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Research report

Adenosine A_1 receptors inhibit Ca^{2+} channels coupled to the release of ACh, but not of GABA, in cultured retina cells

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Abstract

We investigated the effect of adenosine A_1 receptors on the release of acetylcholine (ACh) and GABA, and on the intracellular calcium concentration ($[Ca^{2+}]_i$) response in cultured chick amacrine-like neurons, stimulated by KCl depolarization. The KCl-induced release of $[^3H]$ACh, but not the release of $[^{14}C]$GABA, was potentiated when adenosine A_1 receptor activation was prevented by perfusing the cells with adenosine deaminase (ADA) or with 1,3-dipropyl-8-cycloentylxanthine (DPCPX). The changes in the $[Ca^{2+}]_i$ induced by KCl depolarization, measured in neurite segments of single cultured cells, were also modulated by endogenous adenosine, acting on adenosine A_1 receptors. Our results show that adenosine A_1 receptors inhibit Ca^{2+} entry coupled to ACh release, but not to the release of GABA, suggesting that the synaptic vesicles containing each neurotransmitter are located in different zones of the neurites, containing different VSCC and/or different densities of adenosine A_1 receptors. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Acetylcholine; GABA; Release; Retina cell; Ca^{2+} imaging; Chick

1. Introduction

In the retina, the photoreceptors respond to brightness, whereas the ganglion cells, the axons of which converge to form the optic nerve that transports the visual information to the brain, respond to a variety of different properties of the stimuli. Therefore, elaborated connections are required to provide this variety of responses by ganglion cells. The processing of the visual signal takes place in two stages: (i) in the outer plexiform layer, where photoreceptors connect to bipolar cells and horizontal cells, and (ii) in the inner plexiform layer, where bipolar cells connect to ganglion cells and amacrine cells.

Amacrine cells and their connections play an important role in information processing in the retina [4]. These cells may contain several neurotransmitters, including acetylcholine (ACh) and GABA [16]. In the retina, GABA is found in a subpopulation of amacrine cells, and in horizontal cells, whereas ACh is only located in a subpopulation of amacrine cells named ''starburst'' amacrine cells. ''Starburst'' amacrine cells and GABAergic amacrine cells may also play an important role on retinal directional selectivity, a process whereby directionally selective retinal ganglion cells produce robust responses for motion in certain directions, but not in others [1]. According to one model, the spatially asymmetric release of GABA from certain amacrine cells is responsible for the inhibition of responses to motions in the anti-preferred direction [10]. Another model proposes that an asymmetric cholinergic input from ''starburst'' amacrine cells is also responsible for direction selectivity of retinal ganglion cells [9]. However, the exact nature of the ACh- and GABA-mediated asymmetries is not known at the subcellular level.

We have previously reported that cultures of chick retinal neurons are enriched in cholinergic amacrine-like neurons [19]. Since a subgroup of amacrine cells is virtually the only group of cholinergic neurons in the retina [16], cultured chick retinal neurons are mainly amacrine-like cells [19]. A subpopulation of the neurons present in these cultures (38.5%) was stained with an antibody against GABA, and 87% of the cells containing GABA were found to be cholinergic [19]. This is in agreement with observations in the intact retina showing that a subpopulation of cholinergic amacrine cells can also store [3,13,22] and release [12–14] GABA. Based on the different sensitivity of the Ca^{2+}-dependent release of GABA and ACh to

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the effect of blockers of voltage sensitive Ca^{2+} channels (VSCC), we suggested that ACh and GABA are localized in different zones of the cells [19]. In the present work, we show that activation of adenosine A_1 receptors, a retinal neuromodulator [2], inhibits the release of ACh, but not the release of GABA, through the inhibition of VSCC. This observation supports the hypothesis that in cultured chick amacrine-like neurons, the synaptic vesicles containing ACh or GABA are located in different zones, endowed with different densities of VSCC and adenosine A_1 receptors. This mechanism of controlling the release of the two neurotransmitters may be involved in the asymmetric release of ACh and GABA observed in the direction selectivity mechanism.

2. Material and methods

2.1. Preparation and culture of chick retina cells

Primary cultures of chick retinal neurons from 8-day-old chick (White Leghorn) embryos were obtained as described previously [6]. Briefly, the retinas were dissected free from other ocular tissues and incubated for 15 min, at 37°C, in Ca^{2+}- and Mg^{+}-free Hank's balanced salt solution (CMF) supplemented with 0.1% trypsin (GIBCO, Paisley, UK). The digested tissue was centrifuged at $140 \times g$, for 1 min, and the pellet was mechanically dissociated in Basal Medium of Eagle (BME, Sigma, St. Louis, MO, USA), buffered with 20 mM HEPES and 10 mM $NaHCO_3$, and supplemented with 5% heat inactivated fetal calf serum (Biochrom KG, Berlin, Germany), penicillin (100 U/ml) and streptomycin (100 μg/ml). After the appropriate dilution, the cells were plated at a density of 0.4×10^6 cells/cm^2, in 35 mm plastic petri dishes, or on 16 mm glass coverslips, coated with poly-D-lysine (0.1 mg/ml), for neurotransmitter release or for Ca^{2+} imaging studies, respectively. The cells were then kept in culture for 5 days at 37°C in a humidified atmosphere of 95% air/5% CO_2 before the experiments.

2.2. Release of [^{3}H]ACh and [^{14}C]GABA

The experiments were carried out as described previously [19]. Briefly, the cultured chick retinal neurons were incubated for 45 min, at 37°C, in Na^+-medium (in mM: 132 NaCl, 4 KCl, 1.4 $MgCl_2$, 1.2 H_3PO_4, 1 $CaCl_2$, 6 glucose, 10 HEPES-Na, pH 7.4) containing 25 nM methyl-[^{3}H]Choline (2.5 μCi/ml; 83 Ci/mmol, Amersham International, Aylesbury, UK) and 40 nM [^{14}C]GABA (1 μCi/ml; 228 mCi/mmol, DuPont-NEN, Boston, MA, USA), in the presence of 50 μM eserine sulfate (Sigma) and 10 μM aminooxyacetic acid (AOAA; Sigma), to prevent acetylcholine and GABA degradation, respectively. After loading the cells with the radioactive neurotransmitters they were washed three times with Na^+-medium without radiolabelled compounds, and further incubated for 15 min at 37°C.

The release of neurotransmitters was measured using a superfusion method previously described [5,6]. Briefly, the 35-mm petri dishes were covered with a piece of a nylon mesh (100 μm) and placed on a holder, where the dishes were maintained slightly inclined (5°). By using two peristaltic pumps, pre-warmed (37°C) media with the desired composition was delivered to the top of the dishes (2 ml/min), and the superfusate was collected, at the lower part of the petri dishes, every minute, with a Gilson FC-204 fraction collector. After incubation with the radiolabelled compounds, the cells were perfused, for 10 min, with Na^+-medium containing 20 mM ChCl (NaCl was partially substituted by ChCl, in order to maintain the isoosmolarity), and further washed, for 15 min, with Na^+-medium supplemented with 10 μM NNC-711 (Novo Nordisk, Målov, Denmark) and 10 μM hemicholinium-3 (Sigma), to prevent the GABA and Ch uptake, respectively. The perfusion medium was then changed to media with the desired composition, and 1 min samples were collected during 10 min. At the end of each experiment the cells were perfused with 0.2 M HCl, for 5 min, to collect the remaining intracellular neurotransmitters. From each sample, a 300-μl aliquot was collected to determine the [^{3}H]ACh present, and the remaining radioactivity was measured using Universol scintillation cocktail (ICN) and a Packard 2000 Spectometer provided with dpm correction and dual ([^{3}H] and [^{14}C]) counting. The separation of [^{3}H]ACh from [^{3}H]Ch from the total tritium outflow was carried out by the method of Rand and Jonhson [17], as described previously [19]. Briefly, a 300-μl aliquot collected from each sample of the perfusate was incubated with 60 μl of assay mixture for phosphorylation of choline (80 mM $MgCl_2$, 12.5 mM ATP, 300 mM glycine buffer, pH 8.0, 0.062 U/ml choline kinase), for 30 min at 37°C, and was then transferred into scintillation vials containing a mixture of tetraphenylboron solution (30 g/l in isoamyl alcohol) and toluene cocktail (5 g/l of 2,5-diphenyloxazole [PPO] and 0.2 g/l of 1,4-bis[5-phenyl-2-oxazolyl]-benzene [POPOP]). The [^{3}H]ACh was extracted into the organic phase of the scintillation cocktail by tetraphenylboron, and the radioactivity was counted. The phosphorylated [^{3}H]Ch remains in the water phase and was not counted.

2.3. Fura-2 fluorescence measurements by video imaging

Retina cells cultured on glass coverslips were incubated with 5 μM Fura-2/AM (Molecular Probes, Leiden, The Netherlands) and 0.02% Pluronic F-127 (Molecular Probes) in Na^+ medium supplemented with 10% BSA, for 60 min, at 37°C. The coverslips were then washed and further incubated in the same medium, without Fura-2/AM, for 15 min. After this incubation, the coverslips were mounted in a superfusion chamber and placed on the stage of an

inverted Nikon Diaphot microscope. The cells were then perfused with pre-warmed (37°C) Na^+ medium for about 5 min before data acquisition. The experiments were conducted under continuous superfusion with media with the composition indicated in the figures legends, and the fluorescence changes were recorded with a multiple excitation MagiCal imaging system (Applied Imaging, UK). Retina cells were alternately excited at 340 and 380 nm using a stepping filter wheel, and the emitted fluorescence, collected with a 100 × fluor objective (Nikon), was driven to a Photonics Science SIT camera, after passing through a 510-nm bandpass filter. Four frames were averaged to produce each image. Image analysis was performed with the MagiCal system and with software developed by Dr. Enrique Castro (Faculty of Veterinary Sciences, Complutense University of Madrid, Madrid, Spain). Briefly, the background fluorescence at each wavelength was subtracted and fluorescence images were ratioed on a pixel-by-pixel basis. Ratio data were stored as 8-bit pseudo-coloured images. 2 × 2 pixel areas (0.88 μm) of the neurites were drawn and the averaged value of pixel intensities was evaluated at each time point, in order to obtain ratio vs. time plots for all areas defined. The results were expressed as the ratio of fluorescence intensity during excitation at 340 nm ($F_{340\ nm}$) to fluorescence intensity during excitation at 380 nm ($F_{380\ nm}$).

2.4. Statistical analysis

Results are presented as means ± S.E.M. of the indicated number of experiments, and were analyzed using One-way ANOVA. Differences between treatments were determined using the Dunnett's test.

3. Results

3.1. Differential effects of adenosine A_1 receptors on ACh and GABA release

We have previously reported that activation of adenosine A_1 receptors inhibits $[^3H]$ACh release evoked by depolarization of cultured amacrine-like neurons with KCl [20]. A subpopulation of these neurons (about 40%) also contain GABA and the large majority of the GABAergic cells present in the cultures is also cholinergic [19]. Therefore, we investigated whether the release of GABA was also modulated by adenosine A_1 receptors. The results presented in Fig. 1 show that, in contrast with the effect of adenosine A_1 receptors on $[^3H]$ACh release, no effect on $[^{14}C]$GABA release evoked by KCl was observed. Indeed, perfusion of chick retina cells with adenosine deaminase (ADA, 2 U/ml), which converts the extracellular adenosine into inosine, potentiated significantly the release of $[^3H]$ACh, evoked by 50 mM KCl, up to 132.1 ± 8.7% of control, but it was without effect on the release of $[^{14}C]$GABA. The $[^{14}C]$GABA release experiments were performed in the presence of 10 μM 1-(2-(((diphenylmethylene)amino)oxy)ethyl)-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid (NNC-711), a GABA carrier inhibitor [6], and therefore, the neurotransmitter release in response to KCl depolarization is expected to occur exclusively by exocytosis. The adenosine A_1 receptor selective agonist N^6-cyclopentyladenosine (CPA, 100 nM [15]) completely prevented the potentiation of $[^3H]$ACh release induced by removal of endogenous adenosine with ADA, as previously reported [20], but it was without effect on the release of $[^{14}C]$GABA (99.4 ± 4.2% and 105.8 ± 3.2% of the control, respectively). When the adenosine A_1 receptors were blocked with 50 nM of the specific antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) [11] the release of $[^3H]$ACh induced by KCl depolarization was potentiated up to 137.7 ± 5.8% of the control. In contrast, the release of $[^{14}C]$GABA was, again, not significantly affected ($P >$ 0.05) by adenosine A_1 receptor inhibition (92.0 ± 2.1% of the control).

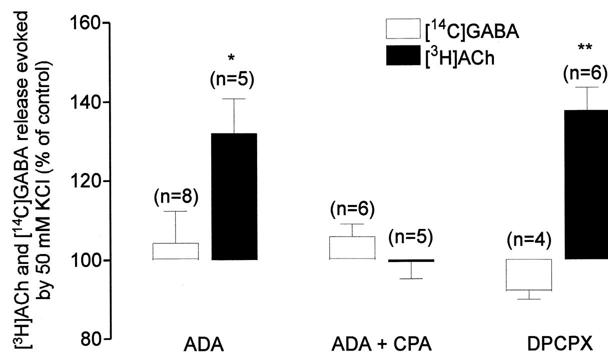

Fig. 1. Effect of adenosine A_1 receptors on $[^3H]$ACh and $[^{14}C]$GABA release from cultured chick retina cells. The cells were stimulated for 1 min with 50 mM KCl in Ca^{2+}-containing Na^+ medium, supplemented with 50 μM eserine, 10 μM hemicholinium-3, 10 μM NNC-711 and 10 μM AOAA. Where indicated, the cells were perfused for 2 min with 100 nM CPA, with 50 nM DPCPX or with 2 U/ml ADA, before and during stimulation. After KCl depolarization, the cells were perfused with Na^+ medium for 5 min, and fractions were collected every minute. The results are expressed as percentage of control, obtained after 50 mM KCl depolarization in the absence of CPA, DPCPX or ADA, and are means ± S.E.M. for the indicated number of experiments. The control release of $[^3H]$ACh and $[^{14}C]$GABA evoked by 50 mM KCl was 15.5 ± 1.1% and 2.1 ± 0.1% of the total content of the neurotransmitters in the cells. $^*P < 0.05$, $^{**}P < 0.01$.

3.2. Adenosine A_1 receptor modulation of Ca^{2+}-influx through VSCC

The observation that activation of adenosine A_1 receptors inhibits $[^3H]$ACh release, but not the release of $[^{14}C]$GABA from cultured chick retina cells, may also suggest that the receptors are heterogeneously distributed within the cells. The results may indicate that the receptors are present only in processes where $[^3H]$ACh is released,

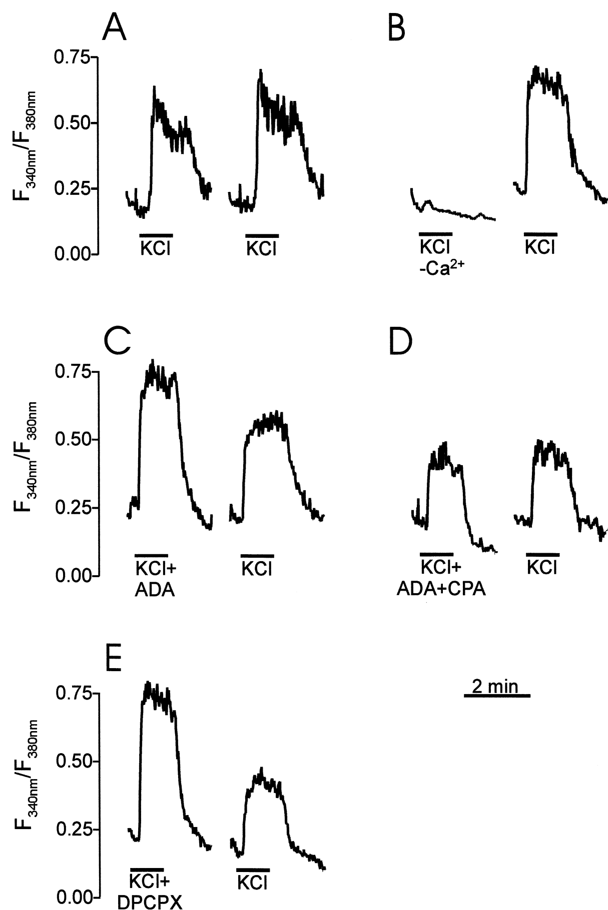

Fig. 2. Modulation by adenosine A_1 receptors of $[Ca^{2+}]_i$ responses induced by KCl depolarization. The cultured chick retina cells were loaded with Fura-2, and placed in a perfusion chamber in the stage of the microscope. The traces are typical $F_{340\ nm}/F_{380\ nm}$ ratio responses of neurite sections (0.88 μm) subjected to the indicated experimental conditions. The neurite sections investigated were selected randomly. The $[Ca^{2+}]_i$ responses shown in each panel (A–E) were recorded from the same neurite segment of retina cells. The cells were perfused with KCl for 60 s, where indicated by bars, and the second stimulus was applied 5 min after the end of the first stimulus. Before stimulation, the cells were perfused with 1 mM Ca^{2+}-containing Na^+ medium, and the first stimulus with 50 mM KCl was applied in the following experimental conditions: (A) Na^+ medium; (B) Ca^{2+}-free Na^+ medium, supplemented with 200 nM EGTA; (C) Na^+ medium supplemented with 2 U/ml ADA; (D) Na^+ medium supplemented with 2 U/ml ADA plus 100 nM CPA; (E) Na^+ medium supplemented with 50 nM DPCPX. In the second stimulation the cells were always depolarized with 50 mM KCl in 1 mM Ca^{2+} containing Na^+ medium. (NaCl was partially substituted by KCl, in order to maintain isoosmolarity.)

or that they are segregated in those domains within the neurites where [^{3}H]ACh release occurs.

Since activation of adenosine A_1 receptors was found to affect [^{3}H]ACh release by inhibiting Ca^{2+} entry through N-type VSCC [19], we investigated the functional distribution of A_1 receptors coupled to the inhibition of the intracellular free calcium concentration ($[Ca^{2+}]_i$) response to depolarization with 50 mM KCl (60 s stimulation), at the neurites, by digital imaging fluorescence microscopy. Perfusion of the cells with KCl increased significantly the Fura-2 fluorescence ratio ($F_{340\ nm}/F_{380\ nm}$) by 0.33 ± 0.02 (ΔS_1; $n = 97$), an effect that was also observed in response to a second depolarization with 50 mM KCl, 5 min later (ΔS_2). The ratio between the fluorescence changes determined in response to the two consecutive stimulations, $\Delta S_1/\Delta S_2$ was 1.04 ± 0.09 (Fig. 3), which clearly shows that after 5 min perfusion in Na^+ medium the cells fully recover the ability to raise the $[Ca^{2+}]_i$ in response to membrane depolarization. Also, 5 min after the first depolarization, the resting $F_{340\ nm}/F_{380\ nm}$ (0.21 ± 0.01) was not significantly different from the initial resting fluorescence ratio (0.21 ± 0.03; Fig. 2A). When Ca^{2+} was omitted from the extracellular medium, no significant increase in the fluorescence ratio was observed upon KCl depolarization (Fig. 2B). This result demonstrates that the increase in the fluorescence ratio observed after KCl depolarization was due to extracellular Ca^{2+} influx through VSCC.

In order to determine whether the $[Ca^{2+}]_i$ response to KCl depolarization was affected by endogenous adenosine acting on A_1 receptors, we compared the maximal $F_{340\ nm}/F_{380\ nm}$ ratio in response to depolarization of the cells with 50 mM KCl in the presence (ΔS_1) and in the absence (ΔS_2) of adenosine deaminase (Fig. 2C, Fig. 3). The average ratio $\Delta S_1/\Delta S_2$ determined in randomly chosen sections of neurites was 1.65 ± 0.06, indicating that adenosine tonically inhibits the $[Ca^{2+}]_i$ responses to KCl depolarization. As shown in Fig. 2D, the excitatory effect of ADA was antagonized ($\Delta S_1/\Delta S_2$ of 1.00 ± 0.02; Fig. 3) by pre-incubation of the cells with the adenosine A_1 receptor agonist CPA (100 nM). The A_1 receptor antagonist DPCPX (50 nM) increased the magnitude of the

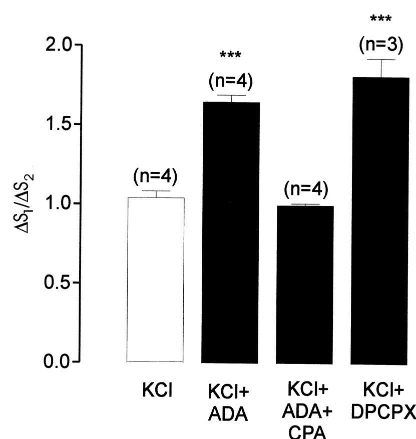

Fig. 3. Summary of the $[Ca^{2+}]_i$ responses induced by KCl depolarization. The experiments were conducted as indicated in the legend of Fig. 2. The results are expressed as the maximal amplitude of the change in fluorescence ratio ($\Delta F_{340\ nm}/F_{380\ nm}$) for the first stimulus divided by maximal amplitude of fluorescence ratio change ($\Delta F_{340\ nm}/F_{380\ nm}$) for the second stimulus ($\Delta S_1/\Delta S_2$), and are means ± S.E.M. for the indicated number of different preparations observed. In each preparation 7–22 segments of neurites were analyzed. ***$P < 0.001$ when compared to KCl.

change on the Fura-2 fluorescence ratio in response to depolarization (Fig. 2E, Fig. 3; $\Delta S_1/\Delta S_2 = 1.81 \pm 0.20$).

4. Discussion

In the present work we found that activation of adenosine A_1 receptors modulates the release of [^{3}H]ACh, but not the release of [^{14}C]GABA, from cultured amacrine-like neurons. The preparation used is enriched in retinal cholinergic neurons [19], and may represent a good in vitro model to study the function of ''starburst'' amacrine cells, a population of cholinergic and GABAergic neurons [3,16,22] which possess regularly spaced, evenly radiating dendrites [7]. Although a subpopulation of the cultured cholinergic amacrine-like neurons also contain GABA [19], we found that only the release of ACh is under tonical inhibition by adenosine acting on A_1 receptors.

The effect of adenosine A_1 receptors on [^{3}H]ACh release was supported by the following observations: (i) removal of the extracellular adenosine with ADA, or blockade of the receptor with the antagonist DPCPX, resulted in a significant potentiation of the ACh release; (ii) activation of the adenosine A_1 receptors with the agonist CPA completely blocked the potentiation of ACh release observed in the presence of ADA. In contrast, none of the above indicated experimental manipulations affected the release of [^{14}C]GABA evoked by membrane depolarization, indicating that either A_1 receptors are excluded from the cellular regions where [^{14}C]GABA was released, and/or that A_1 receptors are not coupled to the inhibition of Ca^{2+} channels present in the active zones where GABA is released.

Based on the differential sensitivities of the release of ACh and GABA to the pharmacological blockade of L- and N-type VSCC, we suggested a differential compartmentalization of the two neurotransmitters inside those amacrine-like neurons that contain simultaneously GABA and ACh [19]. This hypothesis is supported by the results reported here, showing that activation of adenosine A_1 receptors tonically inhibit the release of [^{3}H]ACh, but not the release of [^{14}C]GABA. The extracellular accumulation of adenosine upon KCl depolarization is due to the hydrolysis of ATP released under depolarizing conditions [18], by ectoATPases, and to the release of adenosine through its transporters [19]. At least the latter mechanism is likely to occur in the cholinergic amacrine neurons, since these cells are labeled for adenosine-like immunoreacity in the rabbit retina [2].

In the present work, we show that adenosine modulates the elevation in the $[Ca^{2+}]_i$ in the neurites induced by KCl depolarization. In agreement with the results obtained concerning the effect of A_1 receptors on the release of ACh, the activation of these receptors decreased the influx of Ca^{2+}, whereas the adenosine A_1 receptor inhibition or the removal of the extracellular adenosine, potentiated the elevation of the $[Ca^{2+}]_i$ in the neurites of the cultured retina cells, where neurotransmitter release occurs. This inhibition of Ca^{2+} influx has been described as one of the mechanisms by which adenosine A_1 receptors inhibit the release of several neurotransmitters [8,21,23]. However, the effect of A_1 receptor activation on the $[Ca^{2+}]_i$ response to KCl depolarization was similar in the large majority of the neurite segments analyzed. The differences between the effect of A_1 receptors on the release of [^{3}H]ACh and [^{14}C]GABA could not be resolved in our $[Ca^{2+}]_i$ studies, which revealed that the inhibition of Ca^{2+} entry through VSCC by A_1 receptors occurs along all the neurites. These results indicate that the A_1 receptors are functionally coupled to the inhibition of VSCC in all the neurites of the cells, independently of their neurotransmitter content (ACh and/or GABA).

We have previously shown, in the same preparation, that the release of ACh is triggered mainly by Ca^{2+} influx through ω-Conotoxin GVIA-sensitive N-type VSCC, whereas the release of GABA is more sensitive to Ca^{2+} influx through nitrendipine-sensitive L-type VSCC. However, both types of VSCC contribute to the release of ACh and GABA from cultured retina cells [19]. Furthermore, we have also shown that the effect of ω-Conotoxin GVIA on the release of ACh induced by KCl was not enhanced by activation of adenosine A_1 receptors with CPA [19], suggesting that these receptors are coupled to the inhibition of N-type VSCC. On the contrary, the release of GABA, which is more sensitive to Ca^{2+} influx through L-type VSCC, is not modulated by adenosine A_1 receptors. Since the release of ACh and GABA is triggered by Ca^{2+} entry through the same VSCC, the differential effect of adenosine on the release of the two neurotransmitters cannot be attributed to a specific effect on a particular type of VSCC. Therefore, our results can only be explained based on a spatial segregation of the receptors, which may explain the spatial segregation of neurotransmitter release. This is not in agreement with our $[Ca^{2+}]_i$ imaging studies, which did not reveal a heterogeneous distribution of the effect of adenosine on the $[Ca^{2+}]_i$ signals. One possibility is that there is molecular-level segregation of the release sites mediated by specific coupling of different channels to different release mechanisms, which however, could not be resolved via light microscopic means.

Taken together, the results reported here and those previously published [19], indicate that the release of ACh and GABA from amacrine-like cells is regulated differentially, suggesting that the synaptic vesicles containing the two neurotransmitters may be located in different cellular sites. The differential distribution of active zones containing GABA and ACh may be of physiological significance on the direction selective mechanism in retina function, since it may allow the cells to control differentially the release of the two neurotransmitters, and, therefore, be responsible for the asymmetry of the direction selective mechanism.

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