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Quantitative Relationship between α_1-Adrenergic Receptor Density and the Receptor-Mediated Calcium Response in Individual Astroglial Cells

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SUMMARY

α_1-Adrenergic receptor (α_1-AR) agonists elevate the intracellular calcium concentration ($[Ca^{2+}]_i$) in 60–80% of astroglia *in vitro*. Likewise, 60–70% of astroglia exhibit specific binding sites for the α_1-AR-selective antagonist (±)-^{125}I-[β-(4-hydroxyphenyl)-ethylaminomethyl]tetralone. The density of α_1-AR binding sites varies markedly on individual cells, ranging from a few to >2000 binding sites/1000 μm^2 of surface area. In the present study, we examined the relationship between the density of α_1-AR binding sites on astroglia and their ability to respond to α_1-AR agonists with a rise in $[Ca^{2+}]_i$. A video-based imaging system was used to monitor calcium responses in individual astroglial cells, which were subsequently assessed for their expression of α_1-ARs using receptor binding autoradiography. The ability of a given concentration of phenylephrine (PE) to elicit a calcium response correlated well with α_1-AR density ($r = 0.94$), i.e., the higher the receptor density the greater the probability that a given astroglial cell would respond to α_1-AR agonists. However, the amplitude of the calcium response did not correlate with the α_1-AR density. Cells with low α_1-AR density (<10 binding sites/1000 μm^2) could generate a response with an amplitude comparable to that seen in cells with high α_1-AR density (>1000 binding sites/1000 μm^2). To evaluate the relationship between receptor occupancy and calcium response, PE concentrations and α_1-AR density were varied while the calcium response in individual cells was monitored. Interestingly, for a given cell the amplitude of calcium response reached its maximum with a small step increase in the concentration of PE (<5-fold), whereas the latency of the response decreased when PE concentrations were increased. Irreversible inactivation of α_1-ARs by phenoxybenzamine reduced the potency of PE but not the maximal calcium response. Cells that responded to 100 nM PE were able to generate a comparable response to 10 μM PE after inactivation of 90% of the total α_1-AR binding sites with phenoxybenzamine treatment. In summary, our results indicate that most astroglial cells express a substantial level of "spare" α_1-ARs that increase the sensitivity of these cells to α_1-AR agonists. Once activated, individual astroglial cells tend to generate a maximal $[Ca^{2+}]_i$ elevation that is independent of the total α_1-AR density or the concentration of ligand.

Astroglial cells express α_1-ARs both *in vitro* (1–3) and *in vivo* (4, 5). In primary cultures, 60–70% of astroglia appear to exhibit binding sites for the α_1-AR-selective ligand ^{125}I-HEAT (2). Among astroglial cells that exhibit a detectable level of α_1-ARs, <10% possess a density of >1000 binding sites/1000 μm^2, whereas >50% have a density of <200 binding sites/1000 μm^2 of surface area. Treatment with α_1-AR agonists increases $[Ca^{2+}]_i$ in about 70% of astroglial cells (6). The latency and the amplitude of the astroglial calcium response after the addition of an α_1-AR agonist vary markedly among these cells.

The relationship between α_1-AR density and responsiveness has been studied in many tissue preparations (7). The results of these studies suggest that α_1-AR density determines the sensitivity and the magnitude of responses to α_1-AR agonists. Additionally, tissues often exhibit a substantial number of "spare" α_1-AR binding sites that ensure cellular responses when ligand concentrations are low. Although studies based on tissue preparations have provided valuable information, the complexity of tissues makes it difficult to correlate receptor density and receptor-mediated responses at the single-cell level. Surprises have been encountered where it has been possible to study dose-effect relationships at the single-cell level. For example, Leong (8) reported that individual gonadotropes release LH in an all-or-none manner. Whereas increases in the dose of LH-releasing hormone increase the probability that an individual gonadotrope will release LH, the dose does not affect the amount of LH released. The dose dependence of the amount of LH release in populations appears to result from an increase

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ABBREVIATIONS: α_1-AR, α_1-adrenergic receptor; BME, basal medium-Eagle's culture medium; LH, luteinizing hormone; HBSS, Hanks' balanced salt solution; PE, phenylephrine; GFAP, glial fibrillary acidic protein; PBZ, phenoxybenzamine; HEAT, (±)-[β-(4-hydroxyphenyl)ethylaminomethyl] tetralone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $[Ca^{2+}]_i$, intracellular calcium concentration; IP_3, inositol trisphosphate.

in the number of gonadotropes responding to LH-releasing hormone (8).

In the present study, we combined receptor autoradiography using ^{125}I-HEAT and calcium imaging using fura-2 to evaluate the quantitative relationship between α_1-AR density and α_1-AR-mediated calcium responses in individual astroglial cells. Experiments were designed to examine the relationship between α_1-AR density and (a) the sensitivity of individual astroglia to α_1-AR ligands, (b) the magnitude of the calcium response, and (c) the latency between exposure of astroglia to α_1-AR ligands and their subsequent calcium response.

Materials and Methods

Primary cultures of astroglia. Primary cultures derived from neonatal rat cerebral cortex were established according to the method of McCarthy and de Vellis (9). Cells were plated at low density (10^4/cm^2) on poly-D-lysine-treated coverslips (Corning no. 1) and were maintained in BME containing 10% fetal calf serum, 2 mM glutamine, 0.6% glucose, 50 IU/ml penicillin, and 50 μg/ml streptomycin. Cells were maintained in a humidified atmosphere of 5% CO_2/95% air.

Fura-2 image analysis and ligand application. The image analysis system used to examine changes in $[Ca^{2+}]_i$ has been described previously (6). Cells were incubated for 30 min at 37° with fura-2/acetoxymethyl ester (2 μM) in BME, taken to room temperature, and washed twice with HBSS containing 1.2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 0.2% bovine serum albumin, and 10 mM HEPES, pH 7.4. Cells were held at room temperature for an additional 30 min before analysis. A video-based imaging system was used to detect changes in $[Ca^{2+}]_i$. Images were generated by taking the ratio of fluorescence emission at 510 nm after excitation at 350 and 380 nm; each image was corrected for background levels before the ratio was calculated. The rate of the image acquisition was 3 sec/image. $[Ca^{2+}]_i$ was determined using external calcium standards (10).

PE was used to activate α_1-ARs and was applied by total bath exchange. Before the ligand addition, HBSS was added as a vehicle control. Between each application, cells were incubated in ligand-free HBSS for at least 5 min. At the end of each experiment, cells were rinsed several times and processed for the combined immunocytochemistry and receptor autoradiography, according to the protocol of Lerea and McCarthy (2).

Combined immunocytochemistry and receptor autoradiography. Cells were fixed with 4% paraformaldehyde for 8 min at 4°, rinsed twice in BME supplemented with 20 mM HEPES, and incubated in fresh BME-HEPES for 1 hr at room temperature before the incubation with primary antibodies. In certain experiments, cells were processed without the fixation to assess the number of α_1-ARs on membrane surfaces. Antibody against GFAP was applied at a 1:100 dilution, in BME-HEPES containing 10% fetal calf serum and 0.05% saponin, for 45 min at room temperature. After rinsing, a fluorescein-tagged secondary antibody was applied along with 80 pM ^{125}I-HEAT to label α_1-ARs. Nonspecific binding of ^{125}I-HEAT was determined in the presence of 50 μM phentolamine or 10 μM PE. Incubations with secondary antibody, ^{125}I-HEAT, and appropriate competing ligands were carried out for 60 min at room temperature. Cells were then incubated in BME-HEPES for 10 min, dipped in distilled water to remove salts, and quickly air dried. The culture coverslips were glued on glass slides (70 × 25 mm) and apposed to emulsion (NTB-3)-coated coverslips (70 × 25 mm) in a dark room. The position of the emulsion-coated coverslip was stabilized, and the dry-mounted slides were stored in lightproof boxes for 2 weeks at 4°. After exposure, the emulsion was developed in Kodak D-19 developer (1:1 in water at 15°) for 3 min, rinsed in water, and fixed in Kodak fixer for 10 min at 15°. The cells and emulsion were coverslipped in phosphate-buffered saline/glycerol (1:1, pH 7.6) and sealed with fingernail polish.

Analysis of autoradiography. Autoradiograms were analyzed with a video-based imaging system. GFAP immunoflourescence was visualized using epifluorescence, and silver grains were detected using a dark-field condenser. For each identified field, digitized images of GFAP immunofluorescence and silver grains were taken alternately. Measuring windows were first sized to cover the border of GFAP-stained cells and then the corresponding silver grain images were retrieved and measured. The digitized images of silver grains were highlighted at an intensity of 255 on a 0–255 gray scale, against background at an intensity of 0. The pixel numbers of highlighted silver grains were counted, and the window area was measured at the same time. The total pixel number was divided by the average pixel number of a single silver grain to yield the total number of silver grains in a given area. The grain number was transformed into binding sites according to the equation described by Barnard (11). The binding sites were further divided by measured areas for expression as binding density (binding sites/1000 μm^2 of surface area). Specific binding density was calculated by subtracting nonspecific binding from total binding. Linear regression was used to examine the relationship between the density of α_1-AR binding sites and the calcium response.

PBZ inactivation of α_1-ARs. To estimate functional α_1-ARs, astroglial cells were first activated by various concentrations of PE while the rises in $[Ca^{2+}]_i$ were monitored. After a wash in ligand-free HBSS, PBZ was added for 5 min. The cells were then tested for their ability to respond to PE. The portion of functional receptors remaining after PBZ treatment was calculated according to the equation described by Furchgott (12) and modified by Minneman and Abel (13). They assumed that to obtain the same response before and after receptor inactivation the same absolute number of receptors must be occupied by agonist before and after receptor inactivation. Therefore, $q \approx [A]/[A']$, where q is the fraction of functional receptors remaining after inactivation and $[A]$ and $[A']$ are the agonist concentrations that generate the same maximal response before and after inactivation, respectively.

Materials. Tissue culture chambers were purchased from Falcon and coverslips from Corning. Rabbit immunoglobulin to cow GFAP was purchased from Dako (Carpinteria, CA) and secondary antibody from Cappel Laboratories (Cochranville, PA). HEAT was a gift from Beiersdorf AG (Hamburg, Germany) and was iodinated according to the method of Minneman (14) and Engel and Hoyer (15). NTB-3 emulsion was purchased from Kodak Co. Other chemicals and drugs were purchased from Sigma.

Results

Incubation of astroglia with ^{125}I-HEAT resulted in concentration-dependent binding that was displaced by α_1-AR antagonists, as shown previously in this laboratory by Lerea and McCarthy (2). In agreement with the previous study, individual astroglia were found to vary markedly with respect to their expression of ^{125}I-HEAT binding sites (Fig. 1). Although the majority of astroglia (>50%) had a density of 1–500 binding sites/1000 μm^2, a small percentage (<10%) of the cells exhibited a density of specific binding of >1500 binding sites/1000 μm^2. No clustering of silver grains in different parts of individual cells was apparent. Similar patterns of ^{125}I-HEAT binding were also observed with nonfixed and nonpermeated cells, and such binding was displaced by the hydrophilic agonist PE. These results indicate that ^{125}I-HEAT bound primarily to extracellular membrane receptors on astroglia (data not shown).

To examine the relationship between the density of α_1-AR binding sites and the ability of astroglia to respond to an agonist, astroglia were loaded with fura-2 and their calcium responses to PE were recorded with a video-based imaging system. The density and the spatial distribution of α_1-AR binding sites on the same cells studied by calcium imaging were examined using quantitative receptor autoradiography (16). Six

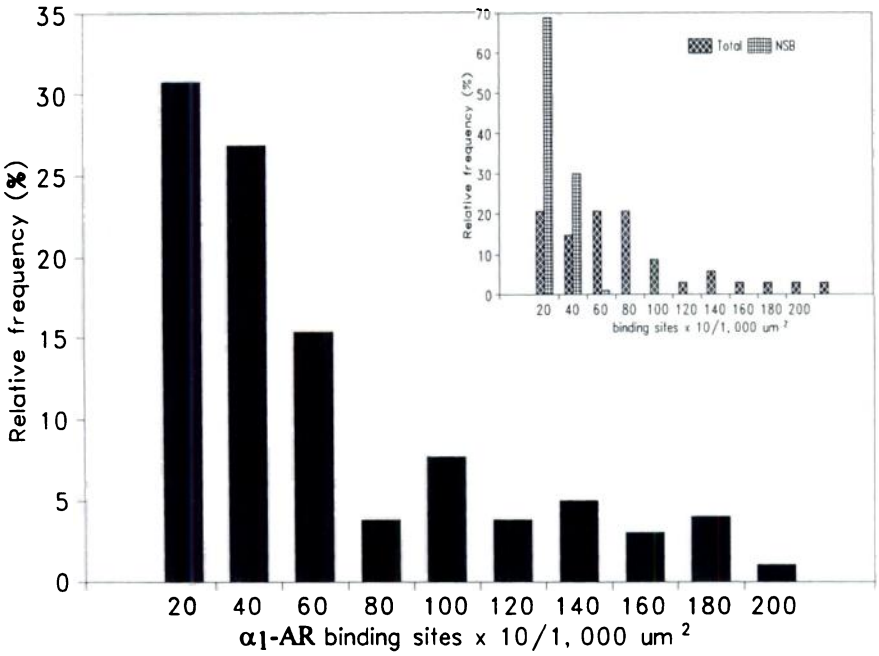

Fig. 1. Distribution of ^{125}I-HEAT binding on astroglial cells. Astroglial cells grown on glass coverslips were first stained with GFAP antibody and then incubated with 80 pM ^{125}I-HEAT. Silver grains in autoradiograms were counted and normalized as binding sites/1000-μm^2 area measured over individual cells (see Materials and Methods). Nonspecific binding (*NSB*) was measured in the presence of 50 μM phentolamine and was substracted from the total binding to yield specific α_1-AR binding (*inset*).

GFAP-immunoreactive cells are presented in Fig. 2; four of these had a high grain density (>1000 binding sites/1000 μm^2) and the other two had a low density (<200 sites/1000 μm^2; Fig. 2, *arrows*). The addition of 10 μM PE increased $[Ca^{2+}]_i$ in the four cells with high α_1-AR densities, whereas the two cells with low densities remained at basal levels. Occasionally, cells with a low density of α_1-ARs also responded to PE with an increase in $[Ca^{2+}]_i$. Among the four cells presented in Fig. 3, one had a high density, two had intermediate densities, and the fourth had a low density; all responded to PE within 5 sec.

The calcium responses of 107 astroglia were plotted as a function of their α_1-AR densities, to examine the relation between these two parameters (Fig. 4). Almost every cell with a density of >1200 binding sites/1000 μm^2 responded to 10 μM PE. Note that a fraction of cells with an α_1-AR density of 1–200 binding sites generated responses with amplitudes comparable to those of cells with high α_1-AR density. No correlation between the amplitude of the calcium response and the α_1-AR

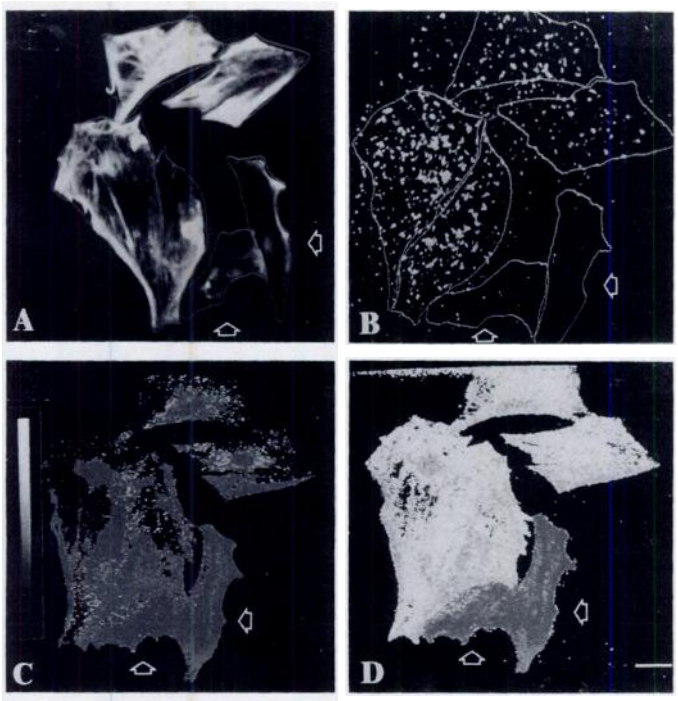

Fig. 2. α_1-AR binding sites and α_1-AR-mediated calcium responses of individual astroglial cells. Digitized images show that, among the six GFAP-positive cells (A), four show higher levels of ^{125}I-HEAT binding and two show low levels (B, *arrows*). After treatment with 10 μM PE, the $[Ca^{2+}]_i$ in the four high density cells was increased from basal levels (C) to >500 nM in 10 sec (D), whereas the two low density cells remained at basal levels (*arrows*). *Bar*, 10 μm.

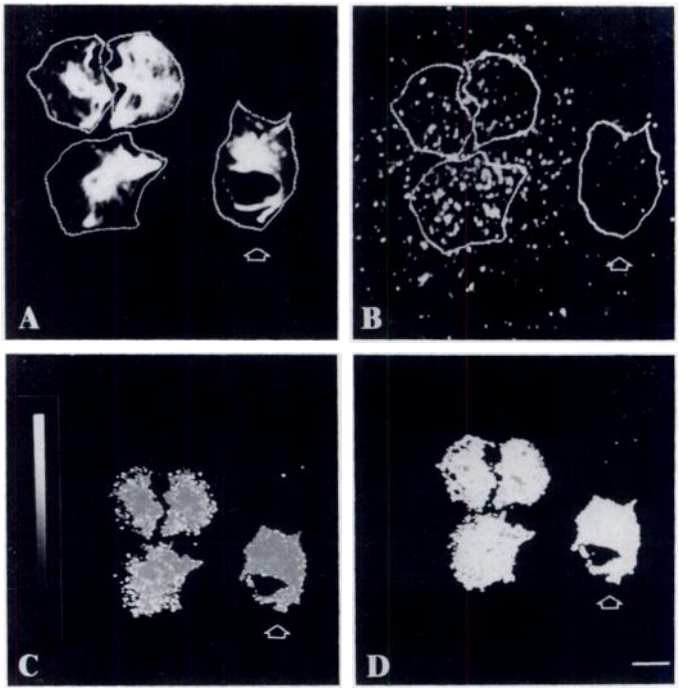

Fig. 3. Calcium responses in astroglia with different α_1-AR densities. The preparation is the same as in Fig. 2. The four GFAP-positive cells (A) show various grain densities (B); each cell responded to 10 μM PE in 5 sec (C, basal level; D, peak response). *Bar*, 10 μm.

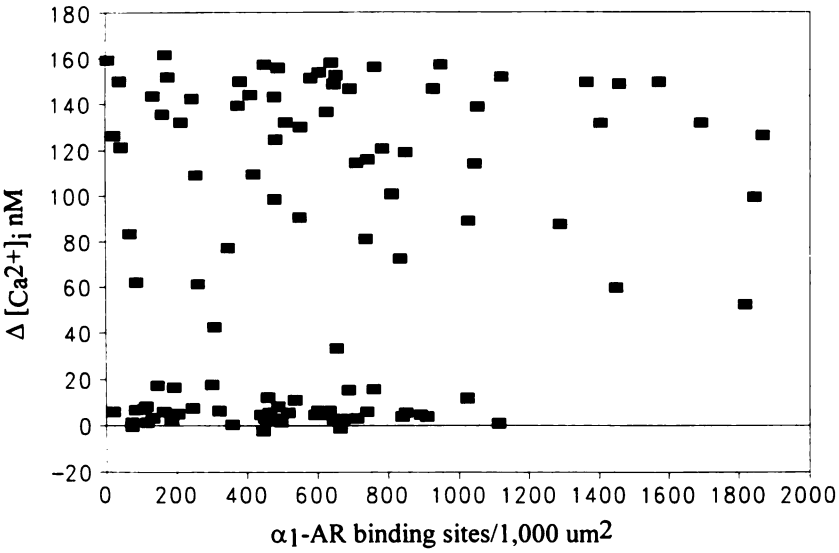

Fig. 4. Calcium responses in individual astroglial cells as a function of α_1-AR density. Each *square* represents a cell that was exposed to 10 μM PE and then processed for α_1-AR binding autoradiography. A total of 107 cells that had an α_1-AR density above the nonspecific level are presented. The *y*-axis represents the increase in $[Ca^{2+}]_i$ above basal levels (peak response − basal level).

density was observed. The results presented in Fig. 5 indicate that the percentage of astroglia that responded with a rise in $[Ca^{2+}]_i$ increased as a function of the density of α_1-ARs expressed by these cells. Thus, the higher the receptor density the greater the probability that an astroglial cell responded to the α_1-AR agonist ($r = 0.94$, linear regression).

PE concentrations were varied to activate different numbers of α_1-ARs. Fig. 6 illustrates the percentage of cells that responded to three different doses of PE. Within each group of α_1-AR density, the percentage of responding cells increased as the PE concentration increased. Similarly, the higher the α_1-AR density the greater the percentage of cells that responded to a given concentration of PE. Table 1 lists 10 cells that responded to at least two doses of PE. Cells 6, 9, and 10 showed higher densities of α_1-AR binding sites, and each responded to 10, 100, and 1000 nM PE with similar $[Ca^{2+}]_i$ peaks. The remaining cells did not respond to 10 nM PE but exhibited increases in $[Ca^{2+}]_i$ in response to 100 and 1000 nM PE. These data again support the lack of correlation between the amplitude of the calcium response and the α_1-AR density.

Table 1 further demonstrates that for many cells the time between ligand addition and onset of a response (latency) decreased as PE concentrations or α_1-AR density increased. The latencies of responses to 10 μM PE are plotted in Fig. 7 as a function of α_1-AR densities. Although some cells with low α_1-AR density generated rapid responses, cells with higher α_1-AR density generally responded more rapidly. These results support an inverse relationship between the response latency and receptor density or agonist concentration.

Dose-effect experiments were performed to examine the relationship between receptor occupancy and calcium responses in individual astroglial cells. The responses of two astroglial cells to different PE concentrations are presented in Fig. 8. Both cells responded to 10 nM but not 1 nM PE and exhibited similar amplitudes of $[Ca^{2+}]_i$ rises in response to 2, 3, 5, 10, 100, and 1000 nM PE. The small variations in the $[Ca^{2+}]_i$ amplitude within such narrow ranges of PE (<5-fold) did not allow the calculation of concentration-dependent parameters such as EC_{50}. Also note that as the PE concentration increased the delay time between PE addition and the $[Ca^{2+}]_i$ rise decreased. This again suggested an inverse relationship between receptor occupancy and the latency of response.

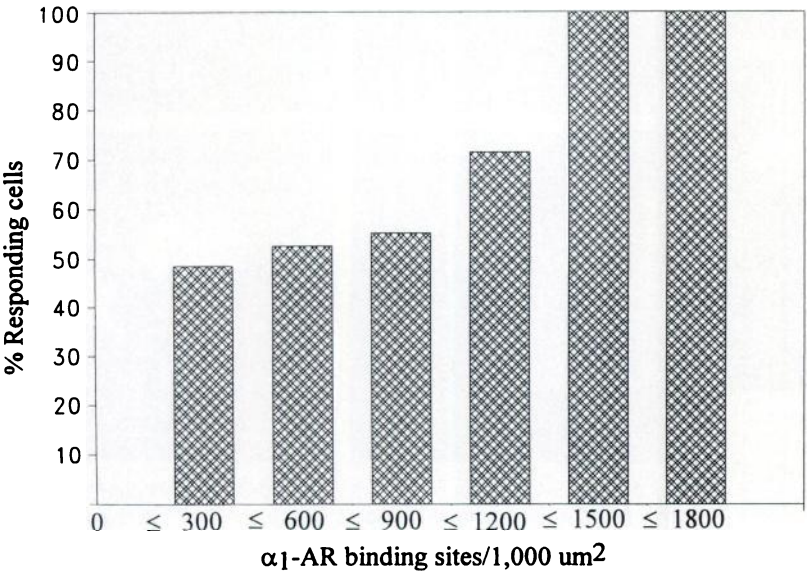

Fig. 5. Relative frequency of astroglial responsiveness in relation to α_1-AR density. This graph was plotted from data for cells presented in Fig. 4. The cells were grouped according to their receptor densities (bin width = 300 binding sites/1000 μm^2). The number of cells that gave a response (>120% of the basal level) was divided by the total number of cells within the group. The correlation coefficient calculated by linear regression was 0.94.

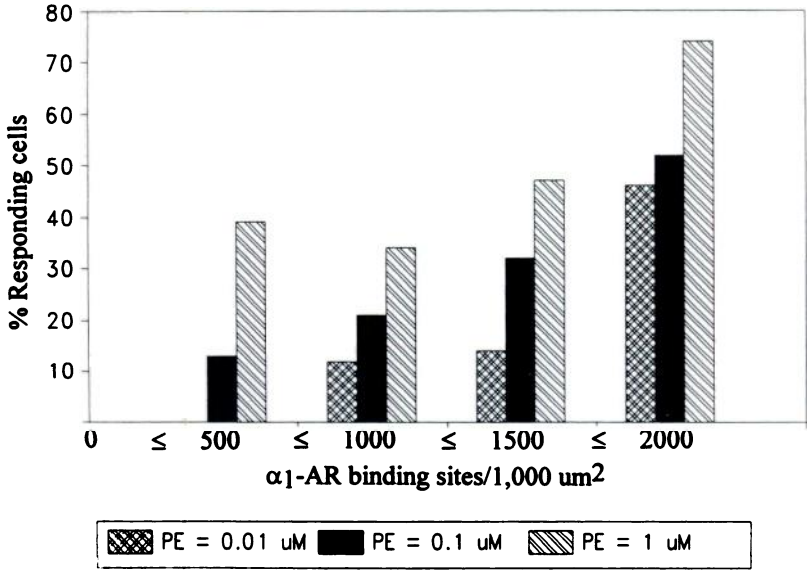

Fig. 6. Relative frequency of astroglial responsiveness to various doses of PE in relation to α_1-AR density. A total of 36 cells were treated with 10, 100, and 1000 nM PE successively and then processed for α_1-AR binding autoradiography. The analysis was the same as in Fig. 5 (bin width = 500 binding sites/1000 μm^2).

TABLE 1

Relationship between α_1-AR density, $[Ca^{2+}]_i$ increase, and response latency

Ten astroglial cells are listed in order of their α_1-AR densities. Their response to each PE concentration is presented as $\Delta[Ca^{2+}]_i$ (peak response − basal level). Latency reflects the number of seconds between drug addition and the onset of the response.

Cell	α_1-ARs/1000 μm^2	PE = 10 nM		PE = 100 nM		PE = 1000 nM	
		$\Delta[Ca^{2+}]_i$	Latency	$\Delta[Ca^{2+}]_i$	Latency	$\Delta[Ca^{2+}]_i$	Latency
		nM	sec	nM	sec	nM	sec
1	33.3	0		34.2	24	52.0	8
2	55.5	0		102.8	9	134.8	5
3	95.0	0		123.2	9	190.0	5
4	351.8	0		142.0	5	191.4	1
5	719.5	0		48.6	24	35.2	6
6	852.3	68.2	1	72.4	1	78.2	1
7	1033.5	0		143.4	1	198.0	1
8	1104.4	0		163.8	5	188.8	1
9	1177.2	51.2	1	58.8	1	56.2	1
10	2008.2	49.8	1	58.6	1	63.0	1

PBZ was used to irreversibly inactivate fractions of α_1-ARs, to test whether astroglia express reserve functional receptors (spare receptors). The two cells presented in Fig. 9 responded to 100 and 1000 nM PE with similar amplitudes. PBZ at 10 nM had no effect on the response to 100 nM PE. When the cells were incubated with 100 nM PBZ, responses to 100 nM PE were diminished. PE at 500 nM was able to generate responses in both cells, with somewhat slower rising times and lower amplitudes. However, 1000 nM PE produced responses comparable to those observed before PBZ administration. The application of 200 nM PBZ blocked the responses to all doses of PE used. The carbachol response at the end of the experiment indicated that the cells were able to respond to an alternative ligand with a rise in $[Ca^{2+}]_i$. According to the assumption of Minneman and Abel (13) that the fraction of functional receptors remaining after inactivation is approximately the ratio of agonist concentrations generating the same maximal responses before and after inactivation, these astroglial cells generated the maximal response when 90% of their α_1-ARs were inactivated (see Materials and Methods).

Discussion

Previous studies from this laboratory indicated that the percentage of type 1-like astroglia that exhibit α_1-AR binding sites is very similar to the percentage of these cells that respond to α_1-AR agonists with an increase in $[Ca^{2+}]_i$ (2, 6). Because the number of astroglial α_1-ARs varied between only a few above background to several thousand per 1000 μm^2, these findings suggest that a small number of occupied α_1-AR binding sites are required to stimulate astroglial $[Ca^{2+}]_i$. The experiments described in this study were designed to examine the relationship between the density of α_1-ARs and the ability of an α_1-AR agonist to increase astroglial $[Ca^{2+}]_i$. Interestingly, the amplitude of the calcium response of individual astroglia did not correlate with the density of α_1-AR binding sites exhibited by the responding cell; that is, cells responded in a nongraded manner to increases in ligand concentration. Conversely, both the probability of responding and the latency between the application of ligand and the calcium response were related to the density of α_1-AR binding sites. Several interesting suggestions arise from these findings. First, the absence of a correlation between the density of receptors and the amplitude of the calcium response in individual cells implies that the signal required to elicit a rise in $[Ca^{2+}]_i$ must reach a threshold and that, once it is reached, a given amount of calcium accumulates. The finding that the latency between the application of ligand and the calcium response is dependent on the density of α_1-ARs suggests that the greater the number of receptors occupied by an agonist the more rapidly the signal responsible for increasing $[Ca^{2+}]_i$ accumulates. The observation that cells with a higher density of α_1-AR binding sites exhibit a higher probability of responding to α_1-AR agonists suggests that the rate at which the signal for an increase in calcium accumulates is important in eliciting a calcium response. Finally, the occurrence of cells that exhibit a very low receptor density but exhibit a rapid and robust calcium response suggests that factors other than receptor density are likely to play a role in determining whether astroglia respond to α_1-AR agonists with a rise in $[Ca^{2+}]_i$.

Two different lines of experimental evidence suggest that α_1-AR agonists induce a nongraded increase in astroglial $[Ca^{2+}]_i$.

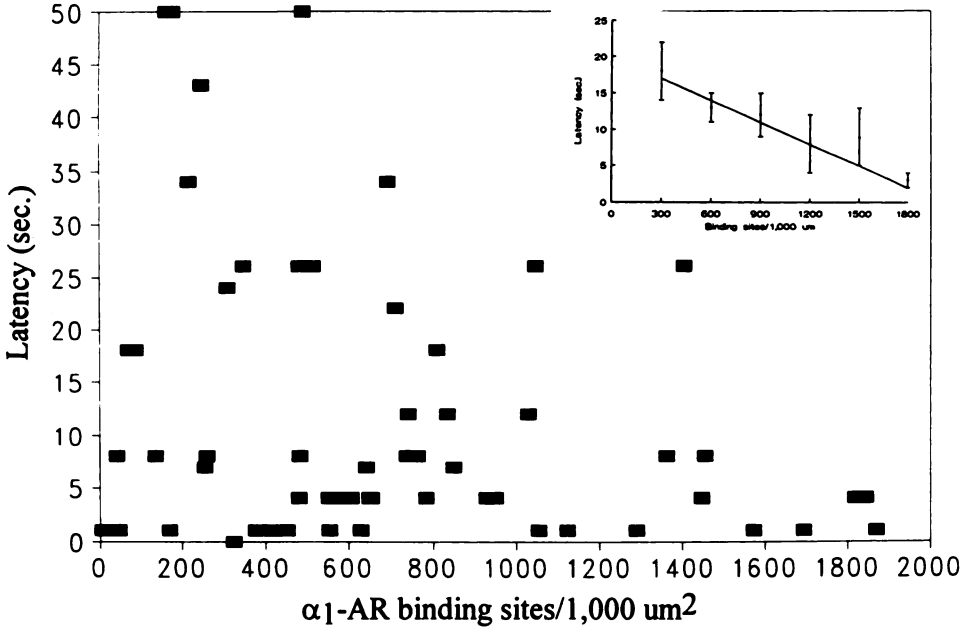

Fig. 7. Relationship between latency of calcium responses and α_1-AR density on individual astroglial cells. The time between the addition of the ligand and the onset of the response was plotted against the α_1-AR density of 51 cells that responded to 10 μM PE. *Inset*, each *point* represents the average time of cells grouped according to their α_1-AR density (bin width = 300 binding sites/1000 μm^2). The *line* is derived from linear regression, with a correlation coefficient of 0.95.

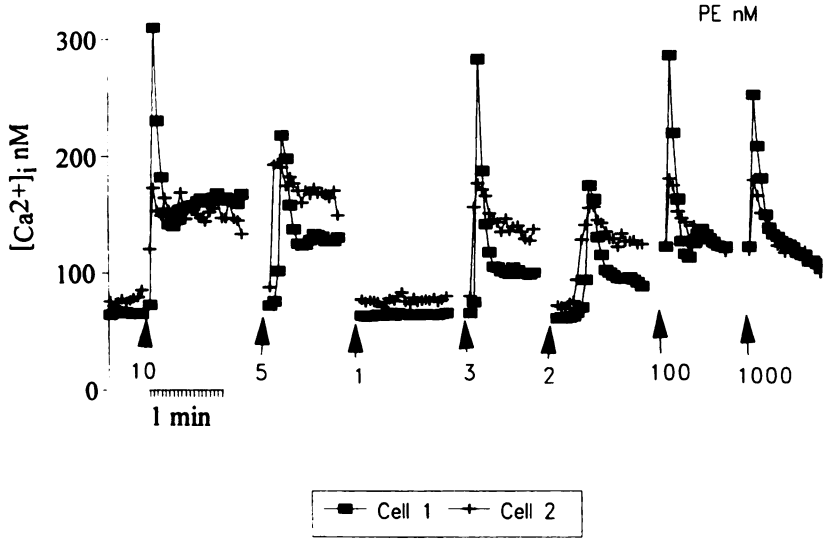

Fig. 8. Dose response of astroglial cells to various concentrations of PE. The two cells were treated with PE by a change of the full bath solution (*arrows*). Between each treatment, the cells were rinsed with ligand-free HBSS three times and incubated for at least 5 min. The sampling rate was 3 sec/measurement.

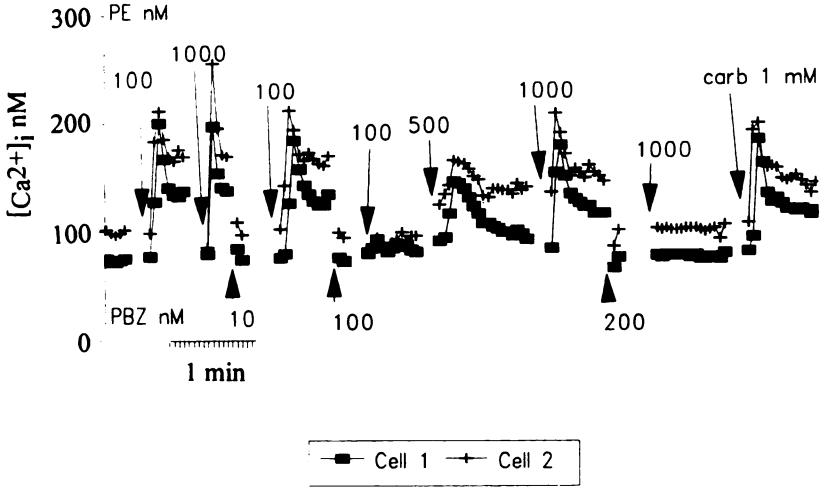

Fig. 9. Responsiveness of individual astroglial cells to PE after irreversible inactivation of α_1-ARs by phenoxybenzamine. The ability of the cells to respond to PE was tested before and after PBZ inactivation of α_1-ARs. The PBZ treatment lasted for 5 min, and free PBZ was removed by excessive washing with HBSS.

First, no correlation was observed between the amplitude of PE-induced increases in $[Ca^{2+}]_i$ and the density of α_1-AR binding sites exhibited by individual astroglia (Fig. 4; Table 1). Because the proportion of α_1-AR binding sites occupied should remain constant in the presence of a fixed concentration of ligand, these findings suggest that the amplitude of the calcium increase was independent of the number of receptors occupied by the ligand. Second, dose-effect experiments indicated that a very small increase (1–3-fold) in ligand concentration resulted in a maximal increase in $[Ca^{2+}]_i$ (Fig. 8). The first argument assumes that the labeling technique used to measure α_1-ARs is valid. Briefly, the labeling of α_1-ARs using this method has been assessed through kinetic and saturation analyses of K_d and competition binding experiments with antagonists, agonists, and stereoisomers; all findings support the view that this method selectively labels α_1-ARs (2). The second argument is dependent on the ability to repeatedly elicit a given calcium response with a given concentration of ligand; in general, this has been problematic. We find marked variation in the amplitude of calcium responses in individual cells with repeated application of a given concentration of ligand. However, through the course of many dose-effect experiments in which the order of the different concentrations of ligand was randomized, we failed to observe a relationship between the dose of the ligand and the amplitude of the calcium response. Although our results are most consistent with a nongraded (all-or-none) calcium response to PE, it remains possible that the calcium response to PE is graded over a very narrow range (1–3-fold) of ligand concentration.

These findings suggest that astroglia respond to α_1-AR agonists with an all-or-none calcium response. The basis for the apparent all-or-none release from stores may involve positive cooperativity in IP_3 binding to its receptor (17). The basis for the all-or-none response from a cell is more difficult to explain. Possibilities include the initial release of calcium from a given store (a) potentiating phospholipase C activity to markedly increase the formation of IP_3, such that levels required to release calcium from additional IP_3-sensitive stores are obtained, or (b) resulting in the overloading and emptying of other calcium stores, or (c) some combination of these processes. The initial release of calcium from a specific IP_3-sensitive store with secondary release from surrounding stores would explain the finding that calcium generally moves through astroglia as a wave originating from a focal point. Although it remains unresolved why a specific IP_3-sensitive calcium store would be released preferentially, our results suggest that it does not involve the restriction of neuroligand receptors to a specific region of the cell surface (2). The idea that the preferential release of calcium from a given store may be due to the density of IP_3 receptors associated with that store is consistent with our findings that the activation of different neuroligand receptors often results in an increase in calcium that originates from the same region of a cell.[1] Our data indicate that activation of α_1-ARs results in a fixed amount of calcium release.

Results presented suggest that both the latency between addition of ligand and the calcium response and the probability of an astroglial cell responding to PE were strongly correlated with the number of occupied α_1-ARs (Figs. 5–7; Table 1). The correlation between the latency to response and the number of occupied receptors suggests that the level of an intracellular signal, most likely IP_3, must reach a threshold concentration before eliciting calcium release. Similar conclusions were drawn by Miledi and Parker (18) after their analysis of latencies of membrane currents evoked in *Xenopus* oocytes by receptor activation or photolysis of caged IP_3. These findings are also consistent with the cooperativity that has been reported for IP_3-mediated calcium release (17). Whether feedforward processes, such as the initial release of calcium from a restricted store positively affecting either phospholipase C or IP_3 binding, play a role in this process remains to be determined. The positive correlation between the number of occupied receptors and the probability of an astroglial cell responding suggests that the rate at which the signal for calcium release accumulates is important in determining whether a cell responds. It seems likely that negative feedback processes (e.g., protein kinase C phosphorylation of a receptor) are activated upon receptor occupation such that, if the threshold of the signal for calcium release is not reached before a given degree of desensitization, the cell fails to respond. The point of desensitization could be at several sites involved in receptor-mediated release of calcium from internal stores. However, the observation that a second ligand can stimulate an increase in calcium release immediately after calcium levels have returned to basal levels after stimulation induced by an alternate ligand (6) suggests that desensitization may be occurring at the level of the neuroligand receptor.

A number of investigators have reported dose-effect increases in calcium levels using calcium indicator dyes (19–21). In general, these studies have been carried out by analyzing the response of cell populations rather than individual cells, as used in our experiments. It is clear that a dose-effect relationship for α_1-AR-stimulated calcium levels would be anticipated with populations of astroglia as well. That is, increasing the number of astroglial α_1-ARs occupied would synchronize the response by decreasing the latency to response and increasing the probability of a response. The net result would be an increase in the amplitude of the response of the population of cells with increasing dose of an α_1-AR agonist. Bootman *et al.* (22) reported that HeLa cells respond to histamine with all-or-nothing mobilization of calcium. Furthermore, there was an increase in the percentage of cells responding as the dose of the ligand was increased (22).

It is interesting that certain cells with a very low receptor density responded with a rapid and robust increase in $[Ca^{2+}]_i$ (Figs. 4 and 7). Although atypical, a sufficient number of these examples were observed to warrant comment. The results of previous studies indicate that there is no obvious clustering of α_1-ARs across the surface of astroglia (2). However, it remains possible that a few receptors spatially localized to a region closely associated with a calcium release site may be capable of stimulating calcium release. The observation that a rapid and robust calcium response can occur in cells with a very low density of receptors suggests that the feedforward process may involve events not at the neuroligand receptor level but rather at the calcium release site. Obviously, additional studies will have to be pursued to address this issue.

In general, the presence of spare receptors is defined as the expression of more receptors than required to elicit a maximal cellular response. The most obvious result of the presence of spare receptors is a decrease in the concentration of ligand

[1] Y. Shao and K. D. McCarthy, unpublished observations.

required to elicit a maximum response. By this definition, with respect to calcium regulation, astroglia exhibit spare α_1-ARs. However, from our findings it is clear that the presence of spare receptors linked to calcium mobilization has effects beyond reducing the concentration of neuroligand required to elicit a maximum response. The spare α_1-ARs of astroglia affect both the probability of the cells responding and the latency to response. *In vivo*, the morphology of astrocytes is extremely complex, and it is easy to imagine that a small fraction of astrocytic neuroligand receptors restricted to a given region of the cell surface respond to a locally released neurotransmitter with a localized rise in calcium, which then moves through the cell as a calcium wave (23). Similar or dissimilar receptor systems may be activated in other regions of the cell. The net result of this type of stimulation is that a given astrocyte receiving a localized signal would transmit the result of that signal throughout the entire cell. In the case of astrocytes connected by gap junctions, an entire group of astrocytes could be activated through the occupation of a small number of receptors spatially restricted on a single astrocyte. Although the calcium response appears to be nongraded, increased receptor density or the presence of multiple receptor systems generating the same signal would integrate to increase the probability of generating a calcium response. Hence, although astroglia exhibit spare receptors (using the definition described above), we would argue that these receptors may be very important in the biology of these cells and in central nervous system physiology.

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