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BACLOFEN INTO THE LATERAL PARABRACHIAL NUCLEUS INDUCES HYPERTONIC SODIUM CHLORIDE AND SUCROSE INTAKE IN RATS

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Abstract—$GABA_A$ and $GABA_B$ receptors are present in the lateral parabrachial nucleus (LPBN), a pontine area involved with inhibitory mechanisms related to the control of sodium appetite. Activation of $GABA_A$ receptors in the LPBN induces strong ingestion of 0.3 M sodium chloride (NaCl) in normonatremic and euhydrated rats. In the present study, we investigated the effects of the $GABA_B$ receptor agonist baclofen, injected alone or combined with $GABA_A$ or $GABA_B$ receptor antagonists into the LPBN on 0.3 M NaCl, water, 0.06 M sucrose and food intake in normonatremic and euhydrated rats. Male Holtzman rats with stainless steel cannulas implanted bilaterally in the LPBN were used. In normonatremic and euhydrated rats, bilateral injections of baclofen (0.5 nmol/0.2 μl) into the LPBN induced 0.3 M NaCl (24.0±3.1 vs. saline: 2.0±0.8 ml/240 min) and water intake (10.6±1.4 vs. saline: 3.5±0.7 ml/240 min) in a two-bottle test. Injections of $GABA_B$ receptor antagonists CGP 35348 (50 nmol/0.2 μl) or 2-hydroxysaclofen (5 nmol/0.2 μl) or $GABA_A$ receptor antagonist bicuculline (1.6 nmol/0.2 μl) into the LPBN reduced 0.3 M NaCl (14.1±4.7 ml/240 min; 9.97±2.5 ml/210 min; 8.8±5.9 ml/240 min, respectively) and water intake induced by baclofen injected into the LPBN. Baclofen (0.5 nmol/0.2 μl) injected into the LPBN also induced 0.06 M sucrose intake (21.8±5.9 vs. saline: 5.0±2.6 ml/180 min). Urinary volume and sodium excretion had a tendency to decrease after baclofen injection into the LPBN, whereas arterial pressure and food intake were not affected. The results show that baclofen injected into the LPBN, in normonatremic and euhydrated rats, produces a natriorexigenic effect dependent on $GABA_A$ and $GABA_B$ receptor activation. The natriorexigenic effect is not secondary to alterations in blood pressure or sodium urinary excretion. In addition, baclofen injected into the LPBN also induces 0.06 M sucrose intake.

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Abbreviations: ANG II, angiotensin II; CCK, cholecystokinin; CRF, corticotropin-releasing hormone; HR, heart rate; K^+, potassium; LPBN, lateral parabrachial nucleus; MAP, mean arterial pressure; MPBN, medial parabrachial nucleus; NAc, nucleus accumbens; NaCl, sodium chloride; Na^+, sodium; PB, parabrachial nucleus; sc, subcutaneous.

Key words: sodium appetite, sucrose intake, water intake, GABA receptors, urinary excretion, blood pressure.

The parabrachial complex (PB) is an important pontine area involved in the control of ingestive behavior receiving visceral and gustatory signals (Rogers et al., 1979; Hermann and Rogers, 1985; Yamamoto et al., 1994; Baird et al., 2001). Main inhibitory mechanisms involved in the control of water and especially sodium intake are present in the lateral parabrachial nucleus (LPBN), the portion of the PB located dorsolaterally to the superior cerebellar peduncle (Ohman and Johnson, 1989; Menani and Johnson, 1995, 1998; Menani et al., 1996; Andrade et al., 2004; Callera et al., 2005; De Oliveira et al., 2007, 2008; De Gobbi et al., 2009).

The LPBN sends projections to forebrain areas involved in the control of fluid-electrolyte balance, like specific hypothalamic nuclei (ventromedial, dorsomedial, paraventricular and supra optic) and amygdala and receives projections from the area postrema and the medial portion of the nucleus of the tractus solitary (Ciriello et al., 1984; Shapiro and Miselis, 1985; Herbert et al., 1990; Krukoff et al., 1993; Jhamandas et al., 1996). Signals from arterial baroreceptors, cardiopulmonary volume receptors and other visceral receptors like taste receptors reach the nucleus of the tractus solitary before ascending to LPBN that in turn may send these signals to forebrain areas involved in the control of fluid-electrolyte balance (Johnson and Thunhorst, 1997).

Bilateral injections of the serotonergic antagonist methysergide into LPBN increased water and hypertonic NaCl intake induced by different treatments, such as central angiotensin II (ANG II) administration, 24 h of sodium depletion produced by the subcutaneous (sc) treatment with the diuretic/natriuretic furosemide combined with 24 h of sodium deficient diet, treatment with the combination of sc furosemide+low dose of captopril (angiotensin converting enzyme blocker), s.c. deoxycorticosterone (Menani et al., 1996, 1998; De Gobbi et al., 2000). Conversely, bilateral injections of the serotonergic agonist DOI (2,5-dimethoxy-4-iodoamphetamine) reduced sodium and water intake induced by furosemide+captopril. These results suggest that an inhibitory serotonergic mechanism involved in the control of water and sodium intake is present in the LPBN. Besides serotonin (5-HT), other neurotransmitters in the LPBN like cholecystokinin (CCK), corticotropin-releasing hormone (CRF), glutamate, opioids, GABA and noradrenaline may modulate the inhibitory mechanisms in the LPBN affecting sodium and water intake (Menani et al., 1996; Menani and Johnson, 1998; Andrade

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et al., 2004; Callera et al., 2005; De Castro e Silva et al., 2006; De Oliveira et al., 2007, 2008; De Gobbi et al., 2009; Gasparini et al., 2009). Voluntary intake of sucrose induced c-fos protein expression in medial and lateral portion of PB and activation of 5-HT_{1B} receptors in the LPBN also reduces food intake, whereas administration of midazolam (benzodiazepine) into LPBN in satiated rats increased food and 0.09 M sucrose intake (Streefland et al., 1996; Higgs and Cooper, 1996; Lee et al., 1998; Simansky and Nicklous, 2002), suggesting that LPBN is also important in the control of food and sucrose intake.

A dense group of immunoreactive varicosities for GABA was described in PB complex/Kolliker fuse nucleus, suggesting that the neuronal process of this area is under an important GABAergic influence, particularly the gustatory and visceral portion of PB (Christie and North, 1988; Araki et al., 1992; Kobashi and Bradley, 1998). GABA is an inhibitory neurotransmitter widely spread in the central nervous system that binds to two subtypes of receptors: $GABA_A$ and $GABA_B$ receptors (Bowery et al., 1987; Araki et al., 1992). The post-synaptic effect of GABA is mediated mainly by $GABA_A$ receptors (bicuculline-sensitive) linked to chloride channels (Bormann, 1988). $GABA_B$ receptors are located mainly on pre-synaptic terminals and belong to G protein family causing membrane hyperpolarization and, therefore, promoting inhibition of neurotransmitter release due to the inhibition of calcium voltage sensitive channels or increased potassium conductance (Bormann, 1988; Zhang and Mifflin, 1998). Both $GABA_A$ and $GABA_B$ receptors are present in LPBN (Christie and North, 1988; Araki et al., 1992) and a previous study showed that muscimol ($GABA_A$ receptor agonist) injected into LPBN induces a strong ingestion of hypertonic NaCl in normonatremic and euhydrated rats (Callera et al., 2005).

Considering the importance of LPBN in the control of sodium and water intake, the existence of $GABA_A$ and $GABA_B$ receptors in the LPBN and the strong ingestion of 0.3 M NaCl intake produced by muscimol injected into the LPBN, in the present study we investigated the effects of the $GABA_B$ receptor agonist baclofen injected alone or combined with $GABA_A$ or $GABA_B$ receptor antagonists into the LPBN on 0.3 M NaCl and water intake in normonatremic and euhydrated rats. In addition, possible changes on food and sucrose intake and on cardiovascular and renal responses to baclofen injected into the LPBN were also investigated.

EXPERIMENTAL PROCEDURES

Animals

Male Holtzman rats weighing 290–310 g were used. The animals were housed in individual stainless steel cages with free access to normal sodium diet (Guabi rat chow, Paulinia, SP, Brazil), water and 0.3 M NaCl (and to 0.06 M sucrose solution when the ingestion of sucrose was tested). Room temperature was maintained at 23±2 °C, and humidity at 55±10% on a 12:12 light-dark cycle with light onset at 7:00 AM. All tests were performed from 8:00 AM to 1:00 PM. The Ethical Committee for Animal Care and Use from Dentistry School of Araraquara—UNESP approved the experimental protocols used in the present study. The experimental protocols followed the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication no. 80-23, 1996). All efforts were made to minimize animal discomfort and the number of animals used.

Cerebral cannulas

Rats were anesthetized with s.c. ketamine (80 mg/kg of body weight, Agener Uniao, Embu-Guacu, SP, Brazil) combined with xylazine (7 mg/kg of body weight, Agener Uniao, Embu-Guacu, SP, Brazil) and placed in a stereotaxic instrument (Kopf, Tujunga, CA, USA). The skull was leveled between bregma and lambda. Stainless steel 23-gauge cannulas were implanted bilaterally into the LPBN using the following coordinates: 9.4 mm caudal to bregma, 2.2 mm lateral to the midline, and 4.3 mm below the dura mater. The tips of the cannulas were positioned at a point 2 mm above each LPBN. The cannulas were fixed to the cranium using dental acrylic resin and watch screws. Stainless steel obturators (30-gauge) filled the cannulas, except during injections. After the surgery, the rats received i.m. injections of the analgesic cetoprophen 1% (0.03 ml/rat) and a prophylactic dose of the antibiotic penicillin (30,000 IU). Rats were allowed to recover for 5 days before starting the tests.

Injections into the LPBN

Bilateral injections into the LPBN were made using 5-μl Hamilton syringe connected by polyethylene tubing (PE-10) to 30-gauge injection cannula (2 mm longer than the guide cannula) that was carefully inserted into the guide cannula 15 s before starting manual injection. For bilateral injections, the first injection was initially performed in one side, the injection cannula was removed and repositioned in the contra-lateral side and, then the second injection was made. Therefore injections were made ~1 min apart. The injection volume into the LPBN was 0.2 μl in each site. After the injections, rats were placed back into their cage.

Drugs

The drugs injected into the LPBN were baclofen and 2-hydroxysaclofen purchased from RBI-Sigma Chemicals (St Louis, MO, USA); p-(3-aminopropyl)-p-diethoxymethyl-phosphonic acid (CGP 35348) and bicuculline purchased from Tocris (Ellisville, MO, USA). Baclofen, 2-hydroxysaclofen and CGP 35348 were dissolved in saline and bicuculline was dissolved in a mix of propylene glycol/water 2:1 (vehicle).

Saline or vehicle (propylene glycol/water) was injected in the LPBN as control.

Water and 0.3 M NaCl intake by satiated, normonatremic and euhydrated rats

After the cerebral surgery, the rats had free access to water, 0.3 M NaCl and food for at least 5 days (period of recovery). The rats were tested in their home cages. Water and 0.3 M NaCl were provided from burettes with 0.1-ml divisions that were fitted with metal drinking spouts. Food was not available for the rats during the tests. Cumulative intake of 0.3 M NaCl and water was measured at every 30 min during 210 or 240 min, starting 15 min after bilateral injections of baclofen (0.5 nmol/0.2 μl) or saline (0.2 μl) into the LPBN. A recovery period of at least 3 days was allowed between tests.

In one group of normonatremic and euhydrated rats, only the effects of baclofen injected into the LPBN on water and 0.3 M NaCl intakes (two-bottle test) were tested. The rats were submitted to two tests. In each test, the group of rats was divided in two. In the first test half of the group received saline and the other half received baclofen (0.5 nmol/0.2 μl) into the LPBN. In the next test, the rats received the same treatments in a counterbalanced design.

In another group of normonatremic and euhydrated rats, the effects of the combination of CGP 35348 ($GABA_B$ receptor antag-

onist) and baclofen injected into the LPBN on water and 0.3 M NaCl intake (two-bottle test) were tested. Saline or CGP 35348 (50 nmol/0.2 μl) was injected into the LPBN 15 min before injection of baclofen (0.5 nmol/0.2 μl) or saline into the same area. These rats were submitted to four tests and received the following combinations of treatments into the LPBN: saline+saline, saline+baclofen, CGP 35348+baclofen and CGP 35348+saline. In each test, the group of rats was divided in two and half of the group received one of the combinations of treatments cited above into the LPBN and the remaining animals received another combination of treatments into the LPBN. The sequence of the treatments in each rat in different tests was randomized and at the end of four tests each rat received all the four treatments. The same protocol was also tested in a different group of rats that received injections of 2-hydroxysaclofen (5 nmol/0.2 μl) into the LPBN instead of CGP 35348.

A previous study showed that $GABA_A$ receptor activation in LPBN induced sodium intake in normonatremic and euhydrated rats (Callera et al., 2005). Therefore, to test a possible participation of $GABA_A$ receptors in the effects of baclofen, another group of rats received injections of the $GABA_A$ receptor antagonist bicuculline (1.6 nmol/0.2 μl) or vehicle combined with baclofen (0.5 nmol/0.2 μl) or saline injected into the LPBN. The protocol was similar to that used to test the effects of pre-treatment with 2-hydroxysaclofen or CGP 35348, except that bicuculline instead of 2-hydroxysaclofen or CGP 35348 was injected into the LPBN. The dose of bicuculline used was an effective dose that blocks $GABA_A$ receptors into LPBN as showed previously (Callera et al., 2005).

Water intake after bilateral injections of baclofen (0.5 nmol/0.2 μl) or saline (0.2 μl) into the LPBN was also tested in group of satiated and euhydrated rats that had access only to water during the test. The protocol used was similar to that used when satiated rats treated with baclofen injected into the LPBN had access to water and 0.3 M NaCl simultaneously.

Food, water and 0.3 M NaCl intake by satiated, normonatremic and euhydrated rats

The effects of baclofen injected into LPBN were also tested in a group of satiated and euhydrated rats that had access simultaneously to water, 0.3 M NaCl and food. Water and 0.3 M NaCl were provided from burettes with 0.1-ml divisions that were fitted with metal drinking spouts. Rats had access also to a pre-weighted amount of regular chow pellets. Water, 0.3 M NaCl and food intake was recorded at every 30 min during 240 min, starting 15 min after bilateral injections of baclofen (0.5 nmol/0.2 μl) or saline (0.2 μl) into the LPBN. All the chow spillage under the cages was recovered at every measurement to calculate food intake.

The rats were submitted to two tests. In each test, the group of rats was divided in two. In the first test half of the group received saline and the other half received baclofen into the LPBN. In the next test, the rats received the same treatments in a counterbalanced design. A recovery period of at least 3 days was allowed between tests.

Water and 0.06 M sucrose intake by satiated, normonatremic and euhydrated rats

Rats had daily *ad libitum* access to food pellets, water and 0.06 M sucrose for at least 5 days before starting the tests. On the days of tests, baclofen (0.5 nmol/0.2 μl) or saline was injected, bilaterally into the LPBN, 15 min before access to burettes with 0.1-ml divisions filled with water and 0.06 M sucrose. Cumulative water and 0.06 M sucrose intake (two-bottle test) was measured at every 30 min during 180 min.

The rats were submitted to two tests. In each test, the group of rats was divided in two. In the first test half of the group received saline and the other half received baclofen into the LPBN. In the next test the rats received the same treatments in a counterbalanced design. A recovery period of at least 3 days was allowed between experimental sessions.

Renal excretion

Renal excretion was tested in a group of euhydrated and satiated rats that received bilateral injections of baclofen (0.5 nmol/0.2 μl) or saline into the LPBN in two different conditions: with and without access to water and 0.3 M NaCl immediately after injections into the LPBN.

To test renal excretion when rats had no access to water and 0.3 M NaCl, in the first test half of the group received bilateral injections of baclofen and the remaining animals received injections of saline into the LPBN. In the next test, the rats received the same treatments in a counterbalanced design. A similar protocol was repeated in the next two tests when rats had access to water and 0.3 M NaCl to ingest. As control, to compare with the renal excretion when rats received baclofen into the LPBN and had access to water and 0.3 M NaCl, rats were submitted to an additional test in which they received injections of saline into the LPBN combined with intragastric loads of water and 0.3 M NaCl at every 30 min at the same volume they had ingested in the previous tests that they received baclofen into the LPBN. In this additional test, the animals received eight intragastric loads of the mixture (water+0.3 M NaCl) at time 0, 30, 60, 90, 120, 150, 180 and 210 min. The amount of fluid in each load was the same that the animals had ingested when they were treated with baclofen into the LPBN in the previous tests at the times 0–30; 30–60; 60–90; 90–120; 120–150; 150–180; 180–210; 210–240 min.

No food was available during renal excretion test. Urine samples were collected at every 30 min during 240 min starting immediately after LPBN injections. Urine samples were analyzed in a Na^+/K^+ analyzer (Nova 1, Nova Biomedical, Waltham, MA, USA). Water and 0.3 M NaCl intake was also recorded at every 30 min during 240 min if rats had access to water and NaCl solution.

Arterial pressure and heart rate recordings

Rats were anesthetized with s.c. ketamine (80 mg/kg of body weight)+xylazine (7 mg/kg of body weight) and a polyethylene tubing (PE 10 connected to a PE 50) was inserted into the abdominal aorta through the femoral artery. The cannula was guided s.c. and exteriorized at the back of the rat. On the next day the cannula was connected to a P23 Db pressure transducer

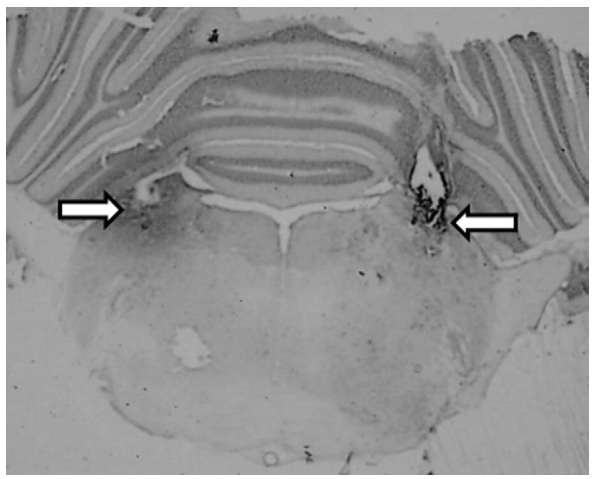

Fig. 1. Photomicrograph showing the sites of injections into the LPBN (arrows).

(Statham Gould, Madison, WI, USA) coupled to a pre-amplifier (model ETH-200 Bridge Bio Amplifier, CB Sciences, Dover, NH, USA) connected to a Powerlab computer recording system (Powerlab 16SP, ADInstruments, Colorado Springs, CO, USA) to record mean arterial pressure (MAP) and heart rate (HR) in unanesthetized rats. One group of rats was tested for the effects of saline and another group for the effects of baclofen (0.5 nmol/0.2 μl) injected into the LPBN on MAP and HR. MAP and HR were recorded for the next 120 min after baclofen injections into LPBN and the maximum changes were analyzed. During arterial pressure and heart rate recordings, water, NaCl and food were not available for the rats.

Histology

At the end of the experiments, the animals received bilateral injections of 2% Evans Blue solution (0.2 μl) into the LPBN. They were then deeply anesthetized with sodium thiopental (80 mg/kg of body weight) and perfused transcardially with saline followed by 10% formalin. The brains were removed, fixed in 10% formalin, frozen, cut in 50-μm serial coronal sections, stained with Giemsa, and analyzed by light microscopy to confirm the injection sites into the LPBN.

Statistical analysis

The results are reported as means±SEM. Two-way repeated-measures (RMANOVA) or two-way ANOVA using treatments and times as factors and Fisher's least significance difference (LSD) tests were used for comparison. Differences were considered significant at $P<0.05$.

RESULTS

Histological analysis

The LPBN injection sites were centered in the central lateral and dorsolateral portions of the LPBN as defined by Fulwiler and Saper (1984). Fig. 1 shows the typical LPBN injection sites. Injections reaching the ventral lateral and external lateral portions as well as the Kolliker-Fuse nucleus were observed in some rats and the results from these rats were included in the analysis. In some rats, injections also spread to the brachium (superior cerebellar peduncle), or slightly ventral to this structure, reaching the dorsal portions of the medial

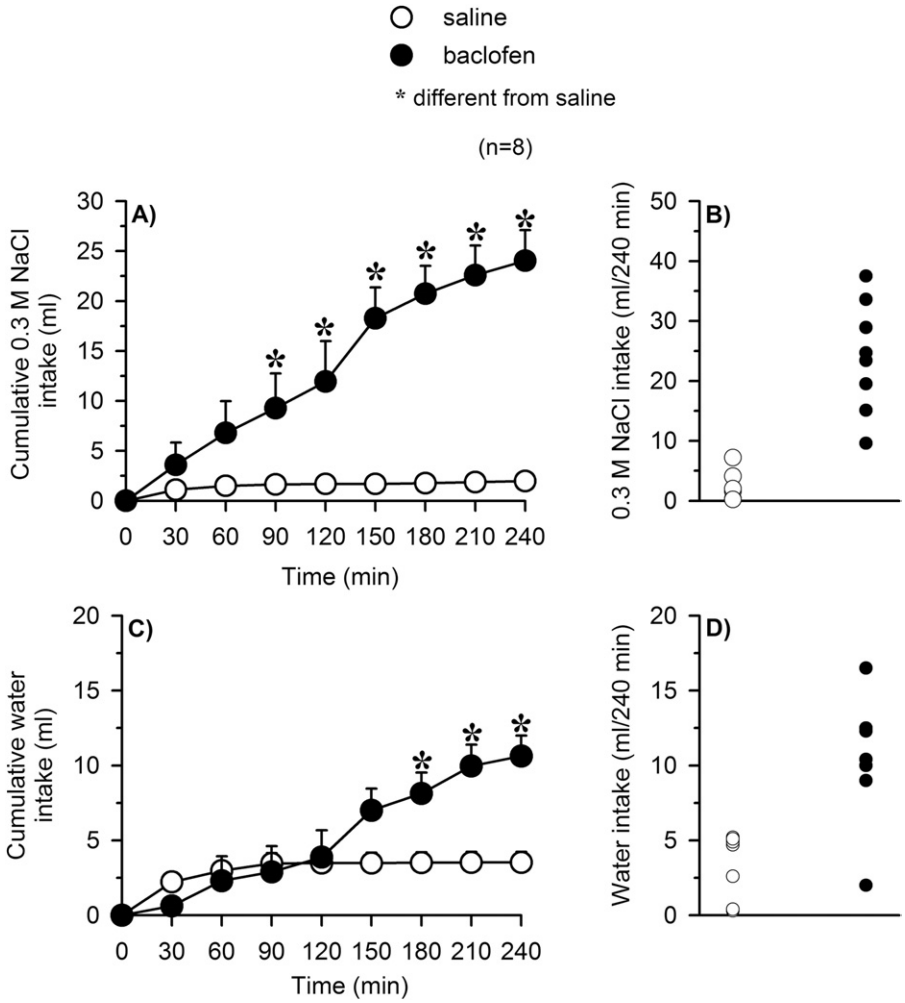

Fig. 2. (A) Cumulative 0.3 M NaCl intake; (B) individual 0.3 M NaCl intakes; (C) cumulative water intake; (D) individual water intakes by satiated, normonatremic and euhydrated rats treated with bilateral injections of baclofen (0.5 nmol/0.2 μl) or saline into the LPBN. (A) and (C) results are expressed as means±SEM; *n*=number of rats.

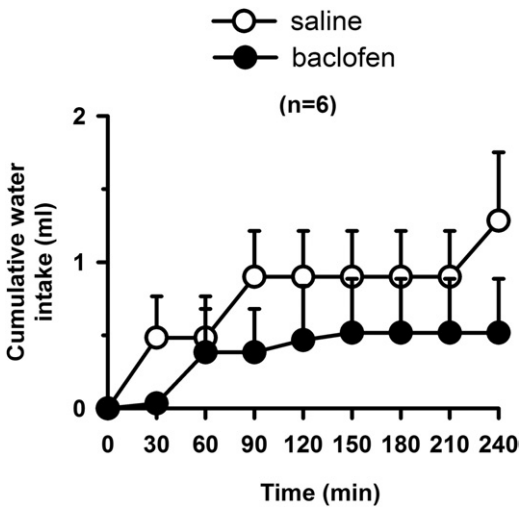

Fig. 3. Cumulative water intake by normonatremic and euhydrated rats treated with bilateral injections of baclofen (0.5 nmol/0.2 μl) or saline into the LPBN. Results are expressed as means±SEM; n=number of rats.

parabrachial nucleus (MPBN) uni or bilaterally. There was no difference in the effects whether injections were restricted to the LPBN or also spread to brachium and dorsal portions of MPBN.

Water and 0.3 M NaCl intake by euhydrated and satiated rats treated with bilateral injections of baclofen into the LPBN

Bilateral injections of baclofen (0.5 nmol/0.2 μl) into LPBN in normonatremic, euhydrated and satiated rats induced 0.3 M NaCl intake, as shown by the significant difference between treatments [$F(1,7)=19.7$; $P<0.05$], and also water intake, as suggested by the significant interaction between treatments and times [$F(7,49)=14.3$; $P<0.05$] when rats had access simultaneously to water and 0.3 M NaCl (two-bottle test) (Fig. 2). Compared to saline injections, the cumulative ingestion of 0.3 M NaCl significantly increased after baclofen injection into the LPBN after 90 min until the end of the test (Fig. 2A), whereas the cumulative ingestion of water significantly increased in the last hour of test (180–240 min) (Fig. 2C).

Baclofen (0.5 nmol/0.2 μl) injected into LPBN did not affect water intake in euhydrated and satiated rats when they had access only to water (one bottle test) [$F(1,5)=0.6$; $P>0.05$] (Fig. 3).

Water and 0.3 M NaCl intake by euhydrated and satiated rats treated with the combination of baclofen and CGP 35348, 2-hydroxysaclofen or bicuculline into the LPBN

Previous injections of the $GABA_B$ receptor antagonist CGP 35348 (50 nmo/0.2 μl) into LPBN reduced 0.3 M NaCl intake [$F(3,18)=9.6$; $P<0.05$] and water intake [$F(3,18)=7.3$; $P<0.05$] induced by baclofen (0.5 nmol/0.2 μl) injected in the same area in normonatremic and euhydrated rats (Fig. 4A, B).

Another $GABA_B$ receptor antagonist 2-hydroxysaclofen (5 nmol/0.2 μl) injected into LPBN also reduced 0.3 M NaCl intake, without changing water intake induced by injection of baclofen (0.5 nmol/0.2 μl) into the same area (Fig. 5A, B). ANOVA showed significant interaction between treatments and times for 0.3 M NaCl intake [$F(18,288)=15.7$; $P<0.05$] and water intake [$F(18,288)=5.52$; $P<0.05$] in rats that received the combination of 2-hydroxysaclofen and baclofen into the LPBN.

Similar to $GABA_B$ receptor antagonists, previous injections of the $GABA_A$ receptor antagonist bicuculline (1.6 nmo/0.2 μl) into the LPBN also reduced the natriorexigenic [$F(3,15)=4.8$; $P<0.05$] and dipsogenic [$F(3,15)=4.0$; $P<0.05$] effects of baclofen injected in the same area (Fig. 6).

To confirm that rats treated with baclofen injected into the LPBN have a preference for NaCl intake and that water intake is a consequence of the ingestion of hypertonic NaCl, the molarity of the cumulative fluid ingested (water+0.3 M NaCl) by rats treated with CGP 35348 and baclofen alone or combined into the LPBN was also analyzed (Table 1). In control test (saline injection into the

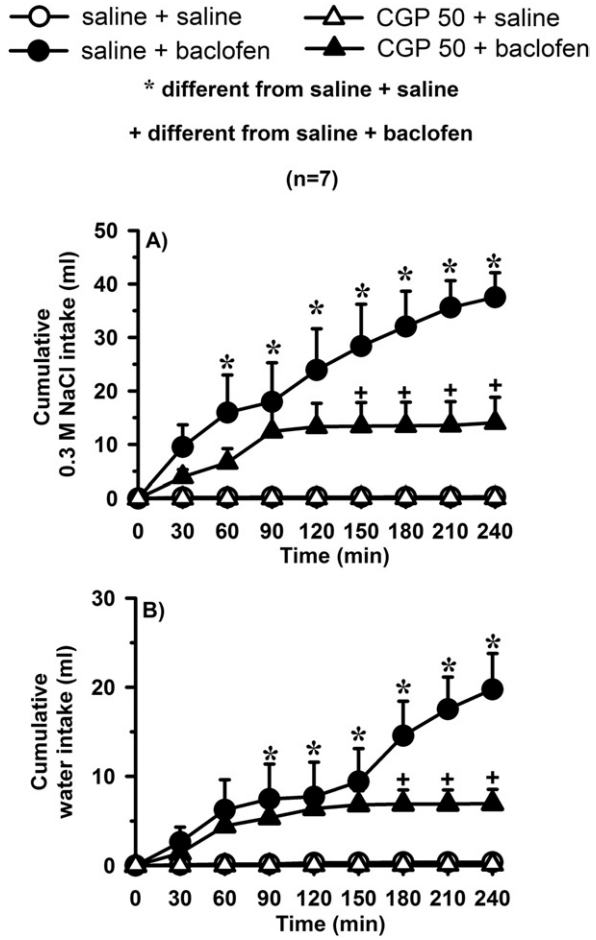

Fig. 4. (A) Cumulative 0.3 M NaCl intake; (B) cumulative water intake by normonatremic and euhydrated rats treated with bilateral injections of CGP 35348 (50 nmol/0.2 μl) or saline combined with baclofen (0.5 nmol/0.2 μl) or saline into the LPBN. Results are expressed as means±SEM, n=number of rats.

LPBN), the fluid ingested was hypo or isotonic (the molarity ranged from 0.04 to 0.16 M from the beginning to the end of the test), whereas after injections of baclofen into the LPBN the molarity of the fluid ingested increased and was iso or hypertonic (the molarity of the fluid ingested was 0.15 M at the beginning of the test, reached 0.22 M from 150 to 180 min of the test and was 0.21 M at the end) [$F(3,18)=4.3$; $P<0.05$] (Table 1). A nearly isotonic fluid was ingested after baclofen injection combined with CGP 35348 injection into the LPBN (molarity ranged from 0.12 to 0.16 M) (Table 1).

Food, water and 0.3 M NaCl intake by euhydrated and satiated rats treated with bilateral injections of baclofen into the LPBN

In rats that had access simultaneously to food pellets, water and 0.3 M NaCl, bilateral injections of baclofen (0.5 nmol/0.2 μl) into LPBN induced 0.3 M NaCl as showed by the significant interaction between treatments and times [$F(7,35)=3.6$; $P<0.05$] (Fig. 7A). However, in these rats, baclofen (0.5 nmol/0.2 μl) injected into LPBN did not affect water [$F(1,5)=0.01$; $P>0.05$] or food intake [$F(1,5)=0.1$; $P>0.05$] (Fig. 7B, C).

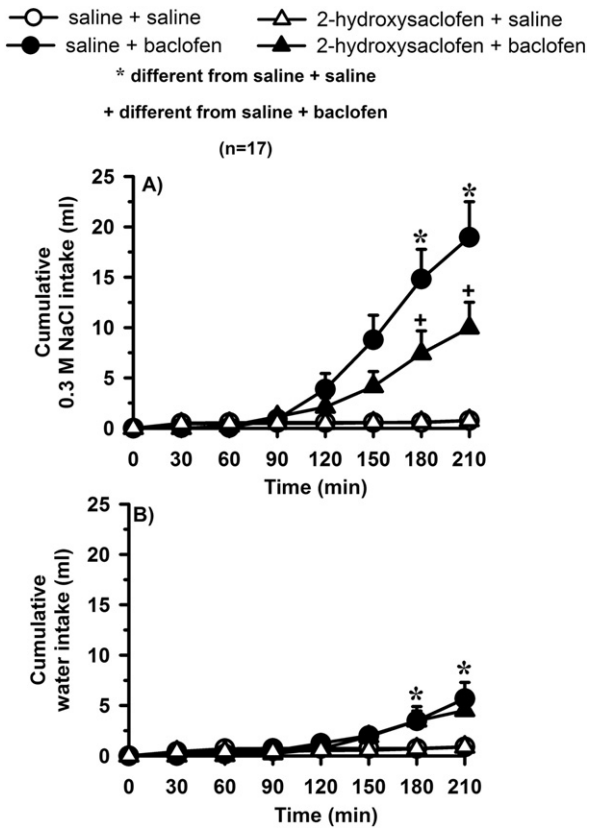

Fig. 5. (A) Cumulative 0.3 M NaCl intake; (B) cumulative water intake by normonatremic and euhydrated rats treated with bilateral injections of 2-hydroxysaclofen (5 nmol/0.2 μl) or saline combined with baclofen (0.5 nmol/0.2 μl) or saline into the LPBN. Results are expressed as means±SEM, *n*=number of rats.

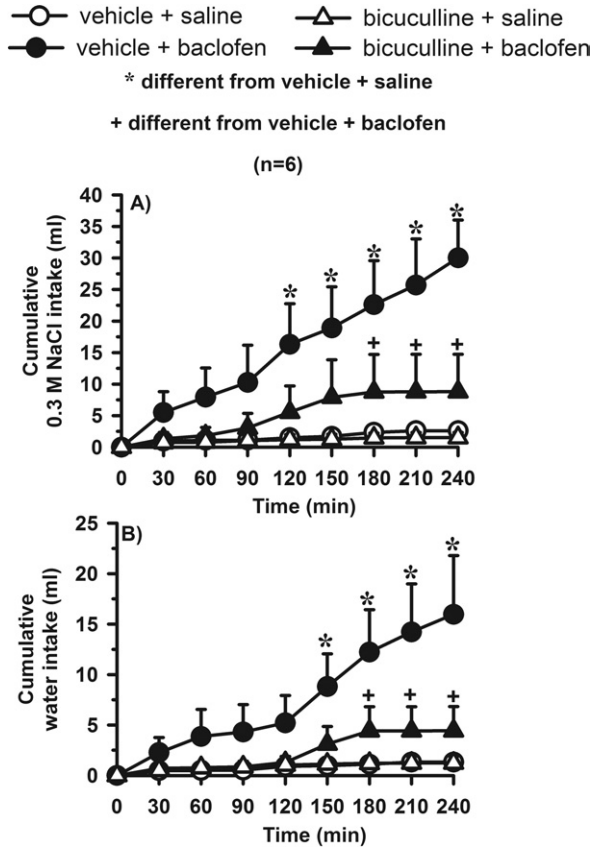

Fig. 6. (A) Cumulative 0.3 M NaCl intake; (B) cumulative water intake by normonatremic and euhydrated rats treated with bilateral injections of bicuculline (1.6 nmol/0.2 μl) or vehicle combined with baclofen (0.5 nmol/0.2 μl) or saline into the LPBN. Results are expressed as means±SEM, *n*=number of rats.

Water and 0.06 M sucrose intake by euhydrated and satiated rats treated with baclofen into the LPBN

Bilateral injections of baclofen (0.5 nmol/0.2 μl) into LPBN in satiated, normonatremic and euhydrated rats

Table 1. Molarity of the fluid (water+0.3 M NaCl) ingested by normonatremic and euhydrated rats treated with bilateral injections of CGP 35348 or saline combined with baclofen or saline into the LPBN

Time (min)	Treatment			
	Saline+saline	Saline+ baclofen	CGP+saline	CGP+ baclofen
30	0.04±0.04	0.15±0.04*	0.04±0.04	0.15±0.04*
60	0.07±0.04	0.17±0.03*	0.04±0.04	0.12±0.03
90	0.11±0.05	0.18±0.03	0.07±0.04	0.16±0.03
120	0.09±0.04	0.22±0.03*	0.04±0.03	0.16±0.03
150	0.09±0.04	0.22±0.03*	0.11±0.04	0.16±0.03
180	0.14±0.05	0.21±0.02	0.11±0.04	0.16±0.03
210	0.14±0.05	0.21±0.02	0.11±0.04	0.16±0.03
240	0.16±0.04	0.21±0.02	0.10±0.04	0.16±0.03

Results are expressed as means±SEM; * different from saline+saline; *n*=7. CGP (50 nmol/0.2 μl); baclofen (0.5 nmol/0.2 μl). Molarity was calculated using cumulative ingestion of water and 0.3 M NaCl at each time.

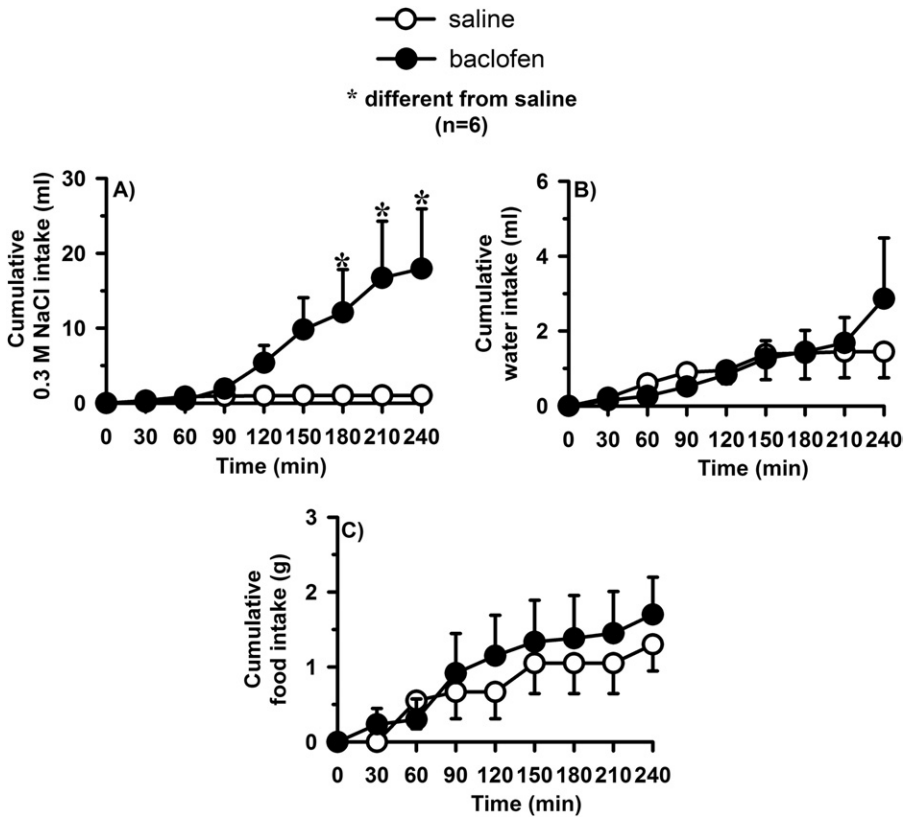

Fig. 7. (A) Cumulative 0.3 M NaCl intake; (B) cumulative water intake; (C) cumulative food intake by satiated, normonatremic and euhydrated rats treated with bilateral injections of baclofen (0.5 nmol/0.2 μl) or saline into the LPBN. Results are expressed as means±SEM, n=number of rats.

induced 0.06 M sucrose intake [$F(5,45)=4.61$; $P<0.05$] and a small ingestion of water [$F(5,45)=3.95$; $P<0.05$] (Fig. 8).

Renal excretion by satiated rats treated with bilateral injections of baclofen into the LPBN

In satiated rats that had no access to water or 0.3 M NaCl, bilateral injections of baclofen (0.5 nmol/0.2 μl) into the LPBN produced no change in urinary volume [$F(1,4)=1.9$; $P>0.05$], sodium [$F(1,4)=1.3$; $P>0.05$] or potassium excretion [$F(1,4)=0.7$; $P>0.05$] (Fig. 9).

In satiated rats that ingested water and 0.3 M NaCl, besides increases in 0.3 M NaCl (27.9±9.5 vs. saline: 0.5±0.3 ml/240 min) and water intake (9.9±3.1 vs. saline: 0.9±0.3 ml/240 min), bilateral injection of baclofen (0.5 nmol/0.2 μl) into LPBN also increased urinary volume [$F(2,8)=9.3$; $P<0.05$], sodium [$F(2,8)=10.8$; $P<0.05$] and potassium excretion [$F(2,8)=12.9$; $P<0.05$] (Fig. 9). However, the urinary volume and sodium excretion after baclofen injected into the LPBN by rats that ingested water and 0.3 M NaCl were lower compared to the urinary volume and sodium excretion of rats treated with saline into the LPBN that received intragastric load of 0.3 M NaCl and water at the same amounts that they ingested after baclofen injection into the LPBN (Fig. 9).

Changes in arterial pressure and heart rate in euhydrated rats treated with bilateral injections of baclofen into the LPBN

Bilateral injections of baclofen (0.5 nmol/0.2 μl) into the LPBN in normotensive rats (MAP: 116±3 mmHg and HR: 399±27 bpm) did not affect MAP (4.2±2.1 vs. saline: 4.0±1 mmHg, n=5–8), [$F(1,44)=0.7$; $P>0.05$] neither HR (−30.5±12.3 vs. saline: −29.1±23.4 bpm), [$F(1,44)=1.7$; $P>0.05$].

DISCUSSION

The present results show that bilateral injections of baclofen into the LPBN in satiated, normonatremic and euhydrated rats induce strong ingestion of 0.3 M NaCl shortly after injections (between 1 and 4 h after injections). In the two-bottle test, at the end of the 4 h, rats also increased water intake and the total of fluid (water+0.3 M NaCl) ingested was above 30 ml, a strong ingestion that in some rats was almost 20% of their 300 g body weight. The natriorexigenic effect of baclofen injected into the LPBN was reduced by the pre-treatment with the $GABA_B$ receptor antagonists CGP 35348 or 2-hydroxysaclofen or by the $GABA_A$ receptor antagonist bicuculline injected into the same area, suggesting that the activation of $GABA_A$ and $GABA_B$ receptors is important for the natriorexigenic effect of baclofen injected into the LPBN. Besides hypertonic

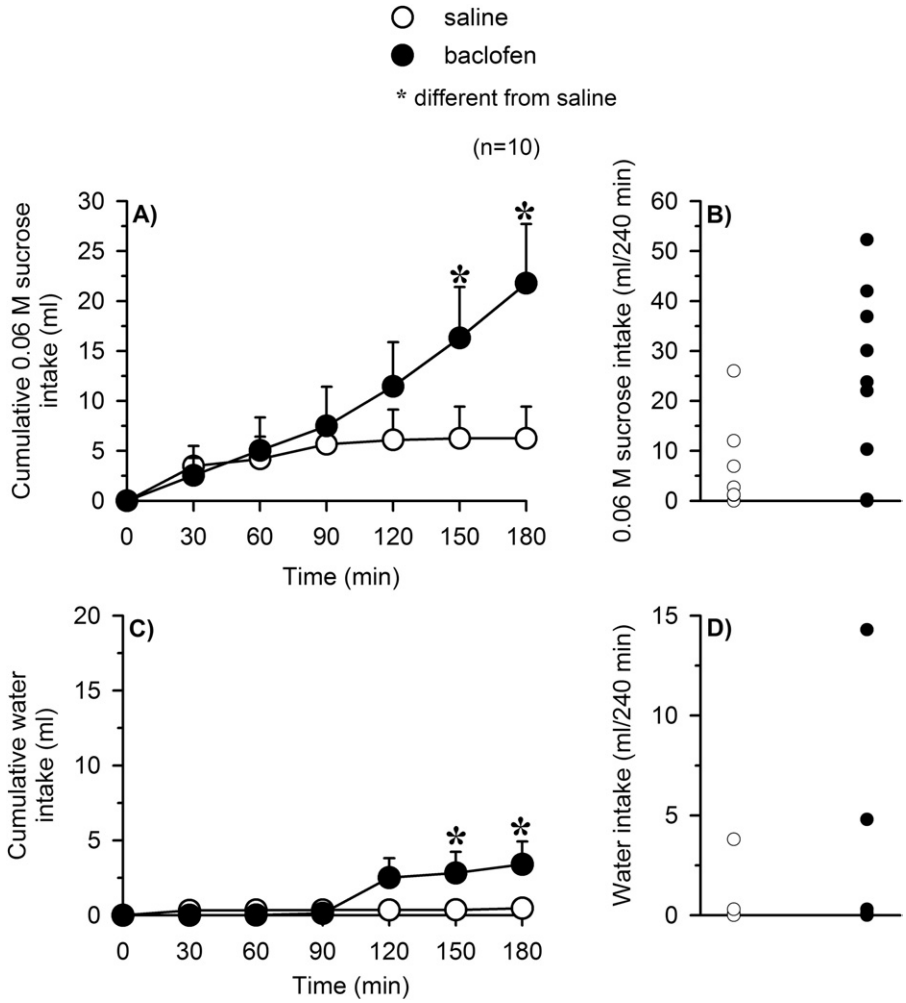

Fig. 8. (A) Cumulative 0.06 M sucrose intake; (B) individual 0.06 M sucrose intakes; (C) cumulative water intake; (D) individual water intakes by satiated, normonatremic and euhydrated rats treated with bilateral injections of baclofen (0.5 nmol/0.2 μl) or saline into the LPBN. (A) and (C) results are expressed as means±SEM; n=number of rats.

NaCl, baclofen injected into the LPBN also induced 0.06 M sucrose intake in satiated rats, which suggests that GABAergic mechanisms in the LPBN are also involved in the control of sucrose intake.

Although injections of baclofen into the LPBN induced no water intake if only water was available, the ingestion of water usually increased after injections of baclofen into the LPBN when rats simultaneously ingested 0.3 M NaCl, probably as a consequence of the increased plasma osmolarity due to the excessive ingestion of hypertonic NaCl. In spite of the simultaneous ingestion of water, this intake is not enough to compensate the increased osmolarity produced by the ingestion of hypertonic NaCl. The fluid ingested (water+0.3 M NaCl), after injections of baclofen into the LPBN, was hypertonic during most of the test (the molarity ranged from 0.15 to 0.22 M). There is no clear explanation, why water intake increased after injections of baclofen into the LPBN when rats also ingested 0.06 M sucrose, however, not when 0.3 M NaCl, food pellets and water were simultaneously available.

Baclofen injected into the LPBN induced no food intake in satiated rats. Therefore, the ingestion of hypertonic NaCl and sucrose solutions induced by baclofen injected into the LPBN is not due to non-specific activation of all ingestive behaviors. In addition, the blockade of 5-HT, activation of α_2 adrenoreceptors or injections of the opioid agonist β-endorphin into the LPBN did not affect 0.06 M sucrose in rats trained to ingest this solution for 2 h daily (Menani et al., 1998; Andrade et al., 2004; De Gobbi et al., 2007; De Oliveira et al., 2008), which suggests that the increased ingestion of 0.06 M sucrose by satiated rats treated with baclofen injections into the LPBN is an effect more specific for baclofen acting in the LPBN.

Injections of baclofen into the LPBN produced no change in arterial pressure and the increase in renal excretion seems to be only the consequence of increased ingestion of water and NaCl, suggesting that the natriorexigenic response to baclofen injected into the LPBN is not secondary to cardiovascular responses or increased renal excretion. Moreover, urinary sodium and diuresis by rats

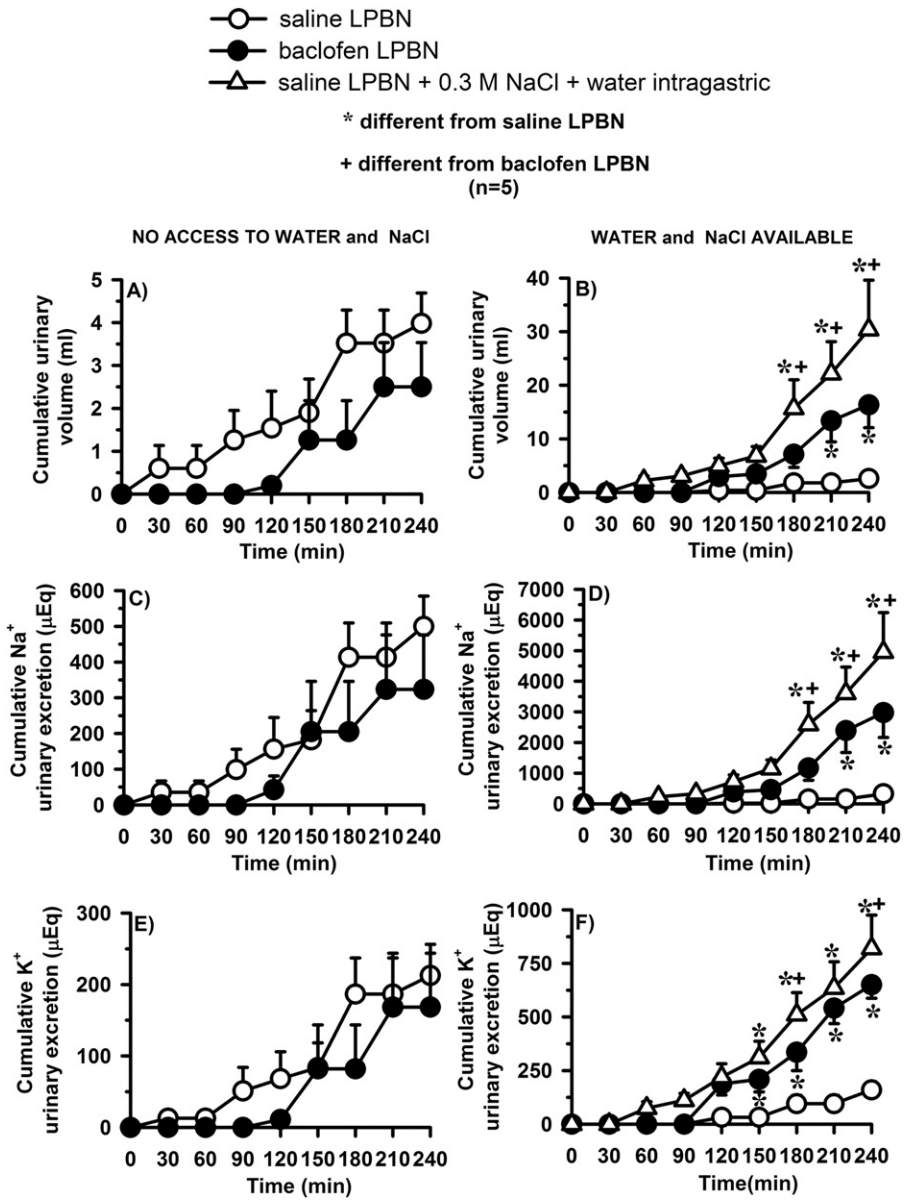

Fig. 9. (A, B) Cumulative urinary volume; (C, D) cumulative urinary sodium excretion; (E, F) cumulative urinary potassium excretion in normonatremic and euhydrated rats treated with bilateral injections of baclofen (0.5 nmol/0.2 μl) or saline into the LPBN when rats had access to water and 0.3 M NaCl (right panels) or had no access to these substances (left panels). Right panels show also urinary volume, sodium and potassium in rats treated with saline into the LPBN combined with intragastric loads of water+0.3 M NaCl at the same amounts that they had ingested after baclofen injection into the LPBN in a previous test. Results are expressed as means±SEM, *n*=number of rats.

treated with baclofen injected into the LPBN that ingested water and 0.3 M NaCl were reduced compared to the renal excretion by the same rats when they were treated with saline injected into the LPBN combined with intragastric load of equivalent amounts of water and 0.3 M NaCl. These results suggest that the blockade of LPBN mechanisms with injections of baclofen at the same time induces sodium intake and reduces urinary sodium excretion and diuresis, all responses consistent with an action of LPBN mechanisms against body fluid volume expansion as previously proposed for the serotonergic mechanism in the LPBN (Margatho et al., 2007).

Previous studies showed that the pre-treatment with the $GABA_A$ receptor antagonist bicuculline injected into the LPBN reduced the natriorexigenic effect of muscimol injected into the same area in satiated rats, whereas the $GABA_B$ receptor antagonist CGP 35348 injected into the LPBN did not modify the effects of muscimol injected into the LPBN (Callera et al., 2005; De Oliveira et al., 2007), suggesting that the activation of $GABA_A$ receptors in the LPBN induces NaCl intake. Differently from the previous results, the present study suggests that the natriorexigenic effect of baclofen injected into the LPBN depends on simultaneous activation of $GABA_A$ and $GABA_B$ receptors.

Perhaps, the activation of $GABA_B$ receptors by baclofen injected into the LPBN together with a baseline activation of $GABA_A$ receptors by endogenous GABA produces a sufficient inhibition of LPBN mechanisms to release sodium intake. Or instead of endogenous GABA, a non-specific activation of a limited number of $GABA_A$ receptors by baclofen together with the activation of $GABA_B$ receptors in the LPBN might inhibit the LPBN mechanisms releasing NaCl intake. In both cases, although only the activation of $GABA_B$ receptors produces no sodium intake, the activation of $GABA_B$ receptors in the LPBN may facilitate the effects of $GABA_A$ receptor activation on sodium intake. An involvement of $GABA_A$ receptors on baclofen effects was already reported. The inhibition of food intake by intraperitoneal administration of baclofen was abolished by previous treatment with bicuculline, suggesting a possible involvement of $GABA_A$ receptors in the effects of baclofen (Zarrindast et al., 1989). Moreover, it is not possible to completely discard the action of bicuculline blocking $GABA_B$ receptors, at least partially, which would reduce the activation of these receptors similarly to the effects of the specific $GABA_B$ antagonists. However, independent on how specific for $GABA_A$ receptors is the antagonism produced by bicuculline, the present and previous studies (Callera et al., 2005; De Oliveira et al., 2007) clearly suggest that both $GABA_A$ and $GABA_B$ receptor activation in the LPBN facilitates the ingestion of hypertonic NaCl.

Differently from early studies (Menani and Johnson, 1995, 1998; Menani et al., 1996; De Gobbi et al., 2000), more recent studies (Callera et al., 2005; De Oliveira et al., 2008) and the present results have suggested that only the blockade of the inhibitory mechanisms with injections of GABAergic or opioid agonists into the LPBN is enough to disrupt the satiety and to drive rats to ingest strong amount of hypertonic NaCl. Specific blockade of 5-HT, CCK, glutamate or CRF in the LPBN combined with the activation of facilitatory stimuli (such as dipsogenic/natriorexigenic treatment) increases NaCl intake and occasionally water intake, whereas, the same blockades in the LPBN are not enough to induce sodium intake in rats that are not stimulated to ingest sodium. Perhaps after removing the action of only one neurotransmitter, the action of the remaining neurotransmitters is still sufficient to restrain the ingestion in satiated rats. It seems that injections of baclofen, similar to the injections of muscimol or β-endorphin into the LPBN, completely and simultaneously block the action of all the inhibitory mechanisms/neurotransmitters in the LPBN, which combined with any residual facilitatory signal still present as the one produced by normal levels of ANG II may facilitate NaCl intake as previously proposed for the drinking induced by intra-median raphe injections of muscimol in normally hydrated rats (Stratford and Wirtshafter, 2000). It is also possible that sodium and sucrose intake induced by baclofen injected into LPBN results from an activation of areas involved with rewarding. The rewarding mechanism for sodium and sucrose intake may involve dopaminergic neurotransmission in the nucleus accumbens (NAc) (Hoebel et al., 1989; Frankmann et al., 1994; Roitman et al., 1999; Hajnal et al., 2004). Opioid receptor activation in the LPBN increases the activity of the NAc and also induces sodium intake in normonatremic and euhydrated rats (Denbleyker et al., 2009; De Oliveira et al., 2008), suggesting that the same LPBN mechanism increases sodium intake and the activity of the NAc, an area involved with rewarding.

The present study suggests that an active LPBN inhibitory mechanism, important for satiety, restrain sodium and sucrose intake in satiated animals. For sodium appetite, the suggestion is that signals from arterial and volume receptors, osmoreceptors or taste receptors reaching the LPBN release different neurotransmitters that may modulate the activity of this inhibitory mechanism. In most of the physiological conditions, deactivation of LPBN inhibitory mechanism is combined with increased facilitatory signals for sodium intake like increased levels of ANG II or aldosterone and, then, rats are easily driven to ingest sodium. However, as rats ingest sodium the activity of the inhibitory mechanism of the LPBN increases, restraining sodium intake again. Conditions that reduce osmoreceptor and/or arterial, volume and/or taste receptor activity may decrease the release of neurotransmitters like 5-HT, CCK, glutamate or CRF in the LPBN, however, the same conditions probably also increase the release of opioids, GABA or noradrenaline in the LPBN reducing the activity of LPBN inhibitory mechanism and increasing sodium appetite (Menani et al., 1996; Menani and Johnson, 1998; Andrade et al., 2004; Callera et al., 2005; De Castro e Silva et al., 2006; De Oliveira et al., 2007, 2008; De Gobbi et al., 2009; Gasparini et al., 2009 and present results). More studies are necessary to understand how different signals affect the activity of the LPBN inhibitory mechanism, the specific role of these different neurotransmitters in the LPBN and how these neurotransmitters interact with each other in the control of sodium appetite.

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