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Research report

Effects of inhibitory avoidance training and/or isolated foot-shock on ectonucleotidase activities in synaptosomes of the anterior and posterior cingulate cortex and the medial precentral area of adult rats

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Abstract

Compelling evidence has indicated the involvement of extracellular ATP and adenosine in the mechanisms of synaptic plasticity and memory formation. In the present study, adult rats were trained in a step-down inhibitory avoidance task (IA) or submitted to isolated foot-shock (IF) (0.4 mA) before measuring ectonucleotidase activities in the synaptosomes of the anterior and posterior cingulate cortex (AC and PC, respectively) and the medial precentral area (Fr2). IA increased ATP and ADP hydrolysis immediately after training in the synaptosomes of PC and AC, respectively, ($P < 0.05$). Foot-shock (independent of occurring during IA or IF) increased ATP hydrolysis in synaptosomes of AC and Fr2 immediately after application and decreased AIP hydrolysis in AC 90 min after application ($P < 0.05$). Foot-shock (independent of occurring during IA or IF) increased ATP hydrolysis in PC immediately and 90 min after application, and in Fr2, but only immediately after application ($P < 0.05$). These results suggest that the ectonucleotidase pathway responds to a mild foot-shock in AC, PC and Fr2 and may be involved in memory consolidation of step-down inhibitory avoidance in the cingulate cortex.

Keywords: Ectonucleotidases; Cingulate cortex; Prefrontal cortex; Medial precentral area; Inhibitory avoidance; Memory; Stress

1. Introduction

ATP is currently recognized as a neurotransmitter and neuromodulator in the central nervous system [15]. ATP is released in the hippocampus following neuronal stimulation and is a potent inhibitor of hippocampal neuronal excitability [14,44]. Effects of ATP may be directly mediated by two subclasses of P2-purinoreceptors, P_{2x} and P_{2y} [38]. Signaling actions induced by extracellular ATP are correlated to the activity of ectonucleotidases, which trigger enzymatic conversion of ATP to adenosine. This group of ectonucteotidases includes an ecto-ATPase (EC 3.6.1.3), an ATP diphosphohydrolase (apyrase, EC 3.6.1.5) and an ecto-5′-nucleotidase (EC 3.1.3.5) [7,47]. Adenosine, formed by the enzymatic action of an apyrase and a 5′-nucleotidase, can act upon P1-purinoreceptors and is rapidly taken up via high-affinity uptake systems [10].

There is increasing evidence to indicate that ATP and adenosine may play a very important role in activity-dependent synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (LTD) [14,43]

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and in memory consolidation [6,7]. Our laboratory has demonstrated that the ectonucleotidase activities in the hippocampus and the entorhinal cortex, but not in the parietal cortex, are significantly decreased after step-down inhibitory avoidance training (IA) [6,7]. In fact, involved in the consolidation of memory for the IA in rats are several cortical structures, such as the hippocampus, the amygdala, the entorhinal cortex, the posterior parietal cortex [24], the posterior cingulate cortex (PC) [33,40] and the medial precentral area (Fr2) of the prefrontal cortex [34]. However, the involvement of the ectonucleotidase pathway in the cingulate cortex and Fr2 during memory processing has not yet been investigated.

The cingulate cortex has a strategic position in the Papez circuit of emotion processing, by which it intermediates signals between the hippocampal formation, the anterior thalamic nuclei, the prefrontal cortex, and neocortical visual, auditory, and motor areas [5,12,20,29,31,46]. In fact, there is increasing evidence that PC is, in a variety of species, involved in processing of memories, mainly those associated with emotional contents [8,17,18,31,33,40,42], and there is also some evidence to show that the anterior cingulate cortex (AC) is involved in memory processing [3,39].

Emotional arousal [11] anxiety and stress [19] strongly modulate both the earliest phase of memory formation and retrieval [23–25]. Furthermore, compelling evidence has shown that stress increases the activity of a variety of modulatory neurotransmitters in several brain regions, such as the prefrontal cortex [1,16,21,22,32]. Since there is evidence to suggest a role of adenosine in the regulation of behavioral responses related to stress [36,45], adenosine might affect memory modulation as a by-product of this regulation. However, adenosine might also be specifically involved in memory modulation via other mechanisms. Therefore, in order to verify this issue and considering that (1) the adenosinergic system of the cingulate and prefrontal cortex may be related to emotional contents and stress; (2) the ectonucleotidases are an important pathway for adenosine production; and (3) the cingulate cortex and Fr2 are involved in memory consolidation, the aim of the present study was to evaluate the effect of inhibitory avoidance training and isolated foot-shock on synaptosomal ATP and ADP hydrolysis in these structures.

2. Material and methods

2.1. Subjects

A total of 370 male Wistar rats (age, 60–120 days; body weight, 250–400 g) from our breeding stock were used in the study. Animals were housed five to a cage with food and water 'ad libitum' under a 12-h light:12-h dark cycle (lights on at 07:00 h) at a temperature of 25 °C. Procedures for the care and use of animals were adopted according to the regulations published by the Brazilian Society for Neuroscience and Behavior (SBNeC).

2.2. Behavioral procedures

2.2.1. Step-down inhibitory avoidance task (IA)

Rats were gently placed on a 2.5-cm high, 7.0-cm wide, 25.0-cm long Formica platform at the left side of a $50 \times 25 \times 25$-cm apparatus, the floor of which was a series of parallel 0.1-cm caliber stainless-steel bars spaced 1.0 cm apart. Immediately after stepping down, placing the four paws on the grid, animals received a 3.0-s, 0.4-mA scrambled foot-shock and were removed from the training apparatus.

2.2.2. Isolated footshock (IF)

Animals were placed directly on the grid and received a 3.0-s, 0.4-mA foot-shock, after which they were removed. A barrier was placed in order to avoid animals seeing an escape route to the platform.

2.3. Subcellular fractionation

Animals were sacrificed by decapitation immediately or 90 min after the behavioral procedure and the brain structures were removed. After removal, brains were placed into an ice-cold isolation medium (320 mM sucrose, 5 mM HEPES, pH 7.5, and 0.1 mM EDTA) before removing the entire cingulate cortex of both hemispheres, followed by a cortical region estimated to be the precentral area (Fr2) [46]. The entire cingulate cortex was divided into two portions corresponding to AC and PC. Pools of two animals were used for each synaptosomal preparation. Structures were gently homogenized in five volumes of ice-cold isolation medium with a motor-driven Teflon-glass homogenizer. The synaptosomes were isolated as described earlier [37]. Briefly, 0.5 ml of the crude mitochondrial fraction was mixed with 4.0 ml of 8.5% Percoll solution and layered onto an isoosmotic Percoll/sucrose discontinuous gradient (10/16%). The synaptosomes that banded at the 10/16% Percoll interface were collected with wide tip disposable plastic transfer pipettes. The synaptosomal fraction was washed twice at $15\,000 \times g$ for 20 min with the same ice-cold medium to remove the contaminating Percoll and the synaptosome pellet was resuspended to a final protein concentration of approximately 0.5 mg/ml. The material was prepared fresh daily and maintained at 0–4 °C throughout preparation.

2.4. Enzyme assays

The reaction medium used to assay ATP and ADP hydrolysis was essentially as described earlier [4] and contained 5.0 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose and 45 mM Tris–HCl buffer, pH 8.0, to a final volume of 200 μl. The synaptosomal preparation (10–20 μg protein) was added to the reaction mixture and preincubated for 10 min at 37 °C. The reaction was initiated by the addition of either ATP or ADP to a final concentration of 1.0 mM and stopped by the addition of 200 μl 10% trichloroacetic acid (TCA). The samples were chilled on ice for 10 min and 100-μl samples were taken for the assay of released inorganic phosphate (Pi) [13]. Incubation time and protein concentration were chosen in order to ensure the linearity of the reactions in both enzyme assays. Controls with the addition of the enzyme preparation after the addition of TCA were used to correct non-enzymatic hydrolysis of the substrates. All samples were run in duplicate. Protein was measured by the Coomassie Blue method [9], using bovine serum albumin (BSA) as a standard.

2.5. Statistical analysis

Data are expressed as mean ± standard deviation (S.D.). Ectonucleotidase activities for each substrate (ATP or ADP) and structure were analyzed by the general factorial analyses of variance (ANOVA), considering TIME (naïve, 0 or 90 min) and TASK (naïve, IA, or IF) as fixed factors, and a post hoc Duncan test, when appropriate. Since there was a redundancy between factors (i.e. naïve was included in both factors), TASK by TIME interactions were not analyzed by the general factorial ANOVA. However, the one-way ANOVA was also used for evaluating differences among groups or factors. $P < 0.05$ was considered to indicate significant difference.

3. Results

3.1. Ectonucleotidase activities in the posterior cingulate (PC)

Significant main effects for TIME [$F(2,26) = 5.87$; $P = 0.023$] and TASK [$F(2,26) = 35.24$; $P < 0.001$] were found when comparing ATP hydrolysis in PC (Fig. 1) (general factorial ANOVA). The one-way ANOVA [$F(4,24) = 18.31$, $P < 0.001$] followed by a Duncan test $\alpha < 0.05$) indicated the following changes in ATP hydrolysis (relative to naïve): first, an increase of 43% immediately after IA, but not IF. Second, a decrease of 23% 90 min after IF but not IA. Third, a trend towards an increase 90 min after IA ($P = 0.062$) (Fig. 1A).

A significant main effect for TASK [$F(2,26) = 5.13$; $P = 0.014$] but not for TIME [$F(2,26) = 2.20$; $P = 0.151$] was found when comparing ADP hydrolysis in PC (general factorial ANOVA). Foot-shock (independent of being IA or IF) increased ADP hydrolysis immediately (57%) or 90 min (44%) after being applied [one-way ANOVA of factors, $F(2,26) = 4.43$, $P = 0.022$; Duncan, $\alpha < 0.05$] (Fig. 1B).

3.2. Ectonucleotidase activities in the anterior cingulate (AC)

Significant main effects for TIME [$F(2,24) = 28.47$; $P < 0.001$] and for TASK [$F(2,24) = 12.60$; $P < 0.001$] were found when comparing ATP hydrolysis in AC (Fig. 2) (general factorial ANOVA). The one-way ANOVA of factors [$F(2,24) = 33.71$; $P < 0.001$] indi-

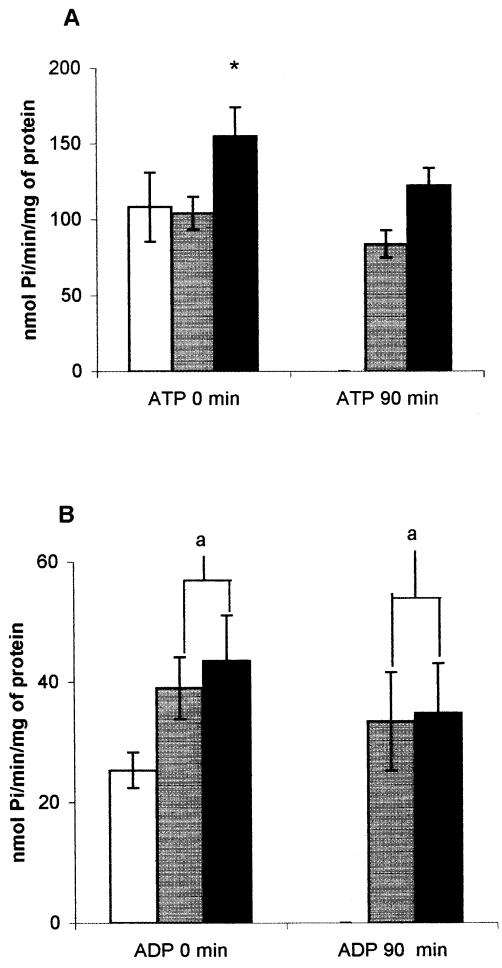

Fig. 1. ATP (A) and ADP (B) hydrolysis in synaptosomes from posterior cingulate cortex of rats sacrificed immediately after either isolated foot-shock (IF) or inhibitory avoidance (IA) training. White, gray and black bars represent naïve, shocked (IF) and trained (IA) animals, respectively. Bars indicate mean ± S.D. N per group was 5–8. * Significantly different from any other group (one-way ANOVA, $P < 0.05$). (a) Significantly different from naïve animals due to a main effect for TASK (general factorial ANOVA, $P < 0.05$).

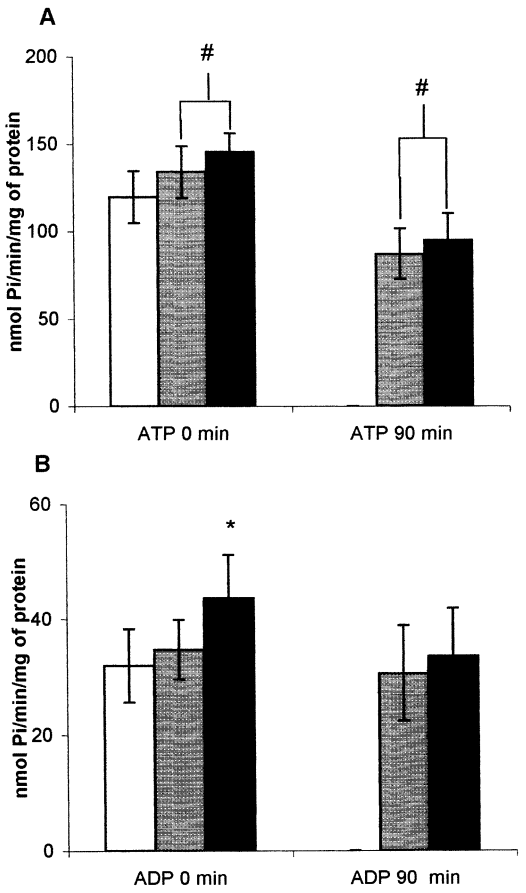

Fig. 2. ATP (A) and ADP (B) hydrolysis in synaptosomes from anterior cingulate cortex of rats sacrificed immediately after either isolated foot-shock (IF) or inhibitory avoidance (IA) training. *N* per group was 5–9. * Significantly different from any other group (one-way ANOVA, $P < 0.05$). # Significantly different from naïve animals due to main effects for TIME and TASK (general factorial ANOVA, $P < 0.05$).

cated that foot-shock (independent of being IA or IF) increased ATP hydrolysis immediately after being applied (18%) and decreased its activity (23%) 90 min after application (Duncan, $\alpha < 0.05$) (Fig. 2A).

A significant main effect for TASK [$F(2,28) = 10.29$; $P = 0.001$] but not for TIME [$F(2,28) = 2.55$; $P = 0.122$] was found when comparing ADP hydrolysis in AC (general factorial ANOVA). The one-way ANOVA of groups [$F(4,26) = 6.35$, $P = 0.001$] followed by a Duncan test indicated an increase of ADP hydrolysis immediately only after IA training (36%, relative to naïve) (Duncan, $\alpha < 0.05$), but not after IF (Duncan, $\alpha > 0.1$) (Fig. 2B).

3.3. Ectonucleotidase activities in Fr2

A significant main effect for TIME [$F(2,28) = 8.35$; $P = 0.008$] and a trend towards a main effect for TASK [$F(2,28) = 3.31$; $P = 0.053$] were found when comparing ATP hydrolysis in Fr2 (Fig. 3) (general factorial ANOVA). The one-way ANOVA of groups [$F(4,26) = 9.82$; $P < 0.001$] indicated that foot-shock (either IA or IF) increased ATP hydrolysis immediately after being applied (35 and 39%, relative to naïve, respectively) (Duncan, $\alpha < 0.05$) (Fig. 3A).

Significant main effects for TIME [$F(2,26) = 10.61$; $P = 0.003$] and TASK [$F(2,26) = 5.55$; $P = 0.010$] were found when comparing ADP hydrolysis in Fr2 (general factorial ANOVA). The one-way ANOVA of factors indicated [$F(2,26) = 18.03$; $P < 0.001$] that foot-shock (independent of being IA or IF) increased ADP hydrolysis immediately after application (47%) (Duncan, $\alpha < 0.05$), but not 90 min later (Duncan, $\alpha > 0.10$) (Fig. 3B).

4. Discussion

Our results indicate that the ectonucleotidase activities in the cingulate cortex and Fr2 respond to a mild

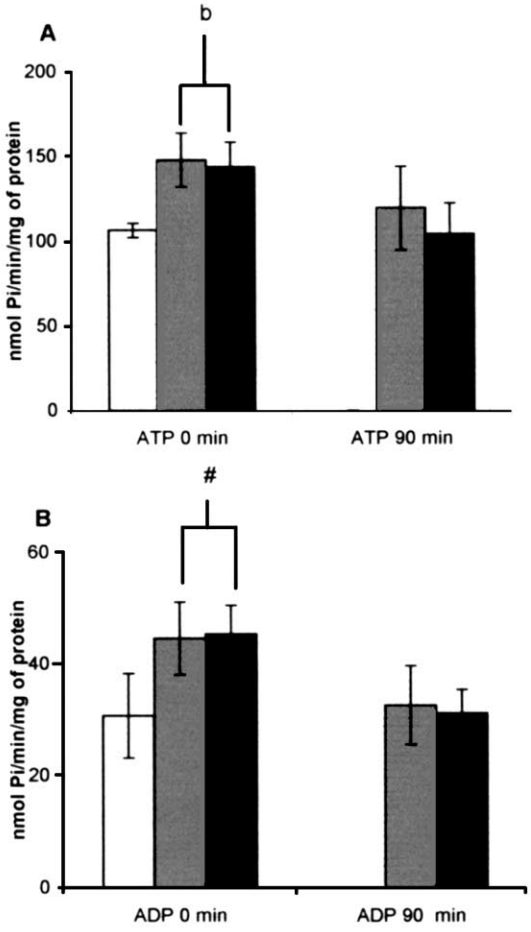

Fig. 3. ATP (A) and ADP (B) hydrolysis in synaptosomes from Fr2 of rats sacrificed immediately after either isolated foot-shock (IF) or inhibitory avoidance (IA) training. *N* per group was 5–7. b and # indicates a significant difference from naïve animals due to a main effect for TIME and for TIME and TASK, respectively (general factorial ANOVA, $P < 0.05$).

foot-shock, even when applied without IA training, indicating that ATP and ADP hydrolysis in AC, PC and Fr2 changes during stress. Furthermore, it was observed a learning-specific increase in ATP and ADP hydrolysis in the posterior and anterior cingulate cortex of rats, respectively.

4.1. Changes in ectonucleotidase activities and their role in stress

The changes of ectonucleotidase activities from the cingulate cortex and Fr2 triggered by foot-shock, independent of occurring during IA or IF, are not due to handling or any specific component of learning, since both groups were different only from naïve animals. This similar effect observed in IA and IF is very likely to be associated not to memory consolidation, but to neurochemical and neurohumoral changes induced by stress after foot-shock. In fact, memory modulation of IA by stress-related neurohumoral changes caused by the unconditioned stimulus has earlier been described [23,25]. This indicates that a mild stress, such as a mild foot-shock (3.0 s, 0.4 mA), is enough to promote specific changes in the ATP hydrolysis and ADP hydrolysis in AC, PC and Fr2. Considering the conditioned (CS-platform) and unconditioned stimulus (US-foot-shock) in inhibitory avoidance task, the US (foot-shock) seems to be the more important component to cause this observed enzymatic changes in IA and IF. It is unlikely that these changes obeyed to contextual fear learning to the shock: at least, three to five 0.4–0.5mA foot-shocks are needed in order to detect such conditioning [2].

Compelling evidence has shown that stress increases the activity of a variety of modulatory neurotransmitters in several brain regions [16,21,22,26,32]. Interestingly, a mild stress, such as handling for 30 s, injection of saline, tail pinch [1], or a mild foot-shock, as shown here, is enough to alter the neurochemistry of the rat prefrontal cortex. However, since AC is involved in nociception and its cognition (in anticipation of pain, for example) [28], we may not rule out the possibility that the observed change in ATP hydrolysis in AC, but not in Fr2, may have occurred due to the processing of pain rather than to an unspecific effect of stress. A further study may clarify this issue.

4.2. Changes in ectonucleotidase activities and their role in memory

Increasing evidence has indicated PC as an important structure in mediating memory processes in several mammalian species [8,17,31,33,40,41] and there is also some evidence to show that AC is involved in memory consolidation [39]. Interestingly, binding of [^{3}H]DPCPX to adenosine A_1 receptors is higher in the more inferior part of PC [41], where LTP and LTD are observed [20]. Similarly to PC, binding of [^{3}H]DPCPX to adenosine A_1 receptors is higher in the more inferior part of AC [41], where LTP is observed [19]. In the hippocampus, high but not low frequency stimulation of the Schaffer fibers releases ATP, which is degraded to generate adenosine [14]. It has been shown that adenosine attenuates LTD and inhibits LTP via A_1 receptors [35]. Our results showing a task-dependent increase in ATP and ADP hydrolysis in PC and AC, respectively, indirectly suggest an increase in adenosine levels after inhibitory avoidance training. For this reason, adenosine A_1 receptors in PC may be activated in order to modulate this process. ATP acts as a neurotransmitter and/or as a substrate for ecto-protein phosphorylation, producing changes in synaptic plasticity [43]. The ATP removal would prevent the modification of too large a number of synapses, allowing only the adequate synapses to be modified by LTP- or LTD-like mechanisms. Further studies are necessary to clarify whether ATP hydrolysis is increased in PC in order to provide enough adenosine to modulate the mechanisms involved in memory consolidation and/or for ATP to become unavailable in the synaptic cleft, or whether it is merely a by-product of these mechanisms. Curiously, the ectonucleotidase activities from the cingulate cortex have an opposite response to inhibitory avoidance training when compared with those from the hippocampus and entorhinal cortex, in which ATP and ADP hydrolysis decreased [6,7].

4.3. Underlying mechanisms involved in ectonucleotidase activities changes

It has been shown that an ecto-ATPase is co-expressed with an ecto-ATP diphosphohydrolase in the rat brain [27]. Furthermore, studies have demonstrated that the ecto-ATPase and the ecto-apyrase may be differently distributed with specialized and different functions in different regions of the same tissue [30]. The differences observed in ATP and ADP hydrolysis in synaptosomes of PC and AC suggests that the two related extracellular nucleotide-hydrolyzing enzymes may be involved in the effects observed here. The increase in ATP hydrolysis in synaptosomes of PC might involve an ecto-ATPase, whereas the increase in ADP hydrolysis in synaptosomes of AC may be related to an ectoapyrase. These findings might indicate a functional compartmentalization of the nucleotide hydrolysis and suggest that these enzymes may be differentially involved in different regions of the cingulate cortex.

The present contribution focuses on the alterations of the ectonucleotidase activities from cingulate cortex and medial precentral area that follow a stress induced by foot-shock accompanied or not by inhibitory avoidance

training. Further studies are necessary to clarify the mechanisms underlying these events and their relevance to the understanding of stress and/or memory mechanisms.

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