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Biochimica et Biophysica Acta 1239 (1995) 67–73

BBA Biochimica et Biophysica Acta

The A_1 agonist CCPA reduced bisoxonol-monitored membrane potential depolarization elicited by high K^+ in cerebrocortical nerve endings

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Received 24 November 1994; revised 12 May 1995; accepted 6 June 1995

Abstract

In this study the effect of the A_1 agonist 2-chloro-N^6-cyclopentyladenosine (CCPA) on bis(1,3-diethylthiobarbituric acid)trimethine oxonol (bisoxonol)-monitored membrane potential in cerebrocortical nerve endings was evaluated. CCPA (30, 100 and 300 μM) caused a dose-dependent decrease of high K^+- and veratridine-induced membrane depolarization. This decrease was counteracted by the A_1-specific antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) (30–100 μM). On the contrary, the A_2 receptor antagonist 9-chloro-2-(2-furanyl)-5,6-dihydro-1,2,4-triazolol-[1,5-*c*]quinazoline-5-imine (CGS 15943) was unable to interfere with the lowering effect exerted by CCPA (100 μM) on K^+-elicited membrane depolarization. Finally, the A_2 receptor agonist 2-[*p*-(2-carboxyethyl)phenethylamine]-5′-*N*-ethylcarboxamidoadenosine (CGS 21680) did not induce any modification of K^+-induced membrane depolarization. The results of the present study suggest that K^+-induced membrane depolarization in cerebrocortical brain nerve endings may be modulated by A_1 receptors.

Keywords: A^1 agonist activity; Agonist; Chlorocyclopentyladenosine; Membrane potential; Bisoxonol monitoring; Potassium ion; Cerebrocortical nerve ending

1. Introduction

There is considerable evidence that adenosine acts as an inhibitory modulator of neuronal activity in the CNS. In fact, it has been shown that it inhibits neuronal firing rates, synaptic transmission and neurotransmitter release [1,2]. Adenosine exerts its modulatory activity by acting on receptors which were initially classified into two subtypes, termed A_1 and A_2, on the basis of their ability to inhibit or stimulate cAMP formation [3]. The advent of radioactive ligands for adenosine binding sites allowed a more definitive receptor classification based on agonist affinity. By means of this methodology it has been shown that the A_1 receptors are widely distributed throughout several regions of the brain [4]. By contrast, A_2 high-affinity binding sites have been found only in some brain areas such as striatum, olfactory tubercle and nucleus accumbens [5,6], although, more recently, a subpopulation of A_2 receptor sites at low affinity, that displays a wide distribution throughout the brain, has been characterized [7].

The activation of A_1 receptors seems to be responsible for the inhibition of neurotransmitter release [8]. It has been suggested that this effect may be due to a blockade of Ca^{2+} influx into nerve terminals [9]. Since a condition of membrane hyperpolarization may also lead to an inhibition of neurotransmitter release, it appeared of interest to evaluate the activity of 2-chloro-N^6-cyclopentyladenosine (CCPA), an adenosine derivative possessing high affinity for A_1 receptors [10], on bisoxonol-monitored membrane depolarization induced by high K^+ concentrations or veratridine in cerebrocortical synaptosomes. In order to verify the specificity of the effect of CCPA on K^+-induced membrane depolarization, its action was evaluated in the presence of the specific A_1 receptor antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) [11]. Furthermore, to rule out any effect of CCPA on A_2 receptors, 9-chloro-2-(2-furanyl)-5,6-dihydro-1,2,4-triazolol[1,5-*c*]quinazoline-5-

Abbreviations: CCPA, 2-chloro-N^6-cyclopentyladenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine.

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SSDI 0005-2736(95)00143-3

imine (CGS 15943) [12], a selective antagonist of these receptor subtypes, was tested on CCPA-induced changes in K^+-elicited membrane depolarization. In addition, the effect of the specific agonist of A_2 receptors, 2-[*p*-(2-carboxyethyl)phenethylamine]-5′-*N*-ethylcarboxamidoadenosine (CGS 21680) [13], was also examined.

2. Materials and methods

2.1. Synaptosomal preparation

Synaptosomal fractions from brain cortex of adult male Wistar rats (150–200 g body wt.) were prepared according to the procedure of Dunkley et al. [14], using a discontinuous Percoll gradient. Briefly, rats were killed by decapitation and the brains were removed and kept on ice. Brain cortex was dissected by means of a fine lancet. Tissue was then homogenized using a Teflon-glass homogenizer in 9 ml/g tissue of cold sucrose medium, whose composition was (in mM): 320 sucrose, 1 EDTA, 0.25 DTT, adjusted to pH 7.4 with 1 M NaOH. The homogenate was centrifuged for 10 min at $1000 \times g$ at 4°C. The supernatant (S_1) was diluted to 14 ml/g of starting tissue with sucrose medium, and 2 ml of S_1 were layered on the top of a tube containing four different Percoll concentrations (from the bottom (in v/v): 23%, 15%, 10% and 3% Percoll sucrose media), prepared using a peristaltic pump in order to achieve flat interfaces among the solutions at different Percoll concentrations. The gradients were then placed in a Sorvall RCSB superspeed centrifuge and spun at 4°C for 5 min at 32000 $\times g$ in an SS-34 rotor. After centrifugation, the fraction at the interface between the 23% and 15% Percoll layers (fraction 4), as described by Dunkley et al. [14], which is highly enriched in viable synaptosomes [14] and which was examined in the present study by electron microscopy (Fig. 1), was removed and diluted 5-times in a solution containing (in mM): 125 NaCl, 2.5 KCl, 1.2 KH_2PO_4. 1.2 $MgSO_4$, 5 $NaHCO_3$, 25 Hepes, 6 glucose (pH 7.4).

Diluted synaptosomes were centrifuged two times at $15000 \times g$ for 15 min at 4°C, in order to remove Percoll. The final pellet was resuspended in a medium containing (in mM): 145 NaCl, 5 KCl, 1.2 $MgCl_2$, 10 glucose, 10 Hepes (pH 7.4) (standard Ca^{2+}-free medium), at a protein concentration of 3–10 mg of synaptosomal proteins per ml, and immediately used for the following experimental procedures.

2.2. Determination of synaptosomal membrane potential variations

The lipophilic anion bisoxonol, which has been used to monitor membrane potential changes in several cell types [15–17] was added, in a concentration of 300 nM from a stock solution of 150 μM in DMSO, to the quartz cuvette of the spectrofluorimeter containing 2 ml of prewarmed medium. Fluorescence intensity of the dye was recorded at an excitation and emission wavelengths of 540 and 580 nm, respectively (5 nm slits for both excitation and emission wavelengths). After 1 min from the addition of bisoxonol, 40–50 μg of synaptosomal proteins were pipetted into the cuvette. Bisoxonol fluorescence intensity variations were not converted into absolute membrane potential values, since the valinomycin nullpoint method [15] could

Fig. 1. Synaptosomal preparation image obtained by electron microscopy.

not be applied due to the formation of complexes between the lipophilic anion bisoxonol and the positively charged molecule of valinomycin.

CCPA, DPCPX, CGS 21680 were dissolved in DMSO at the concentrations of 100, 25 and 100 and mM, respectively. CGS 15943 was dissolved in H_2O at a concentration of 5 mM.

The same volume of solvent utilized to obtain the desired concentration of drugs under investigation did not induce any change of bisoxonol fluorescent intensity signal.

2.3. Protein determination

Synaptosomal protein concentrations were determined by the method of Bradford [18].

2.4. Materials

CCPA and DPCPX were purchased by R.B.I. (Natick, MA, USA). CGS 15943 and CGS 21680 were a gift from CIBA Geigy (Summit, NJ, USA). Bisoxonol was purchased from Molecular Probes (Eugene, OR, USA).

3. Results

3.1. Effect of different concentrations of CCPA on K^+-induced changes of bisoxonol-monitored synaptosomal membrane potential

When synaptosomes, incubated in the standard medium, were exposed to increasing concentrations of K^+ ions, a dose-related depolarization, monitored by the increase of bisoxonol fluorescence emission, was observed (Fig. 2). The addition in sequence to the incubation medium of increasing concentrations (30–100 and 300 μM) of the A_1 receptor agonist CCPA caused a dose-dependent reversion of 35 mM K^+-induced membrane depolarization (Fig. 3). When CCPA (100 μM) was added to synaptosomal prepa-

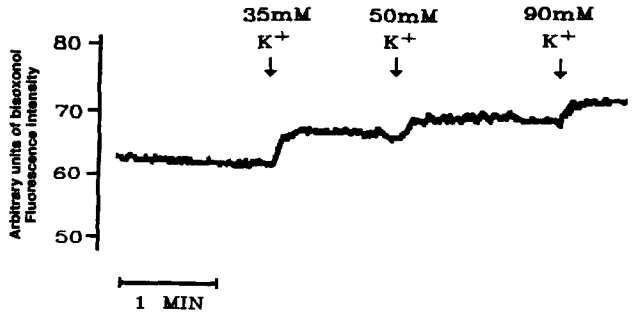

Fig. 2. Dose-effect of K^+-induced synaptosomal membrane depolarization. After isolation, synaptosomes were resuspended in a standard Ca^{2+}-free medium and subjected to a 30 min incubation at 37°C. After this period, they were pelleted, resuspended in the same buffer and kept on ice until their experimental use. Just prior to use, 4–5 μl of synaptosomal resuspension (40–50 μg of synaptosomal protein) was pipetted in the spectrofluorimeter cuvette containing 2 ml of a prewarmed 1 mM Ca^{2+}-containing medium plus 300 nM bisoxonol. Fluorescence emission of the dye was recorded for at least 90 s and, in the absence of any other experimental manipulation, it remained stable for the whole duration of the recording. After 90 s, as indicated by the arrow, 35 mM K^+ was added to the synaptosomes. After a further period, synaptosomes were exposed to 50 mM K^+, as indicated by the second arrow. 110 s later 90 mM K^+ was added to the medium. The results are expressed as arbitrary units of bisoxonol fluorescence intensity, due to the absence of a reproducible calibration procedure for the fluorescent dye, as described in the Materials and methods section. In all figures, each trace is representative of at least three similar independent experiments.

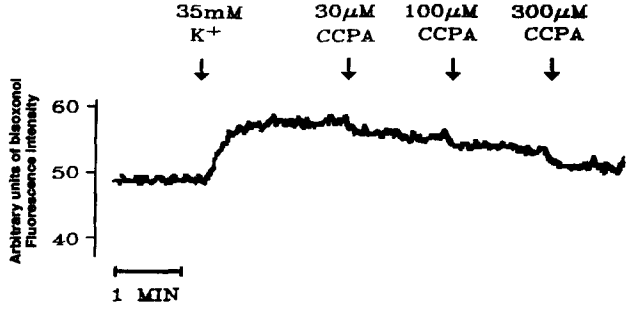

Fig. 3. Effect of increasing concentrations of CCPA on K^+-induced synaptosomal membrane depolarization. The experiment was performed as described in the legend for Fig. 2. Synaptosomes were first exposed to 35 mM K^+ and then to increasing concentrations of CCPA which were added to the medium where indicated by the arrows.

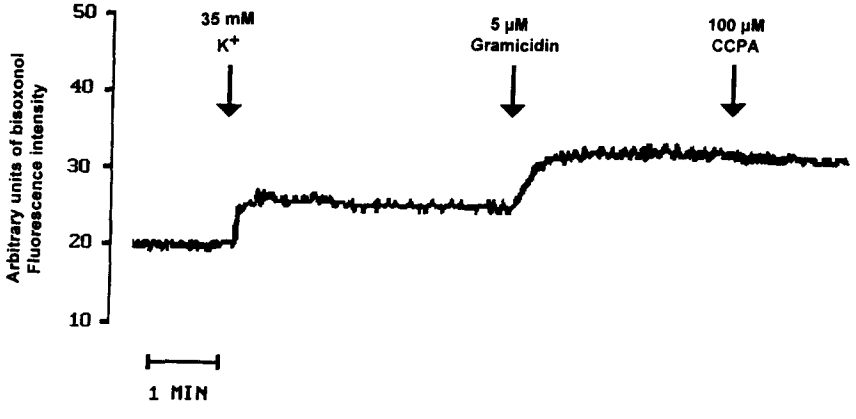

Fig. 4. Effect of CCPA on 35 mM K^+-plus gramicidin-induced depolarization. The experiment was performed as described in the legend of Fig. 2. 35 mM K^+, 5 μM gramicidin and 100 μM CCPA were added to the medium, where indicated by the arrows.

rations after 35 mM K^+ plus gramicidin (which equilibrates internal and external K^+ ion concentrations), it failed to induce any change of membrane potential (Fig. 4), suggesting that any nonspecific optical changes elicited by the compound can be excluded.

3.2. Effect of CCPA on membrane potential titration performed with increasing concentrations of K^+

If CCPA (100 μM) was added to cerebrocortical nerve endings before a titration with increasing concentrations of K^+ (35, 50 and 90 mM) followed by gramicidin (5 μM), the mean of the observed optical changes after addition of each K^+ concentration and gramicidin was not significant from that found in absence of the compound under investigation (data not shown).

3.3. Effect of different concentrations of CCPA on veratridine-induced changes in membrane potential

When synaptosomes were exposed to 30 μM veratridine, a membrane depolarization was observed. Increasing concentrations of CCPA induced a dose-dependent reversion of veratridine-elicited membrane depolarization (Fig. 5). When CCPA (100 μM) was added to cerebrocortical nerve endings before veratridine (30 μM), it blocked veratridine-induced depolarization (data not shown).

3.4. Effect of the A_1 receptor antagonist DPCPX on CCPA-induced reversion of membrane depolarization elicited by 35 mM K^+

CCPA (100 μM) added to the incubation medium 1 min after 35 mM K^+ caused a decrease of K^+-induced depolarization that was of 3 arbitrary units of bisoxonol fluorescence intensity (Fig. 6A). In order to evaluate the specificity of CCPA-induced reversion of membrane depolarization elicited by 35 mM K^+, the A_1 receptor antagonist DPCPX (30–100 μM) was added to incubation medium before the exposure to 35 mM K^+ and to 100 μM CCPA. DPCPX by itself did not induce any change of bisoxonol fluorescence increase elicited by 35 mM K^+, whereas it counteracted, in a dose-dependent way, the decrease induced by CCPA of K^+-elicited depolarization (Fig. 6B and 6C), respectively.

3.5. Lack of effect of the A_2 receptor antagonist CGS 15943 on CCPA- induced inhibition of membrane depolarization elicited by 35 mM K^+

In order to exclude the possibility that CCPA might counteract membrane depolarization elicited by 35 mM K^+ through an unspecific interaction with A_2, the specific A_2 receptor antagonist CGS 15943 was added to the incubation medium before 35 mM K^+ and CCPA. CGS 15943 (1 μM) was unable to interfere with the lowering effect exerted by CCPA (100 μM) on K^+-elicited membrane depolarization (Fig. 7).

3.6. Lack of effect of the A_2 receptor agonist CGS 21680 on membrane depolarization induced by 35 mM K^+

In order to further support the hypothesis that the stimulation of A_2 receptors does not exert an inhibitory effect on K^+-induced membrane depolarization, CGS 21680, a specific agonist of A_2 receptor subtype, was added to the incubation medium 1 min after 35 mM K^+. This compound, at the concentration of 100 μM, did not induce any modification of K^+-induced membrane depolarization (Fig. 8).

4. Discussion

The results of the present study showed that CCPA, a selective agonist of A_1 receptor, is able to cause a dose-dependent inhibition of membrane depolarization induced by 35 mM K^+ and veratridine in cortical brain synaptosomes. It has been reported that this adenosine derivative possesses a selectivity versus A_1 receptor subtype that is

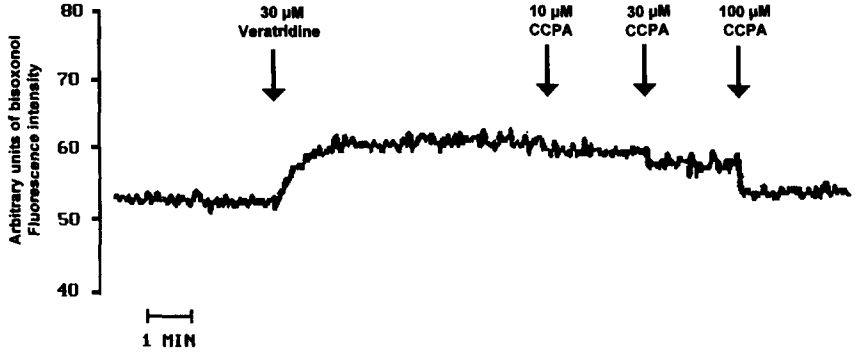

Fig. 5. Effect of increasing concentrations of CCPA on veratridine-induced membrane synaptosomal depolarization. The experiment was performed as described in the legend for Fig. 2. Synaptosomes were first exposed to 30 μM veratridine and then to increasing concentrations of CCPA, which was added to the medium where indicated by the arrows.

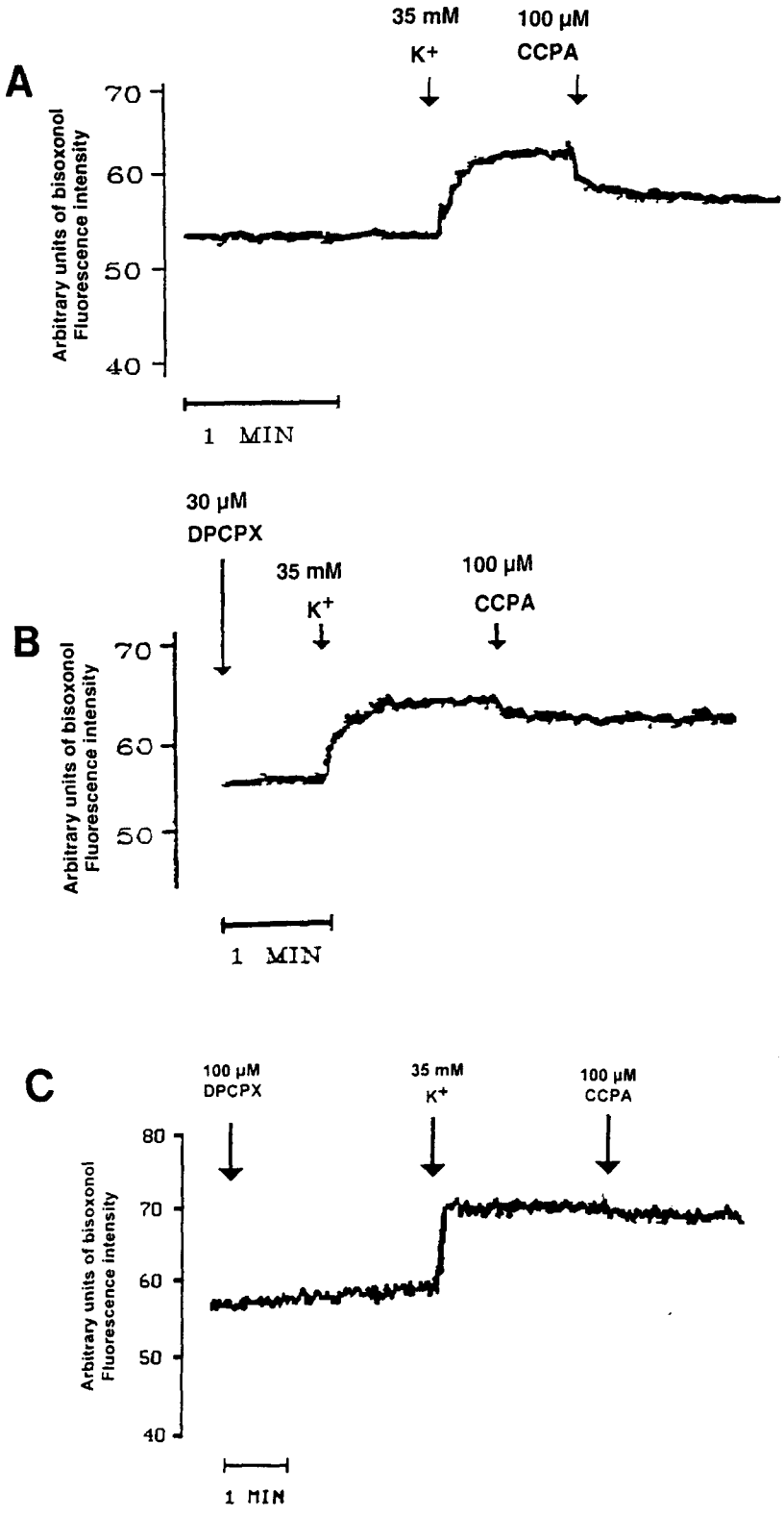

Fig. 6. Effect of different concentrations of DPCPX on CCPA-induced decrease of membrane depolarization elicited by 35 mM K^+. The experiment was performed as described in the legend for Fig. 2. In (A), 35 mM K^+ was added to the medium after 100 s, as indicated by the arrow. After a further period of 55 s, synaptosomes were exposed to 100 μM CCPA. DPCPX (30 and 100 μM) was added to the medium at the beginning of the experiment, as indicated by the arrow. 1 min later, synaptosomes were exposed to 35 mM K^+ (second arrow). 100 μM CCPA was added to the incubation medium (third arrow) after a further period of 90 and 120 s ((B) and (C), respectively). DPCPX (30 and 100 μM), caused a reversion of CCPA-elicited inhibition of K^+-induced membrane depolarization of 33 and 70%, respectively. The means of the fluorescence arbitrary unit values (6 n for each experimental group) obtained with 100 μM CCPA following K^+ addition in the presence of 30 and 100 μM DPCPX were significantly different from the mean of the values obtained with 100 μM CCPA following K^+ addition in the absence of DPCPX ($p \leq 0.05$ ANOVA followed by Newman Keul's test).

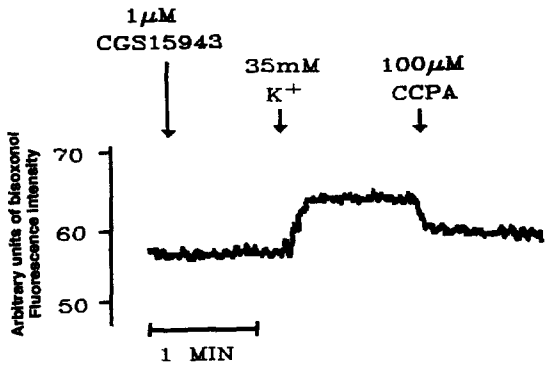

Fig. 7. CCPA-induced decrease of membrane depolarization elicited by 35 mM K^+ is unaffected by the A_2 receptor antagonist CGS 15943. The experiment was performed as described in the legend of Fig. 2. CGS 15943 was added into the medium when indicated by the arrow. After 1 min synaptosomes were exposed to 35 mM K^+, as indicated by the second arrow. 75 s later 100 μM CCPA was pipetted into the couvette (third arrow).

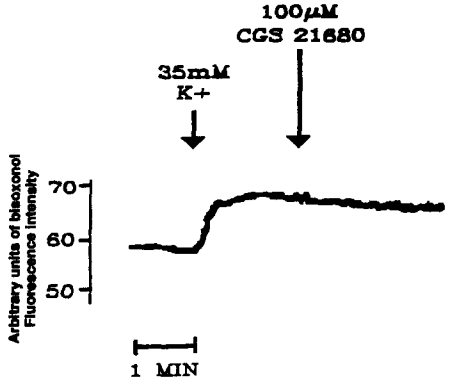

Fig. 8. Lack of effect of the A_2 receptor agonist CGS 21680 on membrane depolarization induced by 35 mM K^+. The experiment was performed as described in the legend of Fig. 2. Synaptosomes were exposed to 35 mM K^+ where indicated by the arrow. 100 μM CGS 21680 was added to the incubation medium, when indicated by the second arrow.

almost 10000-fold higher than versus A_2 subtype [10]. Therefore, it is reasonable to hypothesize that the effect of CCPA on high K^+ and veratridine-induced membrane depolarization is due to the stimulation of the A_1 subtype of receptor. This hypothesis seems to be supported by the findings showing that the effect of CCPA on K^+- induced membrane depolarization was antagonized by the specific A_1 antagonist, DPCPX. This compound can be considered a selective A_1 antagonist, since its A_2/A_1 ratio of affinity is almost 700 [11]. Moreover, it has been recently suggested that DPCPX can be used as probe for in vivo localization of brain A_1 receptors [19]. Further support to the view that the inhibition exerted by CCPA on K^+-induced depolarization is due to a stimulation of A_1 receptors is represented by the results showing that the A_2 antagonist, CGS 15943, [12] was unable to antagonize the effect of CCPA on K^+-induced membrane depolarization. In addition, A_2 receptors do not seem to be involved in the modulation of membrane potential following K^+ exposure since CGS 21680, a compound widely used as A_2 agonist [13], did not induce any change of membrane depolarization elicited by high concentrations of K^+.

At the present time the mechanisms underlying the capability of CCPA to reverse veratridine and high K^+-induced depolarization are unknown; however, since the removal of Cl^- ions did not prevent the CCPA-induced hyperpolarization (data not shown), the reversal of membrane potential induced by CCPA does not involve an increase in Cl^- ion permeability, as occurs in other tissue preparations [4,20]. Furthermore, the fact that CCPA was able to reduce high-K^+-clamped membrane potential depolarization suggests that it is acting by a mechanism other than the activation of K^+ channels. Whatever mechanism is activated by CCPA, it should be operating also in conditions of veratridine-induced depolarization, whereas a blockade of Na^+ channels that could explain this effect does not seem likely, since it does not justify CCPA-induced reduction of K^+-elicited membrane potential depolarization.

It could be observed that the concentrations of CCPA used in the present study were quite high if one considers that its K_i versus A_1 receptors is 0.4 nM [10] and therefore its effect on K^+ and veratridine-induced membrane depolarization cannot be considered very specific. However, this observation does not seem to be in line with the findings showing that the effect of CCPA on K^+-induced membrane depolarization was dose-dependently reversed by the specific A_1 receptor antagonist DPCPX, whereas the selective A_2 antagonist CGS 15943 was ineffective in antagonizing the effect of CCPA.

In conclusion, the results of the present study suggest that K^+-induced membrane depolarization in cerebrocortical brain synaptosomes may be modulated by A_1 receptors and therefore this model may be proposed as a reliable and suitable tool to evaluate the activity of A_1 agonists.

References

[1] Phyllis, J.W. and Wu, P.H. (1981) Prog. Neurobiol. 16, 187–239.
[2] Snyder, S.H. (1985) Annu. Rev. Neurosci. 8, 103–124.
[3] Londos, C., Cooper, D.M.F. and Wolff, J. (1980) Proc. Natl. Acad. Sci. USA 77, 2551–2554.
[4] Linden, J. (1991) FASEB J. 5, 2668–2676.
[5] Yeung, S.M. and Green, R.D. (1984) Naunyn-Schmiedeberg's Arch. Pharmacol. 325, 218–225.
[6] Jarvis, M.F., Jackson, R.H. and Williams, M. (1989) Brain Res. 484, 111–118.
[7] Bruns, R.F., Daby, J.W. and Pugsley, T.A. (1986) Mol. Pharmacol. 29, 331–346.
[8] Dunwiddie, T.N. (1985) Int. Rev. Neurobiol. 27, 63–139.
[9] Ribeiro, J.A., Almeida, A.M. and Namorado, J.M. (1979) Biochem. Pharmacol. 28, 1297–1300.
[10] Lohse, M.J., Klotz, K.N., Schwabe, U., Cristalli, G., Vittori, S. and Grifantini, M. (1988) Naunyn Schmiedeberg's Arch. Pharmacol. 337, 687–689.

[11] Bruns, R.F., Fergus, J.H., Badger, E.W., Bristol, J.A., Santary, L.A., Hartman, J.D., Hays, S.J. and Huang, C.C. (1987) Naunyn-Schmiedeberg's Arch. Pharmacol. 335, 59–63.
[12] Williams, M., Francis, J.E., Ghai, G.R., Braunwalder, A., Psychos, S., Stone, G.A. and Cash, W.D. (1987) J. Pharmacol. Exp. Ther. 241, 415–420.
[13] Williams, M. (1990) in: Adenosine and Adenosine Receptors, pp. 1–5, The Humana Press, Clifton.
[14] Jarvie, P.E., Glenfield, P.E. and Rostas J.A.P. (1988) Brain Res. 441, 59–71.
[15] Waggoner, A. (1976) J. Membr. Biol. 27, 317–324.
[16] Taglialatela, M., Canzoniero, L.M.T., Fatatis, A., Di Renzo, G.F., Yasumoto, T. and Annunziato, L. (1990) Biochim. Biophys. Acta 1026, 126–132.
[17] Rink, T.J., Montecucco, C., Hesketh, T.R. and Tsien, R.W. (1980) Biochim. Biophys. Acta 595, 15–30.
[18] Bradford, M.M.A. (1976) Anal. Biochem. 72, 248–254.
[19] Bisserbe, S.C., Pascal, O., Duckert, J. and Mariere, B. (1992) Brain Res. 599, 6–12.
[20] Rudolphy, K.A., Schubert, P., Parkinson, F.E. and Fredholm, B.B. (1992) Trends Pharmacol. Sci. 13, 439–445.