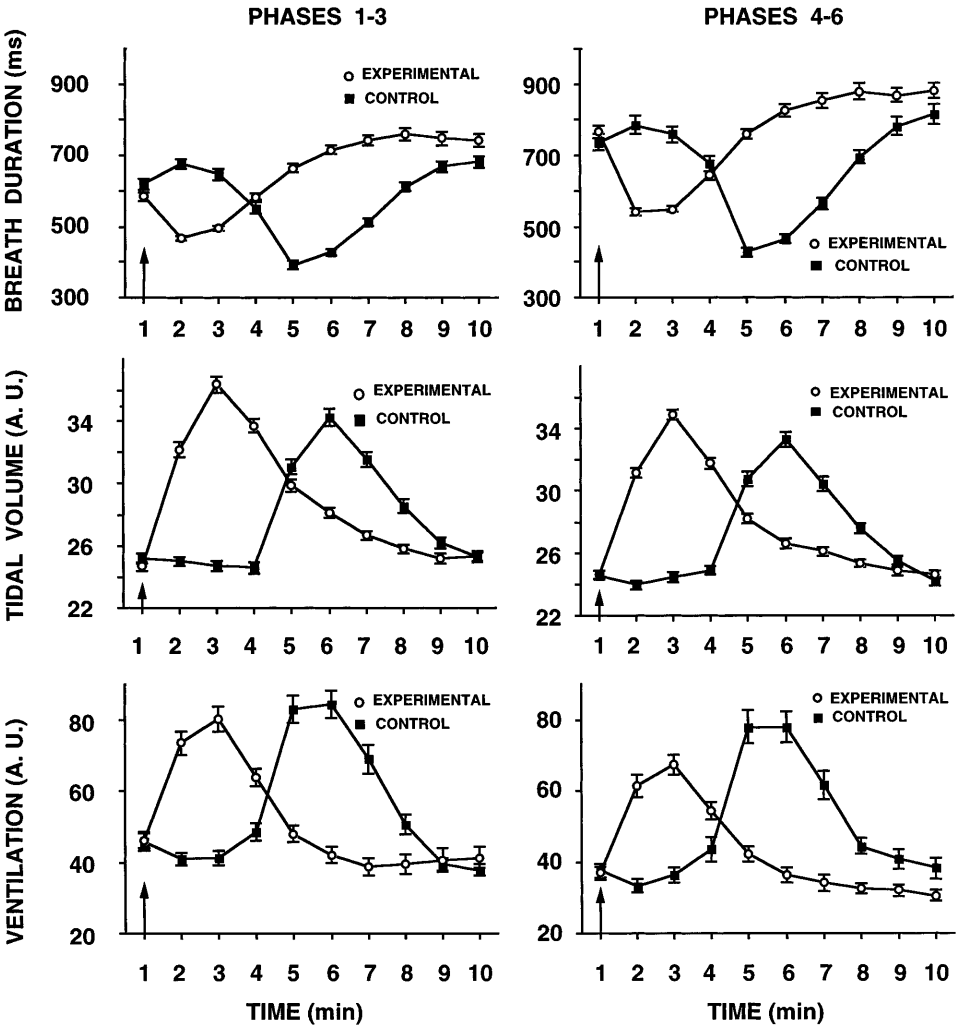

Fig. 2. Responses of breath duration, tidal volume, and ventilation to hypercapnic test in *trial 6*, just before test trial (*trial 7*) during *day 2*. Breath-by-breath values were averaged for each group over successive 1-min periods, over *phases 1–3* or *phases 4–6*. Arrows, presentation of 1-min tone (conditioned stimulus). In experimental group, CO_2 stimulus was superimposed on 1-min tone. In control group, CO_2 stimulus was delivered 3 min after onset of tone. AU, arbitrary units. Values are means ± SE. See text for statistical analyses.

was confirmed by significant group-by-phase interaction [$F(5, 70) = 3.50$, $P < 0.016$]. In fact, in the experimental group, T_T rose significantly over phases [$F(5,40) = 7.52$, $P < 0.0005$]. Contrast analyses between a given phase and all the previous ones showed that the first significant increase in T_T appeared in *phase 4* [$F(1,40) = 5.40$, $P < 0.037$]. The corresponding main effect for phase in the control group was not significant. These results suggest that conditioning occurred in *phase 4*, i.e., after 15–20 paired presentations of the CS and US. We further analyzed the time course of conditioning by averaging T_T over *phases 1–3*, and *4–6*, thus introducing a new within-subject phase block factor (Fig. 4). Phase block-by-group interaction was significant for T_T [$F(1,14) = 6.49$, $P < 0.024$], which confirmed the conditioning effects on T_T as from *phase 4*.

The analysis of $\dot{V}_I$ yielded similar results. Significant group-by-phase interaction was observed for this variable [$F(5, 70) = 2.99$, $P < 0.025$]. In the experimental group, the $\dot{V}_I$ response to the CS alone decreased significantly over phases [$F(5,40) = 13.66$; $P < 0.0001$]. Contrast analyses showed that the first significant decrease in $\dot{V}_I$ was also observed in *phase 4* [$F(1,40) = 20.33$, $P < 0.0001$]. On the other hand, the main effect of phase in the control group was not significant. Averaging $\dot{V}_I$ values over *phases 1–3* and *4–6* yielded the same result as for T_T. Phase block-by-group interaction was significant [$F(1,14) = 7.67$, $P < 0.0151$], which confirmed the effects of conditioning on $\dot{V}_I$. No significant effects were observed for V_T.

Second, we analyzed the response to the CS over the entire 10-min duration of the test trials (*trial 7*). As Fig. 4 shows, the between-group differences in T_T and $\dot{V}_I$ appearing in *minute 1* and lasting until *minute 3* tended to vanish during the remaining period of the trial, from *minute 4* to *minute 10*. This effect was tested by ANOVA on the entire 10-min period: data were pooled over three successive time blocks: *minutes 1–3*, *minutes 4–6*, and *minutes 7–10*, thus introducing a new within-subject time block factor with three levels. Partial analyses of each time block showed that only the first 3-min period yielded significant group-by-phase interactions, i.e., learning effects (this corresponds to results of the above analysis of *minutes 1–3*). By contrast, these group-by-phase interactions were not significant for either the second or third time blocks. This effect was confirmed by a significant group-by-phase-by-time block interaction [$F(10,140) = 2.32$, $P < 0.021$]. The final time block (*minutes 7–10*) lasted 4 min instead of 3 min for the first two time blocks, but

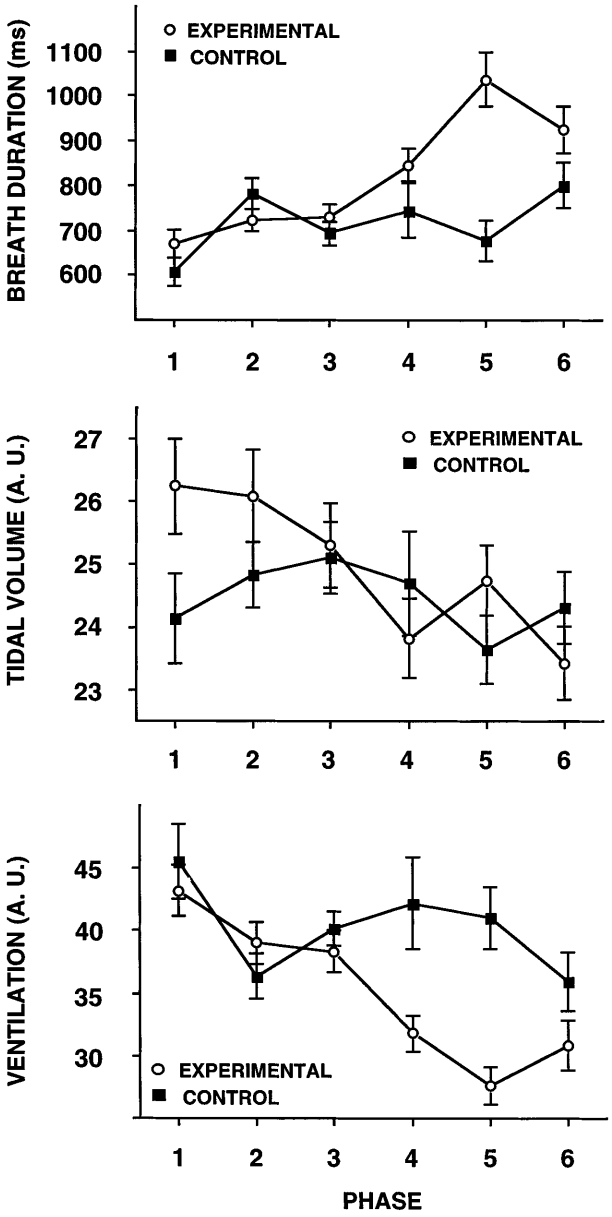

Fig. 3. Responses to conditioned stimulus. Values are means ± SE. Breath-by-breath values were averaged over first 3 min after onset of tone in *trial 7* and then for each group. See text for statistical analyses.

the corresponding analyses for a final time block lasting from *minute 7* to *9* yielded the same results.

The corresponding analyses yielded similar results for $\dot{V}_I$. Partial analyses of each time block showed that significant learning effects appeared during the first 3-min time block only. Group by phase-by-time block interaction was significant [$F(10,140) = 2.34$, $P < 0.020$]. No significant results were found for V_T. Therefore, learning effects were confined to a limited period ~3 min after the auditory stimulus, thus supporting the view that the conditioned changes in T_T were specifically elicited by this stimulus. Direct observation of the animals suggested that the ventilatory CR was not mediated by particular changes in the level of somatomotor activity.

Short-term effects of the CS. The short-term effects of the CS were studied by comparing ventilatory data during the 1-min CS with the data collected during the 1-min period preceding the CS (thus introducing a pre-post factor). In the two groups, the auditory CS elicited an immediate significant decrease in T_T and a significant increase in $\dot{V}_I$, whereas V_T exhibited no significant changes. This response pattern is typical of the ventilatory effects of arousal (29). The main effect of this pre-post factor was significant for T_T and $\dot{V}_I$ [$F(1,14) = 8.14$, $P < 0.013$, and $F(1,14) = 6.86$, $P < 0.020$, respectively]. This immediate increase in ventilation elicited by the CS tended to be lower in experimental than in control rats (6 ± 20 vs. 14 ± 22%), but pre-post-by-group interaction was not significant [$F(1,14) = 2.48$, $P < 0.136$]. The inhibitory CR observed in the experimental group over the 3 min after the onset of the CS was indeed the opposite of the immediate activating effects exerted by the CS.

Extinction of the CR. We observed some between-group differences in $\dot{V}_I$ values during *day 3* (Fig. 5), but their relationship to the CS occurrence was unclear. The ANOVAs carried out on *day 3* did not yield significant results for any of the variables studied.

DISCUSSION

This experiment showed that pairing a hypercapnic and an auditory stimulus elicits an inhibitory CR in rats after ~5–20 paired presentations of these stimuli. This response was characterized by higher T_T and lower $\dot{V}_I$ values in the experimental compared with the control group. No significant effects were observed for V_T. This response contrasted with the immediate response to the CS (a decrease in T_T and an increase in $\dot{V}_I$), which is typical of the physiological component of arousal. Therefore, the inhibitory CR neither potentiated the preexisting stimulating response to the tone nor was similar to the hypercapnic response to the US.

Our contention that the experimental group exhibited inhibitory conditioning in response to the auditory stimulus was based on two main arguments. First, because the only procedural difference between the two groups was the temporal contiguity between the CS and US, we postulated that the differences between the group responses to the CS were due to the learning of the association between the CS and US by the experimental group. Second, we attempted to ascertain whether the CR was specifically triggered by the CS or whether it was caused by contextual factors affecting the two groups differently. This issue is generally investigated by performing direct pre-post comparisons of the effects of the CS. However, in the present experiment, these comparisons were hampered because the breathing pattern of the experimental rats changed just before CS delivery as a result of learning. This is why the pre-CS ventilatory data did not provide a reference level suitable for assessing the effects of conditioning. By contrast, comparison of the breathing variables at CS delivery with the breathing variables for subsequent periods was relevant to the evaluation of the specificity of the CR in relation to the CS. This

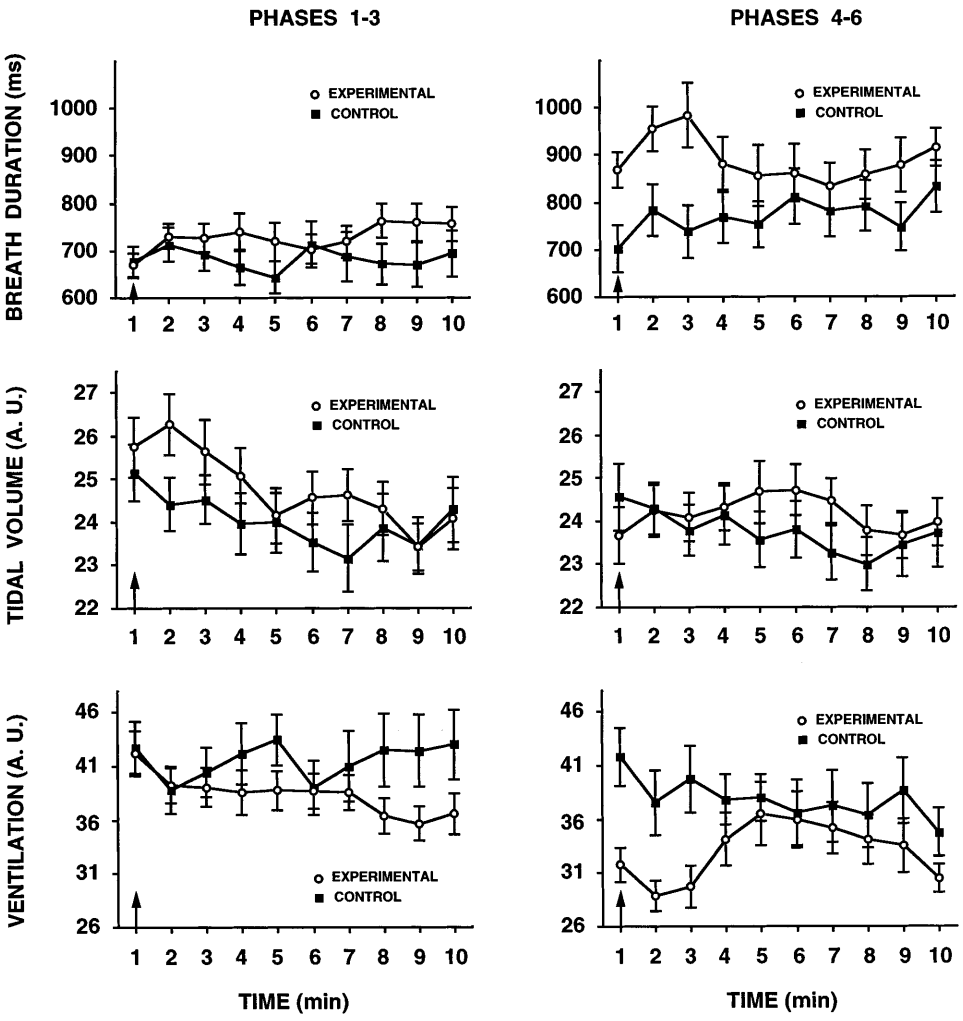

Fig. 4. Responses of breath duration, tidal volume, and ventilation to tone during *day 2* (*trial 7*). Breath-by-breath values were averaged over successive 1-min periods, over *phases 1–3* and *phases 4–6*, and then for each group. Values are means ± SE. Arrows, presentation of tone (conditioned stimulus) (see text for statistical analyses).

comparison showed that the group differences in the breathing patterns during the test trials (*trial 7*) were confined to a period of ~3 min after the CS and then vanished during the remainder of the test trial. It was, therefore, unlikely that the group differences after the CS were caused by a general contextual factor, because this would have affected breathing patterns throughout the entire test trial. Rather, we suggest that our data can be accounted for by specific effects of the CS.

Finally, because T_T and V_I displayed baseline drifts, we addressed the possibility that the group differences observed in the test trials may have arisen because of long-duration changes brought about by the US. These changes may have had different effects in the two groups because, at the time of the test, the US of the preceding trial was given 3 min closer to the CS in the control than in the experimental group. We ruled out this possibility for the following reasons. First, the group differences in T_T and V_I during the test trials were much higher than the baseline changes in these variables. Second, the fact that these group differences in the test trial emerged with practice in the late phases of *day 2* could not be explained by aftereffects of the US, which would have been also observed in the early phases. In addition, had the group differences in the test been due to the fact that the US of the preceding trial was given 3 min closer to the CS in the control than in the experimental group, we would have observed parallelism between the ventilatory curves across the 10 min of the test trials with a lag of 3 min. There was no such trend in the data. Therefore, we ruled out the possibility that the group differences in the test were due to long-duration effects of hypercapnia.

Despite the above arguments in support of the specificity of the CR in relation to the CS, several aspects of our data may still go against this hypothesis. For example, during *day 3*, group differences in breathing patterns seemed unrelated to the CS. The protocols for *day 2* and *day 3* were markedly different. On *day 2*, each phase comprised five CS-US pairings (followed by 1 CS-alone trial), whereas on *day 3*, no US (i.e., no hypercapnia) was delivered. Therefore, there is no contradiction in the fact that *days 2* and *3* data led to different results. In fact, *day 3* data reflected the ventilatory behavior of the animals placed in the plethysmograph in which they had been subjected to repetitive CO_2 stimuli and receiving auditory stimuli. This context was far from neutral, and, as a matter of fact, in *phase 1* the control group exhibited large

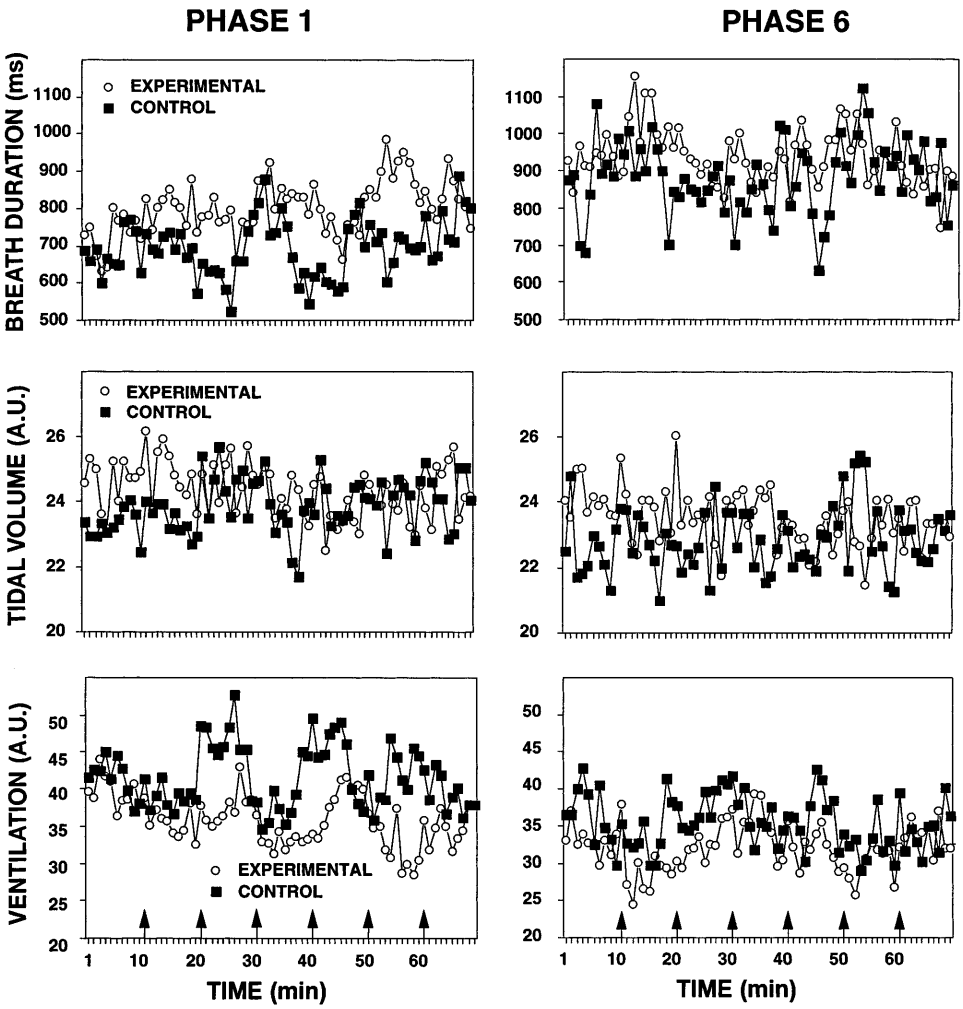

Fig. 5. Responses of breath duration, tidal volume, and ventilation to tone during *day 3* (*trials 1–7*). Breath-by-breath values were averaged over successive 1-min periods and then averaged over each group. Arrows, presentation of tone (conditioned stimulus). No CO_2 was delivered.

transient decreases in T_T and increases in $\dot{V}_I$, which were not observed in the experimental group. However, these differences were not significant and, therefore, provided no further support to the conditioning effects observed on *day 2*. We do not totally rule out the possibility that contextual factors have a role in these nonspecific effects. The recent experiments by Mongeluzi et al. (23) provide a typical example of associations learned between an environment and the aversive effect of a 100% CO_2 stimulus. In these experiments, the conditioning and control environments differed as regards the size and lighting of the experimental room, the presence or the absence of an odor cue (vanilla), and the intensity of background noise. After exposure to a single test with 100% CO_2, freezing periods (i.e., the lack of any detectable body movement except for breathing, which was observed but not measured) were longer in the conditioning than in the control environment. In the present experiment, environmental cues (plethysmograph and visual or auditory cues from the laboratory environment) were strictly identical in the two groups and were, therefore, unlikely to explain between-group differences in breathing patterns. However, other contextual cues may have differently shaped the perceptual experience in the two groups. In particular, the different procedures applied to each group were associated with different levels of wakefulness and aversiveness. First, the sequential exposure to tones and hypercapnia in the control group yielded a higher rate of events, and possibly a greater arousal effect, with concomitant increases in breathing frequency and $\dot{V}_I$. Second, the general aversiveness to the situation may have been greater in the control rats because, unlike the experimental rats, they were not warned of the oncoming aversive hypercapnic stimulus (20). The possibility that the contextual cues were associated with more highly aversive events in the experimental than in the control group may explain why the latter group displayed greater ventilatory activity. We may, therefore, postulate that our data can be explained, at least in part, by the association between the experimental context and stress.

Direct comparison of the hypercapnic response with the responses reported by previous authors was hampered by the fact that, in the present experiment, a tone was delivered during the hypercapnic stimulus. In fact, this response was roughly similar to previously reported responses (17); thus, the 8% CO_2 stimulus used in the present study elicited an ~100% increase in ventilation. However, repetition of the hypercapnic

tests had different effects in the two groups. We observed that the experimental group exhibited higher V_I and lower T_T at the end of the acquisition tests than did controls. The experimental rats may have anticipated the tone, as a result of having learned the association between the CO_2 stimuli and a temporal cue: the constant 10-min interval between two successive stimuli. Previous reports on the conditioning of rats to drug administration provide indirect support for this interpretation in terms of time conditioning. For example, conditioning of body temperature in rats, by using morphine as an US, yielded not only conditioned hyperthermia in response to a CS but also conditioned hypothermia 1 h before the expected morphine injection (10). However, in the present experiment, we feel that this possibility is unlikely because it would have occurred in the control group as well. In fact, we failed to observe any trace of a CR in the controls at the times corresponding to their hypercapnic tests. Alternatively, the changes occurring in the experimental group before the CS may be accounted for by effects of learning on the response to the hypercapnic stimuli. Previous authors have shown that, in some circumstances, the repetition of hypercapnic stimuli may induce changes in the V_T and T_T adopted to achieve a given level of V_I (for a review, see Ref. 14). These changes are poorly understood, but learning has been proposed as one of the underlying mechanisms (21). The respective merits of the two above interpretations are difficult to assess on the basis of the present data. However, this may easily be done in further experiments. A different design, in which the CS would be delivered at random time intervals, would prevent time conditioning from occurring but would not prevent the rats from learning to change their ventilatory response to hypercapnia.

The present finding of an inhibitory CR in the experimental group was consistent with the data of Biryukov et al. (5), although these authors also reported conditioned changes in V_T, contrary to our present data. This difference may be explained by the stronger US used by these authors (50% CO_2), which presumably yielded a stronger conditioned inhibition than did the present 8% CO_2 stimulus. The significant results for inhibitory conditioning found here are at variance with other those of studies, in which the opposite stimulating effect was reported (16, 26), and also with the results of studies that failed to establish any conditioned change (37). The present experiment conditions differed from conditions in these studies in two major respects: the inclusion of a control group and the attempt to mask the onset of the US by acetic acid.

The control procedure consisted of unpairing the CS and US in a sequential control group. Our procedure minimized the total duration of the experiment by presenting the CS only 1 min after the ventilatory effects of the US had vanished. However, a general drawback to the sequential control procedure is that some conditioning may occur in the control subjects if they associate the CS with the US, despite the interval between them. In general, such an interval makes conditioning more difficult but does not necessarily prevent it (20). This possible conditioning of the control group may attenuate the differences between the two groups, thus leading to underestimation of the effects of conditioning. However, in the present study, within-group analysis of the controls did not reveal any conditioning in this group. Another drawback to our control procedure is that it may yield time conditioning, a possibility already considered above. An alternative control procedure would be to deliver the US and CS at random intervals to the control group, while excluding their simultaneous occurrence, to prevent any conditioning ("explicitly unpaired" control group). Under the present conditions, this control procedure would have lengthened the acquisition period, which might have been designed to cover successive days instead of a single day (*day 2*). However, despite the inherent limits of the sequential control used here, the present results show that the ventilatory effects of the tone were different, depending on whether they were previously paired with hypercapnia, which clearly established a conditioning effect.

The choice of acetic acid to mask CO_2 was based on previous studies showing that a somatosensory stimulus such as CO_2 could be masked by an irritant acting on the trigeminal fibers (15, 19). Among the variety of irritants exerting such action, we chose acetic acid, one of the less noxious (3). However, our contention that CO_2 was actually masked by acetic acid under the specific conditions of the present experiment was not based on independent validation studies. Given the rats' sensitivity to somatosensory stimulation (without any masking agent, a rat is able to perceive 0.52% CO_2) (38), we do not rule out that the rats detected the delivery of CO_2, even though this detection was delayed by acetic acid. In addition, CO_2 does not belong to the rats' habitual sensory repertoire, which probably made this stimulus more salient than the tone. Despite this, the present data show, first, that the sensory effects of CO_2 did not overshadow the tone enough to prevent conditioning from occurring. Second, they suggest that the negative findings of the previous experiments using a CO_2 stimulus delivered through the upper airways may be due to the overshadowing of the experimentally controlled CS by the sensory effects of CO_2 (37).

The present result of an inhibitory CR may be due to the inhibitory and aversive effects of CO_2. In addition to its strong activation of breathing through chemosensitivity, the CO_2 stimulus, at least above 8%, has inhibitory effects on breathing, presumably through the upper airway sensory reflex (1). In addition, the increase in V_T, which is one component of the hypercapnic response, elicits an inhibitory vagal reflex by activating stretch receptors in the lung and chest wall. This raises the possibility that these inhibitory effects of hypercapnia were predominantly associated with the CS during the conditioning process. Ventilatory conditioning using aversive stimuli such as inhibitors of breathing has previously been reported. In the experiments of Orem and Trotter (25), the cats were trained to stop breathing in response to a small puff of ammonium hydroxide vapor at the onset of inspiration, and

additional ammonium hydroxide was given if the cat failed to stop inspiration within 500 ms. The major outcomes were that conditioned apneas were associated with the inactivation of cells in the ventral and dorsal ventilatory groups and that some cells within that system became active during these apneas. We believe that these findings may also account for the present inhibitory effect.

We do not deny that the CR might have been mediated by instrumental contingencies between breathing and the aversive CO_2. This possibility is supported by two arguments: first, for rats, CO_2 concentrations are aversive above 8% (35), the level reached in the present experiment. Second, rats are capable of "voluntary" control of breathing to obtain a reward or avoid punishment, as shown by experiments in which changes in the breathing pattern were followed by either rewarding electric brain stimulations (13) or aversive electric shocks (22). The possibility that the present CR was an avoidance response to the aversive CO_2 constitutes an alternative interpretation of our data.

An objection may be raised that the inhibitory conditioning that stems from the aversive sensation caused by CO_2 is poorly suited to model the automatic processes that govern breathing patterns in normal humans. However, it is not impossible that spontaneous breathing patterns may be governed, at least partly, by responses shaped by the optimization of respiratory comfort. This is in line with the notion developed by Chonan et al. (7) that the minimization of respiratory sensations may play a substantial role in the adjustment of breathing patterns. In fact, these authors observed that voluntary changes in breathing frequency or minute ventilation at given levels of P_{CO_2} systematically intensified the sensation of dyspnea, suggesting that spontaneous patterns normally minimize dyspnea. Under natural conditions, respiratory sensations are not necessarily minimized through conscious and voluntary processes but rather through unconscious processes, possibly as a result of some kind of automatization. Within this framework, the conditioned inhibition of breathing as described in the present study may be relevant to the investigation of these processes.

In conclusion, this experiment showed that pairing an auditory stimulus with an 8% CO_2 stimulus led to an inhibitory CR. This conditioning probably resulted from the learning of an association between the auditory stimulus and the aversive or inhibitory effects of CO_2. The present results confirmed the high sensitivity of the respiratory controller to conditioning processes. However, the ability of conditioning by CO_2 inhalation to account for the conditioning processes that may occur under natural conditions requires confirmation by further experiments.

The authors are grateful to Stéphane Delavaud for technical contribution.

This work was supported by Institut National de la Santé et de la Recherche Médicale Grants CJF89-09 and CRI96-03.

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Received 2 April 1996; accepted in final form 5 June 1997.

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